Molecular genetic changes during tumour progression in mouse skin.

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To mum, dad and Pauline, all of whom I

care for very deeply.

Profound quotation page.

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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
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DNase	deoxyribonuclease
RNase	ribonuclease
bp	base pairs
kb	kilobase pairs
M_R	relative molecular weight
mM	millimolar
µl	microlitres
ng	nanogrammes
pmol	picomoles
V/cm	volts per centimetre
W	watts
dpm	disintegrations per minute
OD	optical density (absorbance)
% w/v	weight in grammes per 100ml water
% v/v	volume in ml per 100ml water
BSA	bovine serum albumin
EDTA	ethylenediamine tetra-acetic acid
EtBr	ethidium bromide
Tris	tris(hydroxymethyl)methylamine
PEG	polyethylene glycoll
SDS	sodium dodecyl sulphate
DMBA	7,12-dimethyl benz[a]anthracene
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
HaMSV	Harvey murine sarcoma virus
TPA	12-O-tetradecanoylphorpbol-13-acetate
LOH	Loss of heterozygosity
RFLP	Restriction fragment length polymorphism
BBC	Buckie Bowling Club

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Abstract

This thesis describes the development of a model to analyse the genetic changes associated with tumour progression in mouse skin. Tumours were induced in F1 hybrid mice, thereby permitting the use of heterozygous DNA markers (restriction fragment length polymorphisms) to determine the role of allele loss in papilloma and carcinoma development. Frequently, initiation of mouse skin carcinogenesis involves H-*ras* activation. This gene is located on mouse chromosome 7. The F1 hybrid tumour model was used to demonstrate that tumours with this mutation also show loss of heterozygosity (LOH) or imbalance of alleles on chromosome 7 at a very high frequency. Thus LOH may indicate the presence of an oncogene, although it is often equated with tumour suppressor gene loss. Most frequently the alterations involved non-disjunction, but in some cases mitotic recombination or deletion was detected. These gross chromosome changes were not observed in mouse skin tumours lacking activated H-*ras*. Thus, it is clear that the initiation event can influence the type of alterations which occur at later stages of tumour progression.

In the majority of cases, gross chromosome 7 changes result in an increased copy number of mutant H-*ras* and under-representation or loss of the normal allele, indicating that mutant H-*ras* is involved in both the initiation and progression of mouse skin tumours. It may be that elevation of the mutant signal is required to overcome a suppressive effect of the normal allele. In addition, because elevation of mutant H-*ras* gene copy number occurs by gross chromosomal mechanisms, it is possible that another chromosome 7 gene is also involved in tumour progression. In support of this, mitotic recombination or deletion was detected *distal* to H-*ras* in 4/26 of the chemically induced tumours with activated H-*ras*. In addition, a chromosome 7 alteration was detected in a v-H-*ras* initiated tumour, further evidence that a gene other than H-*ras* on this chromosome is involved in tumour progression. Human tumours frequently demonstrate LOH at the chromosomal region 11p15.5, which is syntenic with the part of mouse chromosome 7 that encompasses the H-*ras* locus. Thus, the homologue of a tumour suppressor gene in this region of human chromosome 11 may be involved in mouse skin tumour development. The Wilms' tumour locus, also on human 11p, is on mouse chromosome 2. RFLP analyses provided no evidence that this gene has a role in mouse skin tumorigenesis.

The non-random nature of chromosome 7 changes was supported by the low frequency of alterations on chromosomes 2 and 11. Two carcinomas did show LOH of a marker on the latter. Interestingly, this chromosome contains a region homologous to human chromosome 17p, which is involved in colorectal cancer. Minisatellite analysis also supported the non-random nature of chromosome 7 changes. Loss or rearrangement of minisatellite bands tended to involve hypervariable loci, suggesting that these were random rearrangements at unstable loci.

In some human cancers genomic imprinting influences the direction of allele loss on 11p. However, this did not appear to be the case with LOH on chromosome 7 in mouse skin carcinomas. The parental strain also did not influence which alleles were under-represented in these tumours.

Some important differences were detected between the genetic changes associated with carcinomas induced by initiation/promotion and those seen in carcinomas obtained by repeated carcinogen treatment. A similar proportion of MNNG/TPA and MNNG/MNNG carcinomas were positive for mutant H-*ras*. However, whereas non-disjunction of chromosome 7 had also occurred in the former, no chromosome 7 changes were detected in carcinomas induced by repeated MNNG treatment. This carcinogen may remove the need for additional chromosome 7 changes by mutating the gene(s) affected by these events in TPA-promoted tumours,

or by altering entirely separate loci. In contrast, tumours induced with repeated DMBA treatment which were positive for activated H-*ras* also had chromosome 7 changes. However, the frequency of events such as mitotic recombination or deletion was much higher in these tumours than in carcinomas induced by an initiation/promotion regime. The major difference between DMBA/DMBA carcinomas and DMBA/TPA carcinomas was that the latter contained a much higher proportion of tumours which lacked activated H-*ras*. Thus it appears that repeated DMBA treatment stimulates the growth of initiated cells which are insensitive to TPA.

Analysis of papillomas showed that gross chromosome 7 changes occur at a premalignant stage of tumorigenesis. This may suggest a tumour promoter-related genetic effect. However, such events are not limited to a single stage of tumour growth since selection for extra copies of mutant H-*ras* was observed in some carcinomas.

The large number of polymorphisms in *M. spretus/M. musculus* hybrids makes them especially useful in the analysis of tumour-related LOH. RFLPs at H-*ras* allow direct identification of the parental origin of the mutant allele and this has been used in conjunction with work by P.Burns to demonstrate the monoclonal nature of mouse skin tumours.

The F1 hybrid model is a novel method of assessing the relevance of allele loss to tumour progression in animal systems. Its application to other models may lead to the identification of new genes involved in tumorigenesis.

Chapter 1

Introduction

1.1 Cancer as a multistep genetic process

The familial nature of certain cancers (Hansen and Cavenee, 1987), the mutagenic capability of many carcinogens (section 1.5.2), and the association of clonal karyotypic changes with most cancers (Heim *et al.*, 1988), support the proposal that a major component of neoplasia is genetic. In the last two decades several genes have been isolated which, on the basis of multiple criteria, have provided conclusive evidence for this hypothesis. These genes fall into two major categories: oncogenes (section 1.2) and tumour suppressor genes (section 1.4). Both classes are implicated in growth control, but contribute to malignancy in separate ways. Oncogenes do so positively, following activation by qualitative or quantitative means (section 1.2.2). Their identification frequently involves assays that detect morphological transformation (section 1.2.1.3). In contrast, tumour suppressor genes are thought to block tumorigenesis and must be functionally inactivated to permit tumour progression (section 1.4). In theory these genes could be identified by their ability to revert the transformed phenotype. However, this has not been a common approach to their isolation because of the difficulties associated with detecting normal cells in a transformed population. Instead, most effort has gone into the mapping of non-random tumour associated deletions, thought to signify the presence of such a gene (section 1.4.2).

It has also been a long-standing notion that cancer is a multistep process (Foulds, 1954 and 1958; Klein and Klein, 1985). The discrete morphological and histological stages of many cancers suggests a stepwise progression towards malignancy, as does the successive emergence of more aneuploid subclones during tumour development (Heim *et al.*, 1988). Mathematical models applied to age-incidence curves have suggested that leukemias require 3-4 mutations, whereas carcinomas develop after 6-7 alterations (Farber and Cameron, 1980; Knudson

1973). These should be regarded as a minimum, since events which are not rate-limiting will not be detected by these methods.

For a growing number of human tumours the type and stage of involvement of non-random gene mutations has been elucidated (Vogelstein *et al.*, 1989; Bouck and Benton, 1989). This has been complemented by *in vitro* and *in vivo* analyses in which the ability of different combinations of oncogenes to effect malignancy have been analysed (section 1.2.3). It is now realistic to speculate that elucidation of all the rate-limiting genetic mutations which are cooperatively responsible for individual malignancies may be possible.

1.2 Oncogenes

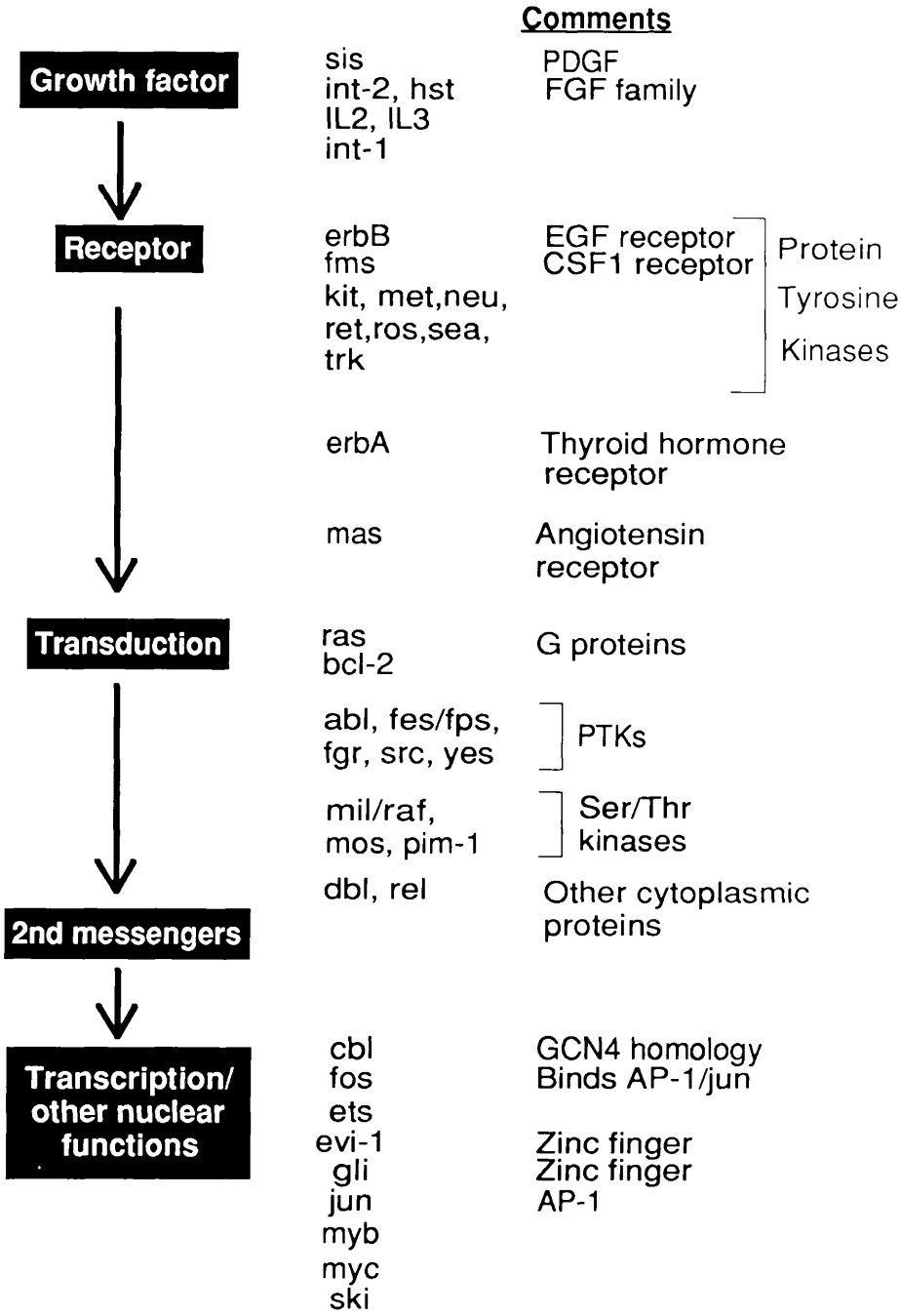
Elucidation of oncogene sequences, and cellular location and biochemical action of the proteins they encode have confirmed their role in the control of differentiation and proliferation (Bishop, 1987). Such studies have facilitated the grouping of these genes into distinct categories (Figure 1). These classes represent many of the known stages of the signal transduction process, from growth factors through to nuclear DNA binding proteins.

1.2.1 The discovery of oncogenes

1.2.1.1 Oncogenes of acutely transforming retroviruses

The very first oncogenes to be discovered were identified as the component of acutely transforming retroviruses responsible for neoplasia (Bishop, 1981; Bishop and Varmus, 1982). Retroviruses have an RNA genome and replicate through a DNA proviral intermediate which integrates into cellular DNA. Over 20 acutely transforming retroviruses have been isolated from a range of species including chickens, turkeys, mice, rats, cats and monkeys. Most of these highly oncogenic

Figure 1 Oncogenes and cellular signalling



The functional components of mitogenic signalling pathways are shown on the left and examples of oncogenes which fulfill these roles are listed on the right. Oncogenes which have yet to be assigned a position include bcl -1 and pvt.

strains are replication-defective as a result of loss or alteration of viral genes, and are found with a helper virus. However, the retrovirus from which the first oncogene, v-*src*, was isolated – Rous Sarcoma virus (RSV) – is an exception; it is replication-competent. Identification of the *src* gene was closely followed by discovery of the *mos* and K-*ras* oncogenes in Moloney and Kirsten murine sarcoma viruses respectively (Bishop and Varmus, 1982).

One of the most significant advances in cancer research to date was the discovery that retroviral oncogenes have normal cellular counterparts; proto-oncogenes (Stehelin *et al.*, 1976; Bishop, 1981). The retroviral versions of these genes lack introns, suggesting that they were transduced from the cellular genome rather than the other way round. However, as discussed below, it is other differences between the cellular and viral forms of these genes which are generally considered to be responsible for their oncogenic properties.

1.2.1.2 Detection by insertional mutagenesis

The majority of replication competent transforming retroviruses do not possess oncogenes. However, these viruses induce malignancy after a long latency period by integrating near or within a cellular proto-oncogene (Nusse, 1986). This brings the gene under the contol of the powerful transcriptional enhancer sequences found in the viral long terminal repeat (LTR) and may also truncate the gene depending on the site of integration. The *myc* oncogene was originally identified in an acutely transforming retrovirus, but is also activated by proviral insertion in the large majority of avian leukemia virus (ALV)-induced bursal lymphomas (Hayward *et al.*, 1981), and proviral activation of c-*myc* is also common in murine B and T-cell lymphomas (Berns *et al.*, 1989). Other genes which were first identified as retroviral oncogenes and then also detected by insertional mutagenesis include c-*erb*B, c-*mos*, c-*myb* and c-H-*ras* (Bishop, 1987). Latent transforming viruses have been used to isolate several novel oncogenes which, to date, have not been detected in acutely

transforming retroviruses. These include five loci frequently activated by MMTV integration: *Int*-1, 2, 3, 4 and *hst* (Dickson *et al.*, 1984; Nusse *et al.*, 1984; Delli Bovi *et al.*, 1987; Peters *et al.*, 1989). *int*-2 and *hst* (also termed KS-3, *hst*F1 and k-FGF) are members of the fibroblast growth factor gene family (Figure 1) and are within 20kb of each other on mouse chromosome 7 (Peters *et al.*, 1989). *Int*-1 is also thought to be a growth factor and is implicated in the control of development (reviewed by Bender and Pelfer, 1987). Activation of more than one of these five genes in a single MMTV-induced tumour has been observed (Peters *et al.*, 1986 and 1989).

Three so called *fim* sites have been identified in FuMLV –induced myeloid leukemias (Sola *et al.*, 1988); *fim*-2 is equivalent to the *fms*/CSF-1 receptor gene, while *fim*-3 is equivalent to the *evi*-1 locus, which encodes a zinc finger protein (Bartholomew *et al.*, 1989). In addition, the IL2 and IL3 genes, which encode haematopoetic growth factors, have been activated by insertional mutagenesis in leukemia cells (Chen *et al.*, 1985; Ymer *et al.*, 1985). Also, retroviral integration at the *pim*-1 and N-*myc* loci is frequently associated with murine T-cell lymphomas (Berns *et al.*, 1989), although unlike c-*myc* these genes have never been detected in acutely transforming retroviruses.

1.2.1.3 Detection of cellular transforming genes by transfection

There is no evidence that the acutely transforming retroviruses isolated from animal tumours are involved in human neoplasia. However, the discovery that retroviral genes were derived from cellular homologues suggested that alteration of proto-oncogenes by non-viral means might induce malignancy (Bishop, 1981 and 1987). After Hill and Hillova (1972) demonstrated the transformation of chicken embryo fibroblasts with DNA from RSV-transformed rat cells, attempts were made to induce transformation of normal cells by transfection of human tumour DNA. Development of the calcium precipitation approach for DNA transfection (Graham

and van der Eb, 1973; Wigler *et al.*, 1977) paved the way for successful transmission of the malignant phenotype from tumour to normal cells (Shih *et al.*, 1979 and 1981; Cooper *et al.*, 1980; Krontiris and Cooper, 1981; Perucho *et al.*, 1981). The human origin of the transforming activity was confirmed by hybridisation of middle-repetitive Alu sequence to DNA from transfectants (Shih *et al.*, 1981, Perucho *et al.*, 1981). Subsequently, viral oncogene probes were used to identify the presence of human H-*ras* in NIH3T3 cells transformed by T24/EJ bladder cell line DNA (Der *et al.*, 1982; Parada *et al.*, 1982; Santos *et al.*, 1982), and the human K-*ras* gene in foci generated by transfection of DNA from the lung carcinoma cell lines A2182 and LX-1, and the colon carcinoma cell line SW480 (Der *et al.*, 1982; Pulciani *et al.*, 1982b; McCoy *et al.*, 1983). That *ras* activation was not only a feature of tumour cell lines was demonstrated by the detection of *ras* oncogenes in foci transformed with DNA from primary tumour samples (Pulciani *et al.*, 1982a).

Of the 10-20% of human malignancies that possess transforming activity in the NIH3T3 assay, the transforming gene in 90% of these is a member of the *ras* family (Balmain, 1985; Barbacid, 1986). In addition, more sensitive detection methods have revealed that the NIH3T3 transfection assay can underestimate the level of *ras* activation in particular tumour types (section 1.3.2; Bos, 1989). Thus in certain cancers, the proportion of tumours with *ras* mutations is as high as 90% (Bos, 1989).

Other transforming genes, which like K- and H-*ras*, have retroviral counterparts include *ros* , *raf*-1, *mos*, *src*, *fes*, and *fos* (Barbacid, 1986). However, several other transforming genes including *dbl*, *hst*, *mas*, *met*, *neu*, *ret* and *trk* do not have viral homologues (Der, 1987). Of all these genes only *ras*, *ret*, *hst* and *neu* have been repeatedly detected in the NIH3T3 transformation assay. The *neu* gene is frequently activated in chemically induced rodent schwannomas (Schechter *et al.*, 1984). The *hst* gene can be activated by MMTV integration (section 1.2.1.2) but was originally identified as a transforming gene in a human stomach carcinoma (Sakamoto *et al.*, 1986). Its role in tumorigenesis is uncertain though since, apart from MMTV related tumours (Peters *et al.*, 1989), there has been no evidence that it

is expressed in the tumours it has been isolated from (Delli Bovi and Basilico, 1987; Adelaide *et al.*, 1988; Tsuda *et al.*, 1989). Furthermore, it was also isolated following transformation of NIH3T3 cells by normal gastric mucosa DNA (Sakamoto *et al.*, 1986). The role of the *ret* oncogene in human cancer has also been uncertain since its activation frequently occurs during transfection. However, recently it has been demonstrated that what was thought to be a novel oncogene in thyroid papillary carcinomas (*PTC*), actually consists of the N-terminal region of an unknown protein fused to the tyrosine kinase domain of the *ret* proto-oncogene (Grieco *et al.*, 1990). The significance of many of the other transforming genes listed above to tumorigenesis remains uncertain because of their limited association with neoplasia.

1.2.1.4 Other methods for detecting cellular oncogenes

Genomic amplification is one mechanism of oncogene activation (section 1.2.2.2; Alitalo and Schwab, 1986). Attempts have therefore been made to isolate new oncogenes from amplified sequences by repeated denaturation and renaturation of tumour DNA. This approach, which results in preferential reassociation of tumour DNA, has been used to isolate the *gli* gene, which was amplified and expressed in a malignant glioma (Kinzler *et al.*, 1987).

Oncogene activation can also be associated with chromosome translocations (sections 1.2.2.1 and 1.2.2.2b). The cellular homologues of the retroviral oncogenes *abl*, *ets* and *myc* are commonly involved in these events, but new oncogenes have also been detected in this way. The *bcl*-1 gene was identified by cloning of the breakpoint on chromosome 11 in a B-cell chronic lymphocytic leukemia (CLL) in which a t(11;14) (q13;q32) translocation was observed (Tsujimoto *et al.*, 1984b). Similarly, *bcl*-2 was identified at the breakpoint on chromosome 18 in a follicular lymphoma which contained a t(14:18) (q32;q21) translocation (Tsujimoto *et al.*, 1984a; Tsujimoto and Croce, 1986).

1.2.2 Oncogene activation

Proto-oncogene activation requires structural alteration (a qualitative change) and/or elevated expression (a quantitative change). Frequently, qualitative changes involve point mutations, but larger scale events such as truncation and gene fusion have also been observed. Transcriptional effects can occur by a number of mechanisms including gene amplification, chromosome translocation, insertional mutagenesis, mutation of existing regulatory sequences and/or epigenetic events. These alterations are presumed to result in the provision of an amplified or inappropriate signal from the oncogene product, thus deregulating normal cell growth and differentiation.

Weinberg (1985) has discussed an intriguing link between the function of oncogenes and the mechanisms by which they are activated. In most cases activation of cytoplasmic oncogenes occurs by a structural change, whereas nuclear oncogenes tend to be activated by increased expression. Accumulated data also places the secreted (growth factor) oncogenes in the latter category. Weinberg (1985) has suggested that this may relate to the level at which the functions of these oncogenes are regulated. Cytoplasmic oncogenes (eg *ras*) are constitutively expressed and appear to be regulated by continual cycles of activation and inactivation at the protein level. Constitutive activation therefore requires some structural change in order to bypass inhibitory conformations or signals. However, the expression of nuclear oncogenes (eg *fos* and *myc*) is tightly regulated and these proteins may be constitutively active once synthesised. Thus, a continuous signal from these gene products requires deregulation of their transcription.

The mechanisms of oncogene-activation are discussed below. Although there is much to support the above theory, several exceptions have also been documented, implying that the mechanisms of oncogene-induced neoplasia are highly complex.

1.2.2.1 Oncogene activation by structural alteration

a) The *ras* family

The alterations responsible for the activation of *ras* genes are now well documented (Barbacid, 1987; Bos 1989). The activating mutation of the c-H-*ras* gene of the T24/EJ bladder carcinoma cell line was first to be determined. It was found to be a single nucleotide substitution (G->T) giving rise to a p21 protein with a val rather than a gly at codon 12 and a reduced electrophoretic mobility (Tabin *et al.*, 1982; Reddy *et al.*, 1982; Taparowsky *et al.*, 1982). Since then, activated *ras* genes have been detected in several naturally occurring and chemically induced tumours which have mutations in codons 12, 13 and 61 (Guerrero and Pellicer, 1987; Barbacid, 1987; Balmain and Brown; 1988; Bos, 1989). In addition, an activating codon 59 mutation has been detected in v-*ras* (Dhar *et al.*, 1982; Tsuchida *et al.*, 1982) but never in a cellular *ras* gene.

In vitro mutagenesis studies have extended the list of activating mutations to include codons 63 (Fasano *et al.*, 1984), 116 (Walter *et al.*, 1986), 117 (Der *et al.*, 1988) and 119 (Sigal *et al.*, 1986b). These mutations have never been detected in human tumours, but codon 117 mutations have been observed in H-*ras* genes isolated from furan and furfural-induced liver tumours of B6C3F1 mice (Reynolds *et al.*, 1987).

In vitro mutagenesis studies have shown that substitution of Gly12 by any other amino acid (except for proline) (Seeberg *et al.*, 1984) and Gln 61 by any other amino acid (except Pro, Glu and Gly) (Der *et al.*, 1986) confers transforming properties on *ras* genes. Mutations at codon 13 have not been comprehensively studied, but all substitutions analysed activate the gene, although to a lesser extent than mutations at codons 12 and 61 (Fasano *et al.*, 1984; Bos *et al.*, 1985).

b) The Protein Kinase family

The first oncogene to be characterised was the v-*src* gene of Rous sarcoma virus (RSV). It was shown to be a kinase almost a decade ago (Collett and Erikson,

1978, Levinson *et al.*, 1978) and two years later it was found that the kinase activity was specific for tyrosine residues. Since then many other v-oncs and their cellular counterparts have been shown to be protein tyrosine kinases (PTKs) (Hunter and Cooper, 1985) and others are known to be serine/threonine kinases (see Figure 1). The activation of these oncogenes is, in the simplest model, considered to increase the phosphorylation of their substrates through increased catalytic activity. Other models include increased protein stability, recognition of new subtrates and stimulation by new signals (Cooper, 1985).

The PTKs are activated by diverse structural alterations. These include point mutation, C- and N-terminal truncation and gene fusion.

Several PTK oncogenes lack C-terminal sequences. These deletions remove tyrosine residues whose phosphorylation appears to regulate kinase activity. For example, the regulatory tyr 527 residue present in $pp60^{c\text{-}src}$ is absent in $pp60^{v\text{-}src}$. Dephosphorylation of this residue (Cooper and King 1986), or substitution for phenylalanine (Kmiecik and Shalloway, 1987) activates the tyrosine kinase activity of c-*src* to levels similar to that of v-*src*. Similar deletions in the plasma membrane bound v-*fgr* and v-*yes* PTKs (Hunter, 1987) and in the growth factor receptor (GFR) PTKs v-*erb*-B (Ullrich *et al.*, 1984), v-*fms* (Coussens *et al.*, 1986), v-*kit* (Yarden *et al.*, 1987) and v-ros (Podell and Sefton, 1987) have removed a conserved tyrosine present in the cellular versions of these oncogenes.

In addition to C-terminal deletions, point mutations are also known to play a role in the generation and enhancement of the *src* gene transforming potential (Levy *et al.*, 1986). An analogous situation has been suggested for the *fms* (Roussel *et al.*, 1987), and *kit* genes (Yarden *et al.*, 1987). Point mutations that activate c-*src* (and are present in one or more forms of v-*src*) fall within the kinase domain, thereby increasing catalytic activity directly, or in the N-terminal half thereby debilitating an inhibitory region of the protein (Hunter, 1987). The rat *neu* gene is an example of a GFR PTK which is activated by a single point mutation (Bargmann *et al.*, 1986).

This results in the substitution of a glutamate residue for valine in the transmembrane domain of *neu* (Bargmann *et al.*, 1986).

Deletion of part of the cellular sequences during transduction of viral PTKs is not confined to the C-terminal domains of these proteins. The GFR PTKs v-*erb*-B (Ullrich *et al.*, 1984) v-*kit* (Yarden *et al.*, 1987) and v-ros (and an activated c-ros isolated from a human mammary carcinoma cell line) (Birchmeier *et al.*, 1986) have N-terminal deletions which eliminate most or all of the ligand binding domains and may lead to deregulation and possibly constitutive activation of the tyrosine kinase activity. In v-*fms* the extracellular domain is intact and cell transformation may depend on autocrine stimulation (Roussel *et al.*, 1984).

The activation of a PTK gene by gene fusion is best characterised by the involvement of c-*abl* in chronic myelogenous leukemia (CML). Over 90% of CML patients possess the Philadelphia chromosome. This arises from a translocation between chromosomes 9 and 22, which places the c-*abl* gene (normally at 9q34) within a 5.8kb region at chromosome 22q11, known as the breakpoint cluster region (bcr) (Groffen *et al.*, 1984). The breakpoint on chromosome 9 is more variable and can occur almost anywhere within the first intron of c-*abl*, which is at least 200kb long (Bernards *et al.*, 1987). The translocation results in the synthesis of a fusion product consisting of 5' bcr sequence and 3' *abl* sequence (Shtivelman *et al.*, 1985). This resembles the v-*abl* oncogene in which the N-terminal end of the protein is replaced by viral sequences (Bishop and Varmus, 1982).

Two other PTKs, *met* and *trk*, are also activated by gene fusion. The *met* gene was isolated from a chemically transformed human cell line, MNNG-HOS (Cooper *et al.*, 1984). It was subsequently shown to be related to the PTK family (Dean *et al.*, 1985). Park *et al.* (1986) then demonstrated that its activation involved fusion of a 3' portion of *met* on chromosome 7 with the 5' region of a chromosome 1 locus termed tpr (translocated promoter region). The MNNG-HOS cell line was obtained by prolonged treatment of a human osteosarcoma cell line with MNNG, suggesting that

the clastogenic, rather than mutational effect of MNNG (Perry and Evans, 1975) was responsible for *met* activation.

The *trk* gene (originally named oncD) was isolated by transformation of NIH3T3 cells with colon carcinoma DNA (Pulciani *et al.*, 1982a). It was derived by replacement of the extracellular domain of a receptor TK by the first 221 amino acids of a non-muscle tropomyosin molecule (Martin-Zanca *et al.*, 1986).

1.2.2.2 Oncogene activation by elevated expression

Oncogenic activation of nuclear genes and growth factor genes frequently involves deregulated expression. For example, activation of the nuclear genes c-*myc* and N-*myc*, and the growth factor genes IL2, IL3 and the *int* series by proviral insertion has already been mentioned (section 1.2.1.2). However, deregulated expression need not involve highly active exogenous promoters. This is exemplified by a specific chromosomal translocation in Burkitt's lymphoma (BL) and mouse plasmacytomas, which place the c-*myc* gene next to a powerful immunoglobulin promoter. Three specific translocations are found in BL. All involve 8q24 with either 14q32 (in most cases), 2p12 or 22q11 (Rabbitts, 1985). These translocations juxtapose *myc* with the Ig heavy chain locus on chromosome 14, the λ light chain locus on chromosome 2, or the κ light chain locus on chromosome 22. The effects of these events can be contrasted with the translocation of the c-*abl* locus in CML (section 1.2.2.1b). In the latter the N-terminal portion of *abl* is replaced by a coding region derived from the bcr locus (Shtivelman *et al.*, 1985). However, translocations detected in BL do not alter the coding region of c-*myc*. Instead it is thought that the loss of the non-coding first exon, and/or the juxtaposition of Ig regulatory sequences are the critical events (Rabbitts, 1985).

One mechanism which can increase the amount of an oncogene product without necessarily altering its own regulatory sequences is gene amplification. Amplification of oncogenes is an occasional feature of some tumours and a

consistent event in others (Alitalo and Schwab, 1986). Amplification of the nuclear oncogenes c-*myc*, c-*myb* and c-*ets*-1 has been observed in a small proportion of various tumours (Alitalo and Schwab, 1986). However, c-*myc* is commonly amplified in small cell lung carcinomas, as are N-*myc* and L-*myc* (Nau *et al.*, 1985). Amplification of N-*myc* has also been observed in 50% of neuroblastomas, an event which has been correlated with advanced stages of the disease (Brodeur *et al.*, 1984).

1.2.2.3 Activation by mechanisms atypical of a class of oncogenes

As mentioned above there are examples which demonstrate that activation of cytoplasmic and nuclear oncogenes is not restricted to qualitative and quantitative events respectively. In particular there are many examples implicating elevated expression in the activation of cytoplasmic oncogenes. The potential relevance of overexpression of cytoplasmic oncogenes is implied from the large number of these genes which have been transduced by retroviruses, in which they are under the control of highly active LTR sequences. Overexpression of mutated *ras* genes is known to potentiate their transforming powers (section 1.3.4.2), and placing a normal *ras* gene under the control of powerful enhancer elements can give it transforming properties (Chang *et al.*, 1982; McKay *et al.*, 1986). The *mos* PTK oncogene also aquires strong oncogenic powers when linked to a constitutive promoter (Blair *et al.*, 1986). The amplification of oncogenes such as *ras*, *neu* and *erb*B in some tumours is further evidence that enhanced stimulation of the pathways these proteins normally activate and/or other pathways might aid tumour progression. Amplification of *neu* and *erb*B genes is particularly significant because of the high frequency of these events in breast carcinoma (Slamon *et al.*, 1987) and squamous cell carcinomas and brain tumours (Alitalo and Schwab, 1986; Libermann *et al.*, 1985) respectively. It has also been shown that overexpression of normal c-*neu* results in the malignant transformation of NIH3T3 cells (Paolo di fiore *et al.*, 1987).

There is less evidence for the role of qualitative changes in nuclear oncogene activation. Although viral oncogene products are frequently fused to viral proteins (Bishop and Varmus, 1982) the relevance of this to malignancy is not clear. For example, the gag moiety of $p75^{gag\text{-}fos}$ does not appear to influence transforming potential (Miller *et al.*, 1984). The function of the point mutations in v-*myc* is also not clear (Bishop and Varmus, 1982). One nuclear oncogene which is definitely activated by point mutations is the p53 gene. However, this gene may be unique in that it can be classified as both an oncogene and a tumour suppressor gene (section 1.4.5b).

1.2.3 Cooperation of oncogenes

The discovery that certain tumour-derived oncogenes could transform NIH 3T3 cells gave rise to speculation that cancer might be caused by activation of a single oncogene (Parada *et al.*, 1982). This contradicts the evidence suggesting that cancer is a multistep process and has been repeatedly rejected on the grounds that NIH3T3 cells, although non tumo rigenic, are immortal (therefore partially transformed) aneuploid cells and may require only one more genetic change for conversion to malignancy. Additionally, genes introduced into cells may integrate in multiple copies so that even in a partially transformed cell an oncogene may induce transformation only if it is present in elevated amounts (Sager, 1986). Furthermore, fusion of normal fibroblasts and tumo rigenic fibroblasts, transformed by the introduction of an H-*ras* gene, resulted in suppression of the tumourigenic phenotype implying that oncogene transformation is dependent on the loss of certain cellular functions (Craig and Sager, 1985).

A more realistic model involves the cooperative action of at least two oncogenes in the malignant transformation of a cell (Land *et al.*, 1983a and b). This hypothesis was based on studies with early passage rat embryo fibroblasts in which it was shown that although transfection with *ras* induces anchorage independence

(ability to grow in soft agar), malignant transformation requires transfection with a second oncogene (Land *et al.*, 1983b). This, and other studies, led to the proposal that malignant transformation by cytoplamic oncogenes, of which *ras* is the paridigm, is dependent on the complementing action of a nuclear oncogene, such as *myc*, *myb*, p53, *ski*, *fos*, SV-40 large T, polyoma large T or adenovirus E1A (Weinberg, 1985). One of the main effects of oncogene cooperation is that it allows a cell to overcome the inhibitory influence of surrounding normal cells (Land *et al.*, 1986). *In vivo* this event is crucial, since benign expansion of an "initiated" cell dramatically increases the probability that further events, necessary for malignant growth, will occur.

That oncogene cooperation is not merely an *in vitro* phenomenon has been endorsed by the detection of multiple activated oncogenes in single tumours (Murray *et al.*, 1983; Taya *et al.*, 1984; Suarez *et al.*, 1987). However, the list of examples is not long, and has prompted re-evaluation of the oncogene cooperation model (Weinberg, 1989). In this, the events which cooperate with *ras* activation are mutations in the tumour suppressor genes (section 1.4) that negatively regulate the synthesis and/or function of *myc*-like genes.

Analyses involving transgenic mice have provided direct evidence that oncogene cooperation is possible *in vivo*. For example, transgenic mice expressing both a *ras* and a *myc* oncogene under the control of the MMTV LTR show a higher incidence of mammary carcinomas than mice bearing the *ras* oncogene alone (Sinn *et al.*, 1987). Similar observations were made for the expression of *myc* and *ras* in other cellular compartments such as B cells and the pancreas (Quaife *et al.*, 1987).

Other studies have attempted to identify cooperating oncogenes by infecting transgenic mice with a retrovirus, on the basis that a second oncogene might be activated by insertional mutagenesis. Bern's group have used this approach to show that in T-cell lymphomas induced by MuLV in *pim*-1 transgenics the c-*myc* and N-*myc* genes are consistently activated by proviral insertion (van Lohuizen *et al.*, 1989). Similarly, *pim*-1 activation by MuLV integration was detected in pre-B cell

lymphomas obtained by infecting c-*myc* transgenics with MuLV (Berns *et al.*, 1989). These experiments not only demonstrate the relevance of oncogene cooperation to malignancy, but also that changing the order of oncogene activation alters the affected target cell population.

In some experiments involving transgenic mice, expression of certain oncogenes appears to induce polyclonal tumours in a single step manner (Quaife *et al.*, 1987; Bautch *et al.*, 1987; Williams *et al.*, 1988; Muller *et al.*, 1988). However, the powerful promoter sequences used in these analyses, and expression of the oncogene in every cell of the affected tissue (thereby overcoming the inhibitory effect of normal cells) probably potentiates tumour progression. Nevertheless, the sensitivity of some tissues to transformation by a single, highly expressed oncogene is intriguing.

In most cases, transgenes induce tumours after a long latency period which are monoclonal in nature (Stewart *et al.*, 1984; Adams *et al.*, 1985; Lacey *et al.*, 1986; Sinn *et al.*, 1987; Schonenberger *et al.*, 1988; van Lohuizen *et al.*, 1989). This suggests that additional changes are required for malignant growth. This almost certainly also applies to the malignant transformation of early passage fibroblasts by two oncogenes. For instance, the malignant conversion of primary Syrian hamster cells transfected with *ras* and *myc* oncogenes is consistently associated with the loss of a single copy of chromosome 15 (Oshimura *et al.*, 1985). A substantial body of evidence has accumulated suggesting that these additional changes involve the inactivation of tumour suppressor loci (section 1.4).

1.3 The *ras* gene family

In view of the important role oncogenic activation of H-*ras* plays in mouse skin tumorigenesis (section 1.5) it is appropriate that the biochemical and cellular functions of this gene and its close relatives should be reviewed in more depth.

1.3.1 Structural features

Three functional mammalian *ras* genes have been identified: H, K and N-*ras*. Several groups have cloned these genes from a variety of species, and determined their nucleotide sequence and chromosomal location (Barbacid, 1987). The coding sequences are derived from 4 exons and express closely related proteins of M_R 21000 known as p21. The K-*ras* gene has two alternative fourth coding exons (IVA and IVB) that allow synthesis of proteins 188 and 189 amino acids long that differ in their C-terminal residues (McGrath *et al.*, 1983; Capon *et al.*, 1983).

The promoter region of cellular *ras* genes resemble so-called housekeeping genes, in that they lack a TATA box but possess multiple copies of the sequence GGGCGG or its complement; the SP1 binding site originally identified in the SV40 promoter region (Dynan, 1986).

The amino acid sequences of *ras* proteins are very similar. In fact, the first 85 amino acids are identical and there is only a slight divergence (85% identity) in the next 80 residues. This is followed by a short heterologous region, but the homology returns in the last 4 residues where the sequence Cys-A-A-X-COOH (where A is an aliphatic amino acid) is present in all mammalian *ras* genes (Barbacid 1987). This pattern of domains is mirrored in *ras* genes of low eukaryotes, although the sequence identity is naturally less. Conservation of *ras* genes across a wide range of species suggests they have an important cellular function. They have been identified in species as distant from mammals as molluscs (*Aplysia*: Apl-*ras*) (Swanson *et al.*, 1986); slime moulds (*Dictyostelium discoideum*: Dd*ras*) (Reymond *et al.*, 1984) and yeasts (*Saccharomyces cerevisiae*: *RAS*1 and *RAS*2; *Schizosaccharomyces pombe*: *SPRAS*) (Defeo-Jones *et al.*, 1983; Powers *et al.*, 1984; Fukui and Kaziro, 1985). In addition several *ras*-related genes have been identified which are around 30-50% homologous to *ras* proteins (Barbacid, 1987).

1.3.2 Incidence of activated *ras* genes

Of the range of point mutations known to confer transforming activity on *ras* genes only codon 12, 13 and 61 mutations have been observed in human tumours (section 1.2.2.1; Bos, 1988 and 1989). Although *ras* mutations have been detected in a wide variety of malignancies, the frequency among different tumour types is extremely variable (Bos, 1989). For example, almost all pancreatic adenocarcinomas (malignant tumours of the exocrine pancreas) harbour a mutated K-*ras* gene (Almoguera *et al.*, 1988; Smit *et al.*, 1988; Grunewald *et al.*, 1989) and the incidence of mutations in this gene is also high in colon adenocarcinomas (40-50%) (Bos *et al.*, 1987; Forrester *et al.*, 1987; Vogelstein *et al.*, 1988) and lung adenocarcinomas (20-30%) (Rodenhuis *et al.*, 1987 and 1988). In addition, several studies have detected a high incidence of *ras* gene mutations in certain thyroid carcinomas (50%), myeloid neoplasias (50%) and seminomas (40%) (Bos, 1989; Mulder *et al.*, 1989). However, in several other tumour types, such as breast and ovary cancer, the incidence is very low or even zero (Bos, 1989). The reason for the high incidence of *ras* mutations in certain tumours and their absence in others may relate to the tissue distribution of carcinogens and/or the sensitivity of individual tissues to *ras*-induced transformation. For example, transgenic mouse studies have revealed that the pancreas is particularly sensitive to *ras*-induced neoplasia, whereas tumours arising in mammary (Sinn *et al.*, 1987) or lung tissue (Suda *et al.*, 1987) occur after a long latency period.

The causal nature of *ras* activation, and the activation of other oncogenes in neoplasia has been questioned on several occasions (Rubin, 1984; Barbacid 1986; Paul, 1987). The detection of *ras* mutations in animal tumours, which are coincident with the known activity of the carcinogens used to induce these tumours, suggests that this oncogene does participate in neoplasia (section 1.5.2.1). Direct evidence for the causal role of *ras* (and other) oncogenes in tumorigenesis has been provided by studies using transgenic mice (c.f section 1.2.3). For instance, transgenic mice

carrying a mutated H-*ras* gene regulated by the pancreas-specific elastase I promoter develop neoplasia of the fetal pancreas directly after onset of elastase expression (Quiafe *et al.*, 1987). In addition, an H-*ras* transgene regulated by either the SV40 early gene promoter or the Ig enhancer was expressed predominantly in the lung and resulted in the development of adenomatous tumours in this tissue (Suda *et al.*, 1987). These results suggest that *ras* mutations detected in human pancreas and lung (see above; Bos, 1989) are causal. Equivalents of all the human tumours in which *ras* mutations are frequently detected have not been observed in transgenic mice. This may require the use of appropriate transcriptional promoters. Alternatively, cross-species comparisons may be inappropriate in some cases. For example, N-*ras* and K-*ras* activation is common in both human and rodent haematopoetic neoplasia, but although H-*ras* mutations are frequently detected in chemically induced rat (Zarbl *et al.*, 1985) and mouse (Dandekar *et al.*, 1986) breast carcinomas, *ras* activation is rare in the analogous human condition (Bos, 1989). As with tissue specificity, the reasons for species specificity are unknown. Differences in carcinogen metabolism, gene sequence, function and expression are some possibilities.

A third type of specificity concerns the activation of a particular member of the *ras* family in certain tumours. The association between K-*ras* mutations and colon, lung and pancreatic tumours was mentioned above. In addition, activated N-*ras* genes predominate in myeloid neoplasias (Bos, 1989) and melanomas (Van't Veer *et al.*, 1989; Albino *et al.*, 1989). In animal model systems only H-*ras* mutations have been observed in rodent skin and breast tumours, and in mouse liver tumours it is also H-*ras* mutations which predominate (Balmain and Brown, 1988; Balmain *et al.*, 1990). One explanation for this is that the three *ras* genes have separate functions. However, the putative effector domain is identical in all three proteins (Sigal *et al.*, 1986a), suggesting that activation of any one of these genes would have the same effect in all cells. The ability of the H-*ras* oncogene to induce tumours in transgenic mice resembling neoplasias associated with K-*ras* mutations in humans (see above) further supports this conclusion. An alternative explanation is that members of the

ras family are differentially expressed, although expression of all three *ras* genes is detectable in most tissues (Muller *et al.*, 1983; Leon *et al.*, 1987). A systematic comparison of *ras* gene expression has not been carried out in humans. However, a study of adult mouse tissues (Leon *et al.*, 1987) showed that in most tissues the levels of H-, K- and N-*ras* expression are dissimilar. In the case of the thymus and skin high expression of N- and H-*ras* respectively is consistent with the preferential activation of these genes in tumours derived from these tissues (Guerrero and Pellicer, 1987; Balmain and Brown, 1988). However, in other cases (e.g lung and liver) there was no correlation between expression and mutation patterns. Thus, other explanations for preferential *ras* activation, such as the effects of DNA sequence on mutational spectra (Topal *et al.*, 1986; Burns *et al.*, 1987), may be applicable in some cases.

1.3.3 Biochemical properties and the effect of activating mutations

The homology between *ras* and G proteins (Hanley and Jackson, 1987), and their association with the plasma membrane (Willingham *et al.*, 1980; Willumsen *et al.*, 1984) suggests that they are involved in the transduction of signals across the cell membrane. The analogy with G proteins is extended by the observation that *ras* proteins bind guanine nucleotides (Scolnick *et al.*, 1979; Shih *et al.*, 1980) and have GTPase activity (McGrath *et al.*, 1984; Sweet *et al.*, 1984). The latter studies also demonstrated that activated forms of *ras* proteins have decreased GTPase activity, and gave rise to the current model of *ras* activation (Barbacid, 1987). In this, normal *ras* proteins cycle between inactive GDP-bound and active GTP-bound states, but because activated *ras* proteins do not hydrolyse GTP efficiently they remain in an active state. One difficulty with this was that although the majority of *ras* oncogenes were shown to have decreased GTPase activity, exceptions were noted (Colby *et al.*, 1986; Der *et al.*, 1986; Lacal *et al.*, 1986). An explanation for these discrepancies has been suggested by Trahey and McCormick (1987). They found that the *in vitro*

GTPase activity of normal p21 was similar to that of two transforming proteins, derived from genes with codon 12 mutations. However, when comparisons were made using an *in vivo* assay (hydrolysis of labelled GTP in xenopus oocytes) the GTPase activity of normal p21 was over two orders higher than that of the mutant forms. They ascribed this increase to the presence of an *in vivo* factor which was absent from the *in vitro* assay. The factor has been termed GAP: GTPase activating protein.

Recently it has been suggested that GAP may be an effector molecule downstream of *ras*, since mutations in the *ras* effector domain (Cales *et al.*, 1988) and antibodies which bind to this domain (Rey *et al.*, 1989) inhibit p21 and GAP interaction. That GAP is a candidate effector does not exclude the possibility that other molecules interact with *ras*. Recently de Gunzburg *et al.* (1989) have used cross-linking to identify a protein of M_R 60000 whose association with *ras* is potentiated by serum stimulation.

Regulation of *ras* activity may not be a function of GTPase activity alone. Although membrane binding was originally considered to be a straightforward event, it is now known to be a more complex process. It is thought that removal of three residues from the Cys186-A-A-X C-terminal sequence is followed by polyisoprenylisation and carboxymethylation of the Cys186 residue (Hancock *et al.*, 1989). This allows weak association of p21 with cell membranes, an affinity which is markedly increased following palmitoylation (Hancock *et al.*, 1989). Palmitoylation occurs on cys residues close to cys184, but only after polyisoprenylation of cys186, thus explaining why mutation of this residue blocks palmitoylation (Willumsen *et al.*, 1984). Reversible palmitoylation (Magee *et al.*, 1987) may serve to regulate *ras* activity. Recently it has been shown that irreversible myristylation of normal p21*ras* activates the transforming properties of this protein (Buss *et al.*, 1989).

1.3.4 Characteristics of transformation by *ras*

1.3.4.1 Timing of *ras* activation

As discussed in section 1.5.2.1, there is convincing evidence that *ras* activation occurs at initiation in certain chemically induced animal tumours. However, in some animal tumours *ras* activation appears to be a late event (Vousden and Marshall, 1984; Diamond *et al.*, 1988). A role for *ras* oncogenes in both the early and late stages of malignancy also seems to apply to human tumours. The detection of *ras* mutations in benign lesions such as keratoacanthomas of the skin (Leon *et al.*, 1988), adenomatous polyps of the colon (Bos *et al.*, 1987; Forrester *et al.*, 1987) and in myelodysplastic syndrome (preleukemia) (Janssen *et al.*, 1987b; Lui *et al.*, 1987; Hiria *et al.*, 1987; Browett and Norton, 1989) is evidence that *ras* activation can be an early, possibly initiating event in human tumorigenesis. However, the simultaneous presence of two different *ras* mutations in two colon tumours (Forrester *et al.*, 1987) and in five cases of acute myeloid leukemia (Janssen *et al.*, 1987a; Farr *et al.*, 1988) suggests that it can also occur after initiation. In addition although a high percentage of seminomas possess activated *ras*, in some only a fraction of the tumour cells contain the oncogene, suggesting that *ras* mutation occurs after initiation in these cases (Mulder *et al.*, 1989). Similarly, although *ras* mutations are detectable in a subset of melanomas, this oncogene does not appear to be involved in the premalignant stage of this tumour (Albino *et al.*, 1989). Finally, the claim that mutant *ras* can confer metastatic properties on various cell lines (Bernstein and Weinberg, 1985; Waghorne *et al.*, 1987; Collard *et al.*, 1987) further supports the idea that this oncogene affects many stages of malignancy.

1.3.4.2 Levels of *ras* expression during tumour progression

Several experiments involving the transfection of cultured cells with *ras* oncogenes have provided evidence that elevated expression levels affect its transforming potency. For example, the tumorigenicity of Rat 4 cells transfected with a K-*ras* oncogene is greater in clones which have more copies of the oncogene (Winter and Perucho, 1986), and the levels of p21*ras* correlate with the extent of morphological transformation and the degree of DNA synthesis in other fibroblast cell lines (McKay *et al.*, 1986; Reynolds *et al.*, 1987). In addition, the *ras*-induced growth factor independence of MCF-7, an estrogen dependent cell line (Kasid *et al.*, 1987), and PB-3c, an immortalised IL3-independent mouse mast cell line (Andrejauskas and Moroni, 1989) is dependent on high expression of the oncogene. Furthermore, transformation of early passage rodent fibroblasts by mutant *ras* is only possible if it is regulated by a powerful transcriptional enhancer (Spandidos and Wilkie, 1984). Thus, elevated expression of a single oncogene appears to substitute for the activation of a second cooperating oncogene (section 1.2.3; Land *et al.*, 1983a). A potential role for elevated *ras* oncogene expression is also supported by the observation that when *ras* is used to induce metastasis of some cell lines the metastatic potential is greatest in cells expressing high levels of the oncogene (Egan *et al.*, 1987 and 1989). However, this was not true of an adenocarcinoma cell line (Waghorne *et al.*, 1987).

Very little data is available on mutant *ras* expression in human and animal tumours. Forrester *et al.* (1987) used RNase mismatch analysis to locate *ras* mutations in colorectal cancer and to determine the levels of mutant:normal gene expression; they found no difference in the expression of these alleles. However, analysis of some tumour cell lines suggests that the levels of mutant *ras* transcripts may be important. For instance, Cohen and Levinson (1988) have identified a point mutation in the final intron of the H-*ras* oncogene of the T24/EJ bladder carcinoma cell line which enhances both expression and transforming activity of the gene. This

mutation abolishes the synthesis of an alternative transcript, and releases the mutant allele from this putative negative control (Cohen *et al.*, 1989). Preferential expression of the mutated *ras* allele has also been observed in the lung carcinoma cell line, Calu-1 (Capon *et al.*, 1983). This, taken together with studies which have detected amplification of mutant *ras* genes during tumour progression (McCoy *et al.*, 1983; Taya *et al.*, 1984; Winter *et al.*, 1985; Alitalo and Schwab, 1986; Quintanilla *et al.*, 1986) suggests that the effects of elevated mutant *ras* gene expression observed *in vitro* (see above) may have relevance to the development of some tumours *in vivo*. A comprehensive analysis of primary tumour material, using the RNase mismatch assay (Forrester *et al.*, 1987), is important in the proper assessment of this claim.

Most of the reports on levels of *ras* expression in various tumours have not used approaches which distinguish between mutant and normal *ras* transcripts/proteins. Consequently, interpretation of increased expression is limited. Some authors have found that elevated *ras* expression is a common feature of malignancy (Slamon *et al.*, 1984; Gallick *et al.*, 1985; De Biasi *et al.*, 1989), others that it is not (Chesa *et al.*, 1987). Detection of high levels of *ras* expression in tumours which do not commonly contain *ras* mutations may reflect non-specific deregulation. However, since normal *ras* genes controlled by powerful transcriptional enhancers can transform established cell lines (Chang *et al.*, 1982; Pulciani *et al.*, 1985; McKay *et al.*, 1986) a potential role for the wild type allele in tumorigenesis should not be discounted. Significantly, Westaway *et al.* (1986) have identified a provirally activated c-H-*ras* gene in a chicken nephroblastoma, suggesting that overexpression of normal *ras* can contribute to tumorigenesis *in vivo*.

1.3.4.3 Biological aspects of *ras* transformation

The link between *ras* activation and neoplastic transformation suggests that this family of proteins may positively regulate cell growth. Indeed, microinjection of mutant p21 proteins into NIH3T3 fibroblasts induces transient morphological

transformation and proliferation (Stacey and Kung, 1984; Feramisco *et al.*, 1984) and microinjection of antibodies against p21*ras* proteins can temporarily revert the neoplastic phenotype of *ras* transformed rodent cells (Feramisco *et al.*, 1985). More specifically, it has been demonstrated that antibody binding prevents quiescent NIH3T3 fibroblasts entering S phase (Mulcahy *et al.*, 1985).

There is also evidence that in some cell types *ras* may promote transformation by inhibiting differentiation. For example, the normal differentiation programme of skeletal myoblasts (Olsen *et al.*, 1987) and mouse keratinocytes (Yuspa *et al.*, 1983 and 1985) is blocked by the introduction of *ras* oncogenes into these cell types.

ras function is not exclusively associated with stimulating proliferation and/or blocking differentiation. For example, nerve growth factor (NGF)-induced differentiation of PC12 pheochromocytoma cells can be mimicked by microinjection of p21*ras* protein (Noda *et al.*, 1985; Bar-Sagi and Feramisco, 1985). The high amounts of p21*ras* expressed in brain tissue (Furth *et al.*, 1987; Chesa *et al.*, 1987) further supports a role for this protein in neural differentiation, and offers an explanation for the lack of association between *ras* activation and tumours of neuroectodermal origin (Bos, 1989). Expression studies have in fact shown that *ras* is expressed in both proliferating and differentiated cell types (Furth *et al.*, 1987; Chesa *et al.*, 1987) suggesting that this proto-oncogene can interact with a diverse range of intracellular signalling pathways.

1.3.4.4 Interactions between *ras* and cellular signalling pathways

Some of the biochemical properties of the *ras* gene family suggest that they encode G protein which transduce growth factor signals across the cellular membrane (section 1.3.3). Several growth factor receptors may interact with, or indirectly require *ras* function. For example, micro-injection of the *ras*-neutralising antibody Y13-259 blocks the induction of DNA synthesis by PDGF and EGF (Mulcahy *et al.*, 1985; Yu *et al.*, 1988). Insulin-induced Xenopus oocyte maturation

is also dependent on p21*ras* function (Korn *et al.*, 1987) and overexpression of H-*ras* in rat-1 cells increases their sensitivity to insulin, insulin like growth factor 1 (IGF-1) and bombesin (Burgering *et al.*, 1989). Similarly, the sensitivity of NIH3T3 cells to bombesin is enhanced by over expression of N-*ras* (Wakelam *et al.*, 1986). The interaction of p21 with the insulin and epidermal growth factor receptors may be direct since there is evidence to suggest that both these proteins phosphorylate *ras* (Korn *et al.*, 1987; Fujita-Yamaguchi *et al.*, 1989; Kamata and Feramisco, 1984).

The biological events which are downstream of *ras* are discussed in section 1.3.4.3. Several biochemical changes can accompany these events. These include increased synthesis of glucose transporter protein (Flier *et al.*, 1987), protease secretion (Joseph *et al.*, 1987), altered gene expression (Jaggi *et al.*, 1986; Sistonen *et al.*, 1989) and the secretion of growth factors such as TGF-α (De Larco and Todaro, 1978; Anzano *et al.*, 1985), PDGF (Bowen-Pope *et al.*, 1984), and IL3 (Andrejauskas and Moroni, 1989). The molecular links between *ras* activation and these complex effects are poorly understood. Hopes that analysis of simple eukaryotes like yeast might help identify mammalian effectors have not been fulfilled. The yeast *S. cerevisiae* has two *RAS* genes, *RAS*1 and *RAS*2. Mutants lacking either one of these genes are viable, but *ras*1$^-$*ras*2$^-$ mutants are not (Tatchell *et al.*, 1984). The functional relationship between yeast and mammalian *ras* proteins has been established by experiments in which the yeast proteins have been shown to complement mammalian *ras* function and vice-versa (DeFeo-Jones *et al.*, 1985; Papageorge *et al.*, 1985). However, while yeast *RAS* proteins are mainly involved in the activation of adenylate cyclase (Toda *et al.*, 1985) this is not their function in mammalian cells (Beckner *et al.*, 1985). Despite this disappointment, yeast studies may yet uncover mammalian *ras* effectors, since there is evidence that *RAS* proteins have functions other than activation of adenylate cyclase (Michaeli *et al.*, 1989). Another yeast, *S. pombe*, may provide additional clues since the single *RAS* gene found in this species (*SPRAS*) does not interact with adenylate cyclase (Russell and Nurse, 1986).

One of the candidate intracellular pathways which mammalian *ras* proteins may influence is the phosphoinositide system. This pathway regulates several processes including metabolism, secretion, neural activity and cell proliferation (Berridge and Irvine, 1989). It involves transduction of a variety of signals from the cell surface, via a G protein, to the enzyme phospholipase C (PLC). This enzyme hydrolyses phosphatidyl inositol 4,5-bisphosphate (PIP2), thereby releasing the second messengers inositol 1,4,5 trisphosphate (IP3) and diacylglycerol (DAG). IP3 induces an increase in the level of intracellular Ca2+ (Berridge and Irvine, 1989), while DAG, in conjunction with Ca2+, activates PKC (Nishizuka, 1986 and 1988). Evidence that *ras* may be the G protein linking cellular receptors to PLC was inferred from the observation that *ras*-transformed cells contain elevated levels of IP3 (Fleischman *et al.*, 1986; Hancock *et al.*, 1988). Other experiments suggested that different *ras* proteins couple different receptors to PLC; N-*ras* to the bombesin receptor (Wakelam *et al.*, 1986) and H-*ras* to the PDGF receptor (Marshall, 1987). However, elevated IP3 hydrolysis in these experiments may have been an overspill effect caused by abnormally high amounts of p21 *ras* and/or an indirect effect on PLC activity. In this respect, several groups have noted that mutant p21*ras* induces an increase in DAG in the absence of, or well above, any increase in inositol phosphates (Lacal *et al.*, 1987a and b; Wolfmann and Macara, 1987; Seuwen *et al.*, 1988; Morris *et al.*, 1989). It has therefore been suggested that *ras* may mediate the breakdown of other phospholipids, such as phosphatidylcholine (PC), phosphatidyl ethanolamine (Lacal *et al.*, 1987a), or the substrates of phospholipase A2 (Bar-Sagi and Feramisco, 1986). However, *ras*-induced PC hydrolysis and elevation in DAG levels is blocked if PKC is down regulated by prior treatment with phorbol esters (Price *et al.*, 1989). This suggests that the DAG increase is downstream of PKC and that *ras* activates this enzyme by a novel mechanism.

There is also evidence that *ras* stimulates a PKC-independent pathway. Lloyd *et al.* (1989) have shown that scrape loading mutant H-*ras* into Swiss 3T3 cells induces DNA synthesis in a PKC-dependent manner. However, other events,

including induction of c-*myc* expression and phenotypic transformation, were induced even in the absence of PKC.

1.3.4.5 Interaction of *ras* with other oncogenes

One approach employed to determine the pathways with which *ras* interacts has been to identify oncogenes in the same functional complementation group. Noda's group have studied this by fusing cells transformed by a range of oncogenes with a flat revertant of a KiMSV transformed line (Noda *et al.*, 1983). They found that the revertant line could suppress transformation by v-*src* and v-*fes*, but not v-*mos*, v-*fms* or v-*sis*. Another approach involved micro-injection of *ras* antibodies into cells transformed by different oncogenes to determine those that require functional *ras* for transformation (Smith *et al.*, 1986). It was found that transformation by v-*src*, v-*fes* and v-*fms*, but not v-*mos* and v-*raf* (both cytoplasmic ser/thr kinases) was *ras*-dependent. These two studies present a strong case that *ras*, *src* and *fes* are involved in the same or interconnected pathways. The *fms*/CSF-1 receptor may also be in this category. The fact that the *ras* revertant line did not suppress transformation by this oncogene implies that the aspect of *ras* function required by v-*fms* is not suppressed by these cells.

1.4 Tumour suppressor genes

Given that most genetic mutations are probably deleterious, it is possible that the loss of genetic information may be more important to the development of malignancy than oncogene activation. Although few tumour suppressor genes have been isolated (Friend *et al.*, 1986; Finlay *et al.*, 1989; Kitayama *et al.*, 1989), many more probably exist (Hansen and Cavenee, 1987; Bouck and Benton, 1989).

Evidence for the existence of tumour suppressor genes (also termed anti-oncogenes, emerogenes and onco-suppressors) derives from various observations

(Klein, 1987), of which the analysis of normal x tumour (NxT) somatic cell hybrids was the first (Harris *et al.*, 1969) and actually predates the discovery of cellular oncogenes (Stehelin *et al.*, 1976). The main conclusions reached in studies of this type are summarised below

a) Re-expression of the tumorigenic phenotype (assessed by subcutaneous injection of NxT hybrids into nude mice) **is frequently associated with the loss of a specific chromosome from the hybrid.** Some examples are listed in Table 1. These studies have used both intra- and interspecies hybrids. Intraspecies hybrids have the advantage of stability; chromosome loss is rapid in interspecies hybrids. However, identification of the N and T chromosomes is straightforward in the latter, whereas polymorphic markers are required for this purpose in intraspecies crosses.

b) Suppression may be dosage dependent. In several cell fusion studies (Evans *et al.*, 1982; Benedict *et al.*, 1984; Srivatsan *et al.*, 1986) elimination of only one of the pair of suppressor chromosomes contributed by the normal fibroblasts was sufficient to permit reexpression of tumorigenicity. Harris (1988) has reported that there is further selection *in vivo* in favour of cells that have lost both normal chromosomes. This further supports the idea that tumorigenicity increases with falling levels of the suppressor gene.

It is not possible to establish the exact nature of the tumour suppressor responsible for reversion in TxN crosses. However, one argument holds that it may be the wild-type allele of an activated oncogene. In the study by Evans *et al.* (1982) they not only detected loss of a normal mouse chromosome 4 in retumorigenic hybrids, but sometimes observed an increase in the copy number of the tumour cell chromosome 4. This may reflect competition between an activated oncogene and its normal counterpart. However, it may simply be due to the requirements of the NxT hybrids for a certain copy number of chromosome 4; if tumorigenicity is regained by loss of the normal copy, duplication of the mutant may be needed for genetic stability.

Table 1 **Suppressor chromosomes identified by somatic cell hybridisation.**

	Cells		Suppressor chromosome	Reference
	Normal x	Tumour		
Intra-species hybrids	Mouse fibroblasts	Melanoma Lymphoma Sarcoma Carcinoma	Mouse 4	Evans et al., 1982
	Human fibroblasts	Fibrosarcoma (HT1080)	Human 1,4	Benedict et al., 1984
	Human fibroblasts	Cervical carcinoma (HeLa)	Human 11	Srivatsan et al., 1986
Inter-species hybrids	Human fibroblasts	Malignant hamster cells (BHK)	Human 1	Stoler & Bouck, 1985
	Rat fibroblasts	Mouse hepatoma	Rat 5	Islam et al., 1989
	Rat hepatocytes	"	Rat 8	Szpirer et al., 1988

The association between tumorigenicity and loss of chromosome 1 in HT1080 cells (Benedict *et al.*, 1984) is particularly interesting in this respect. N-*ras* is also located on this chromosome and is activated in this cell line. Recently, Paterson *et al.* (1987) have shown that reversion of HT1080 is associated with a drop in the level of mutant N-*ras*, and that tumorigenicity can be restored by transfection with activated *ras* genes. However, introduction of normal p21*ras* could not suppress the effect of the mutant allele. Thus it appears that the level of mutant *ras* is the sole determinant of tumorigenicity. What then is the significance of the correlation between loss of a copy of chromosome 1 and reexpression of tumorigenicity in HT1080 x normal hybrids? One possibility is that there is a linked suppressor gene on chromosome 1 which suppresses the transforming properties of N-*ras* in a dose-dependent manner. Thus loss of this suppressor and elevated levels of mutant N-*ras* may be essential for tumorigenicity. An alternative explanation is that there is selection in the HT1080 x normal hybrids for three copies of chromosome 4, purely for stability purposes. However, the correlation between loss of the normal human chromosome 1 and reexpression of the transformed phenotype in interspecies crosses (Stoler and Bouck, 1985), and the fact that allele loss on this chromosome is associated with the development of various human tumours (Table 2, section 1.4.3) both suggest that there may be a tumour suppressor gene on this chromosome.

The issue of whether the normal copy of an oncogene may suppress the effect of its activated counterpart is discussed further in section 1.4.5e.

c) A single tumour suppressor gene may be involved in diverse tumour types. For example, mouse chromosome 4 suppresses several different malignancies (Evans *et al.*, 1982) as do human chromosomes 1 and 11 (Tables 1, 2 and see below). This is supported by RFLP analysis (section 1.4.3a) in which diverse tumour types lack alleles from the same chromosome.

In addition, suppression of the transformed phenotype in interspecies crosses suggests that related genes may function as tumour suppressors in different species. Mapping studies of human chromosome 1, mouse chromosome 4 and rat

chromosome 5 have identified conserved linkage groups (Lalley *et al.*, 1978; Harris, 1988) and each of these chromosomes is implicated in tumour suppression (Table 1).

d) Non-tumorigenic NxT hybrids retain the transformed phenotype *in vitro* (Stanbridge *et al.*, 1982). Thus, tumorigenicity and morphological transformation may be separate events, an observation that supports the concept of multistage tumorigenesis.

e) Non-tumorigenic NxT hybrids adopt the differentiation characteristics of the normal cell (Stanbridge and Ceredig, 1981; Peehl and Stanbridge, 1982; Harris, 1985). This is consistent with the suggestion that malignancy stems from a block in normal cellular differentiation (Klein, 1987; Harris, 1988).

1.4.1 Suppression by single chromosomes

The association between particular chromosomes and suppression of tumorigenicity has been further strengthened by introducing single normal chromosomes into tumour cells. This technique, involving microcell fusion, has been used to demonstrate the suppressing powers of a normal human chromosome 11 in HeLa cells (Saxon *et al.*, 1986) and a Wilms' tumour cell line (Weissman *et al.*, 1987). The link between chromosome 11 and the latter tumour was originally established by cytogenetic and RFLP analyses (section 1.4.3a).

These results are apparently paradoxical in that whole cell hybrids do not have to lose both copies of the normal chromosome to reexpress tumorigenicity; hybrids that retain one copy are tumorigenic (see above). However, Stanbridge (1989) has recently reported that HeLa cells suppressed by microcell fusion do form tumours in nude mice, but after a long latency period compared with the parental line (cf point b in section 1.4).

1.4.2 Loss of heterozygosity and tumorigenesis

The ability of normal cells to suppress the tumorigenic phenotype suggests that tumour cells have lost an essential regulatory function. In support of this, a large body of evidence has accumulated demonstrating that non-random loss of genetic information is a feature of most (if not all) human tumours (Hansen and Cavenee, 1987; Green, 1988; Bouck and Benton, 1989).

This particular branch of tumour suppressor research can be traced back to the statistical analysis of the childhood oc ular cancer retinoblastoma. In this, Knudson (1971) proposed that two "hits" were responsible for the development of both sporadic and familial cases of this tumour. He predicted that the second event was random in both sporadic and familial forms, and that the first event was also random in sporadic cases but inherited in familial cases. Inheritance endows every cell with the first hit thereby predisposing the patient to retinoblastoma. This explains the appearance of tumours at an earlier age in familial cases, and their growth at multiple rather than single sites.

Knudson's analysis could not determine whether the initial hit was an activating or inactivating mutation. Subsequently, the discovery of constitutional deletions in several retinoblastoma patients, all of which included the chromosomal region 13q14, suggested that the first mutation might involve inactivation at this locus (Knudson *et al.*, 1976; Yunis and Ramsay, 1978). Circumstantial evidence that the second hit might be loss of the remaining functional allele at this locus was provided by a series of reports in 1983. First, Sparkes *et al.* (1983) showed that the enzyme esterase D (ESD) was closely linked to the putative retinoblastoma (*RB*) locus. In an adjacent publication the same group reported a familial case of retinoblastoma in which they detected 50% ESD activity in normal cells, but no activity in a retinoblastoma from this patient (Benedict *et al.*, 1983). This supported the concept that the two hits predicted by Knudson involved homozygous inactivation of a single locus. In addition Godbout *et al.* (1983) analysed

polymorphic forms of ESD in other familial cases and detected loss of one enzyme form in 4/6 retinoblastomas, consistent with the loss of a single remaining normal *RB* allele linked to the absent ESD allele. Following this, Cavenee *et al.* (1983) pioneered the use of restriction fragment length polymorphisms (RFLPs) to identify the mechanisms by which loss of the putative normal *RB* allele occurred. Using a bank of polymorphic chromosome 13 probes they found that markers heterozygous in lymphocytes were often either homo- or hemizygous in tumour cells. In some cases loss of heterozygosity (LOH) was detected at every locus tested, consistent with chromosome loss with or without duplication of the remaining homologue. In others LOH was observed at only some loci, with a doubling in intensity of the remaining allele at these loci, suggestive of mitotic recombination. In those cases in which LOH was not detected it was considered that a subchromosomal mechanism, such as deletion, gene conversion and/or point mutation may have caused homozygous loss of the *RB* gene. In those cases involving complete chromosome loss, retention of the homologue from the affected parent further suggested that the second hit involved loss of the inherited normal allele (Cavenee *et al.*, 1985).

Final proof that retinoblastoma involved loss of function at both *RB* alleles required isolation of the gene itself. This followed the fortuitous discovery that a DNA probe (H3-8) located at 13q14 (Lalande *et al.*, 1984) was deleted in 2 retinoblastomas in which other linked markers were present (Dryja *et al.*, 1986). A short chromosome walk away from this was a probe which recognised a cDNA clone (4.7R) now known to encode the product of the *RB* locus (Friend *et al.*, 1986). Homozygous and heterozygous deletions (some internal) have been identified in retinoblastomas and osteosarcomas; a tumour often contracted by familial *RB* patients who survive the primary tumour (Friend *et al.*, 1986; Lee *et al.*, 1987a; Fung *et al.*, 1987). In one study a large number of retinoblastomas were analysed and found to express the *RB* gene normally (Goddard *et al.*, 1988). This implied that subtle mutations may have been present in these cases. Subsequently the same group

confirmed this by RNase mismatch analysis and DNA sequencing (Dunn *et al.*, 1988 and 1989).

Loss of the *RB* gene is also considered to have a role in several tumour types not associated with the retinoblastoma trait including breast (T'Ang *et al.*, 1988) and lung cancer (Harbour *et al.*, 1988). It is not known why *RB* patients are not predisposed to these tumours, nor why retinoblastoma does not occur in animals, even though it is expressed in most murine tissues (Bernards *et al.*, 1989). The induction of retinoblastoma in transgenic mice carrying the SV40 large T antigen (Windle *et al.*, 1990) may provide more insight into the latter problem.

The *RB* gene encodes a nuclear phosphoprotein of 110-114K (depending on the phosphorylation state) which binds DNA (Lee *et al.*, 1987a and b). It has been suggested that the unphosphorylated form may suppress proliferation since SV40 large T antigen binds this form of RB and maintains cells in a proliferative state (DeCaprio *et al.*, 1988). This was supported by the finding that the RB protein is unphosphorylated during the G1 phase of the cell cycle and phosphorylated during the remaining S, G2 and M stages which involve cell duplication (Buchkovich *et al.*, 1989; DeCaprio *et al.*, 1989; Chen *et al.*, 1989).

1.4.3 Loss of heterozygosity is a common feature of tumorigenesis

Karyotyping and RFLP analysis have shown that non-random LOH is a common feature of most tumours (Table 2). The main points arising from these studies are summarised below.

a) A single locus may be involved in the development of diverse tumours (c.f. point c in section 1.4). Losses involving 1p, 3p, 11p, 13q, 17p and 22q are each implicated in multiple tumours (Table 2a). Analysis of the *RB* gene has presented a strong case that this locus on 13q is involved in tumours other than retinoblastoma (see above). However, proof that there is only one tumour suppressor locus on the other chromosome arms mentioned above requires finer mapping and, ultimately,

Table 2 Examples of non-random allele loss in human tumours

A)

Chromosome region	Tumour	Reference
1p	Multiple endocrine neoplasia (Type 2)	Mathew et al., 1987b
3p	Uterine cervix carcinoma	Yokota et al., 1989
	Renal cell carcinoma	Zbar et al., 1987 Kovacs et al., 1988
	Small cell lung carcinoma	Naylor et al., 1987 Dobrovic et al., 1988 Mori et al., 1989
	All lung cancers	Kok et al., 1987
	Mesothelioma	Popescu et al., 1988
5q	Colorectal adenocarcinoma	Solomon et al., 1987 Law et al., 1988
9p	Lymphoid neoplasia	Diaz et al., 1988
10	Glioma	James et al., 1988
11p	Wilms' tumour	Koufos et al., 1984 Orkin et al., 1984 Reeve et al., 1984 Fearon et al., 1984
	Rhabdomyosarcoma	Scrable et al., 1987
	Hepatoblastoma	Koufos et al., 1985
	Adrenal carcinoma	Henry et al., 1989
	Breast carcinoma	Ali et al., 1987
	Bladder carcinoma	Fearon et al., 1985
	Ovarian carcinoma	Lee et al., 1989
11q	Multiple endocrine neoplasia (Type 1)	Larsonn et al., 1988 Yoshimoto et al., 1989
13q	Retinoblastoma	Cavenee et al., 1983
	Osteosarcoma	Hansen et al., 1985
	Soft tissue sarcomas	Weichselbaum et al., 1988 Stratton et al., 1989
	Small cell lung carcinomas	Harbour et al., 1988 Mori et al., 1989
	Breast carcinoma	T'Ang et al., 1988 Lee et al., 1988

14q	Neuroblastoma	Suzuki et al., 1989
17p	Colorectal adenocarcinoma	Fearon et al., 1987 Law et al., 1988
	Osteosarcoma	Toguchida et al., 1988
	Glioma	James et al., 1988
	Small cell lung carcinoma	Mori et al., 1989
18q	Colorectal adenocarcinoma	Law et al., 1988 Vogelstein et al., 1989
22q	Colorectal adenocarcinoma	Okamato et al., 1988
	Acoustic neuroma	Seizinger et al., 1986
	Meningioma	Seizinger et al., 1987

B)

Tumour	Main sites of allele loss
Neuroblastoma	1p, 14q
Small cell lung carcinoma	3p, 13q, 17p
Colorectal adenocarcinoma	5q, 17p, 18q, 22q
Glioma	10, 17p
Breast cancer	11p, 13q
Osteosarcoma	13q, 17p

isolation of the critical gene(s). Mapping studies of this type have suggested that there are at least two such genes on the short arm of chromosome 11.

Wilms' tumour is a childhood cancer which affects the kidney. Familial cases are associated with the WAGR syndrome (Wilms' tumour, Aniridia, Genitourinary defects, mental Retardation), but the majority of tumours are sporadic. In the familial version tumours develop bilaterally and at an earlier age than sporadic cases, consistent with Knudson's two-hit hypothesis for retinoblastoma. As with retinoblastoma, the link between Wilms' tumour and a specific chromosomal region (11p13) was originally made by cytogenetic studies (Riccardi *et al.*, 1978; Kaneko *et al.*, 1981). RFLP analysis demonstrated LOH for 11p markers in approximately 50% of Wilms' tumours (refs in Table 2a). The probes in these studies map to 11p15 and so it could only be assumed that allele loss extended to 11p13. Several subsequent studies used a variety of 11p13 probes to confirm the involvement of this region in Wilms' tumour and to more tightly map the relevant locus (Glaser *et al.*, 1986; Porteous *et al.*, 1987; Compton *et al.*, 1988; Davis *et al.*, 1988). However, in a separate study Mannens *et al.* (1988) found that 3/5 Wilms' tumours, removed from children with no other symptoms of the WAGR syndrome, were heterozygous for 11p13 markers but no longer heterozygous at 11p15.5. Thus, while LOH at 11p13 is clearly involved in many Wilms' tumours, 11p15.5 appears to contain an alternative Wilms' locus.

Additional evidence that there are at least two tumour suppressors on 11p has been provided by analysis of other tumour types. When Koufos *et al.* (1985) discovered LOH at 11p15 in Wilms' tumour, rhabdomyosarcoma and hepatoblastoma the 11p13 Wilms' locus was naturally implicated in the genesis of all three childhood malignancies. Subsequently however, Scrable *et al.* (1987) analysed one rhabdomyosarcoma in which LOH was restricted to the most distal region of 11p. These tumours are sometimes observed in patients with Beckwith-Wiedemann syndrome (BWS), which is a growth malformation syndrome. BWS patients may also contract Wilms' tumour, adrenocortical carcinoma and hepatoblastoma. These

observations are consistent with the idea that 11p harbours two tumour suppressor genes, one at 11p13 which is kidney specific (tumours other than Wilms' tumour have never been observed in WAGR patients), and the other(s) at 11p15.5 which contains a non-tissue specific gene, or several closely linked cancer genes. In support of this, LOH limited to 11p15.5 has been reported in an analysis of adrenocortical adenocarcinomas (Henry *et al.*, 1989) and breast cancer (Ali *et al.* 1987).

b) Multiple losses are implicated in the development of some tumours. The most comprehensive assessment of this phenomenon has been carried out by Vogelstein *et al.* (1989). By using a large bank of probes they were able to determine the frequency of loss of every human chromosome arm in several colon carcinomas (excluding the acrocentric arms which are thought to contain only ribosomal genes). Considering the high frequency of K-*ras* mutations in these tumours (section 1.3.2) their results suggest that the development of this common malignancy involves the interaction of a complex series of genetic mutations. The variety of potential tumour suppressor loci involved in this and other cancers is summarised in Table 2b.

The loss of alleles from multiple loci is predominantly seen in adult rather than childhood malignancies (Hansen and Cavenee, 1987). This is in accordance with the prediction that the number of steps required to reach malignancy is different for separate tumour types (section 1.1). Furthermore, combined histo pathological and molecular genetic analyses suggest that these steps may occur in a particular order. For example, the early stages of colon carcinoma (benign adenoma) appear to involve *ras* activation and 5q loss, wheras allele loss on 17p and 18q occurs as tumours progress to malignancy (Vogelstein *et al.*, 1988).

c) In familial cancers the disease locus may or may not map to the chromosomal region associated with LOH in the tumours. By using polymorphic enzyme markers and RFLPs to analyse large families with inherited cancer syndromes, several groups have been able to determine which markers segregate with the disease locus, and thereby locate its position in the genome. For example, familial adenomatous polyposis (colorectal cancer) has been mapped to 5q (Bodmer *et al.*,

1987), Von-Hippel-Lindau syndrome (associated with renal carcinoma) to 3p (Seizinger *et al.*, 1988), multiple endocrine neoplasia type 1 (associated with pituitary and pancreatic tumours) to 11q, retinoblastoma to 13q14 (Benedict *et al.*, 1983) and neurofibromatosis (associated with acoustic neuroma and meningioma) to 22q (Rouleau *et al.*, 1987). These findings are consistent with the notion that predisposition to cancer stems from inheritance of one of the defects commonly observed in the tumours. However, this is not alway the case. For example, although LOH at 11p13 is commonly observed in Wilms' tumour, the disease locus did not map to 11p in linkage studies involving three families predisposed to this cancer (Huff *et al.*, 1988; Grundy *et al.*, 1988). In addition, multiple endocrine neoplasia type 2 (MEN2) (associated with medullary thyroid carcinoma, phaeochromocytoma and parathyroid adenomas) maps to chromosome 10 (Mathew *et al.*, 1987a; Simpson *et al.*, 1987), even though tumours from MEN2 patients show allele loss on 1p (Mathew *et al.*, 1987b). Thus, the inherited mutations may a) complement the deletions frequently observed in these tumours; b) represent an alternative route to malignancy; or c) regulate the function of the loci commonly deleted in these tumours. One mechanism by which the latter may occur is discussed in the next section.

1.4.4 Genomic imprinting and tumour suppressor genes

Genomic imprinting is an epigenetic phenomenon which results in the differential expression of genes on paternally and maternally inherited chromosomes. Evidence for this process comes from the observation that embryos in which some, or all, of the chromosomes are from one parent are nonviable, and that the expression of transgenes and penetrance of some mutations is dependent on whether transmission is maternal or paternal (reviewed by Reik, 1989). One of the mechanisms which may control the differential expression of parental alleles is DNA methylation. This derives from the fact that the expression of transfected genes *in vitro*, and of

transgenes *in vivo*, correlates with the levels of DNA methylation, and that exposure of cell lines to 5-azacytidine (which prevents cytosine methylation) can reactivate many silent genes (reviewed by Holliday, 1987).

The first mutation at a tumour suppressor locus is frequently a subchromosomal event (point mutation etc), whereas the second event in the majority of Wilms' tumours and retinoblastomas tends to be a gross chromosomal event (mitotic recombination, non-disjunction or large deletion). It was originally thought that the initial mutation could occur with equal frequency at either allele, thus predicting that the number of tumours containing only maternal or paternal alleles would also be equal. However, in certain tumours, including Wilms' tumour (Reeve *et al.*, 1984; Schroeder *et al.*, 1987; Mannens *et al.*, 1988), osteosarcoma (Toguchida *et al.*, 1989) and rhabdomyosarcoma (Scrable *et al.*, 1989), preferential loss of maternal alleles has been detected, implying that the retained paternal homologue sustained the initial defect. The most straightforward explanation for these results is that the first allele is silenced by an ab erant imprinting gene which acts preferentially on the paternal allele, thereby explaining preferential loss of the maternal chromosome in these tumours (Scrable *et al.*, 1989). This model can explain a familial case of Wilms' tumour (Grundy *et al.*, 1988) and a phaeochromocytoma from a MEN2 family (Mathew *et al.*, 1987b) in which the paternal alleles of chromosomes 11 and 1 respectively were retained, even though the trait had been inherited from the mother. In these types of cases the imprinting gene, located on a different chromosome from the disease locus, is thought to be inherited from the mother. Significantly, this explanation resolves the problems raised by family studies which have failed to identify genetic linkage between Wilms' tumour or MEN2 with loci commonly deleted in these tumours (see Table 2a).

The model suggested by Scrable *et al.* (1989) predicts that there is no requirement for mutation of the paternal suppressor allele. This can be determined in the case of osteosarcomas (Toguchida *et al.*, 1989) since the critical locus , the *RB* gene, has been isolated. Significantly, in 4/9 osteosarcomas the retained paternal

chromosome carried a deletion at the *RB* locus. Models which can explain this finding are described below.

a) Reik and Surani (1989) have discussed a model in which the maternal *RB* allele is imprinted, but only partially down- regulated. Deletion of the paternal allele might permit benign tumour growth, with malignant progression resulting from loss of the maternal chromosome. This model has three steps compared to only two in the simpler model described above.

b) In a model based on studies of Wilms' tumour, Wilkins (1988) has suggested that the maternal allele of a linked transforming gene becomes imprinted. Deletion of the paternal Wilms' locus, followed by loss of the maternal chromosome causes up-regulation of the remaining transforming allele, which is regulated by the Wilms' locus. Again, this model involves three steps.

c) It is also possible that imprinting of the paternal allele of a linked tumour suppressor locus is followed by deletion of the Wilms' paternal suppressor allele and subsequent simultaneous loss of the maternal suppressor alleles at the Wilms' locus and the linked locus. The presence of at least two suppressor loci on 11p (section 1.4.3a) supports this particular three-step model.

Finally, it is important to note that for some of the tumours in which preferential loss of maternal alleles has been observed, an entirely different explanation is possible. Because spermatogenesis involves many more cell divisions than oogenesis, it is likely that the number of mutations is also much higher. Thus, tumours that are sporadic and bilateral (i.e the result of a new germ line mutation) may show a high frequency of maternal chromosome loss simply because the first mutation occured in spermatogenesis. This possibility can be tested by comparing chromosome loss in sporadic bilateral tumours with sporadic unilateral cases. For example, retinoblastomas of the latter type show no preferential loss of maternal or paternal chromosome 13 alleles, whereas new germ line mutations are most almost exclusively confined to the paternal allele (Dryja *et al.*, 1988; Zhu *et al.*, 1988). In contrast, 9/10 sporadic, solitary osteosarcomas (Toguchida *et al.*, 1989) and 5/5

sporadic unilateral Wilms' tumours (Reeves *et al.*, 1984; Schroeder *et al.*, 1987) were found to have retained paternal alleles. It is therefore likely that an imprinting mechanism is involved in these tumours, and that this applies to bilateral cases too.

Although imprinting is clearly an important phenomenon, the exact mechanisms by which it operates and the range of tumour types it affects remain to be determined.

1.4.5 The function of tumour suppressor genes

A diverse range of functions have been postulated for tumour suppressor genes (Sager, 1986; Bouck and Benton, 1989). These are summarised below.

a) Differentiation stimuli

It has been argued that cancer stems from a defect in the normal differentiation programme of a cell (Klein, 1987; Harris, 1988). Klein (1987) has highlighted a number of studies which have employed temperature sensitive retroviruses to show that transformation is reversible, effective only within a particular differentiation window and can be overcome by potent differentiation stimuli. If oncogenes act to block the action of differentiation genes then transformation could also occur by the loss of the latter. This is supported by the finding that non-tumorigenic somatic cell hybrids between normal and tumour cells frequently take on the differentiation characteristics of the normal cell (section 1.4e).

The molecules which regulate differentiation include many classes, from growth factors to DNA binding proteins. The receptor for TGF-β , which is implicated in keratinocyte differentiation, is absent in some carcinomas, and in others the receptors are present but resistant to TGF-β (section 1.5.3.1a). In these cases there appears to be a defect downstream of the receptor.

It seems unlikely that deletion of a negative autocrine growth factor gene would have a significant effect on cell growth since neighbouring cells should be able to secrete enough to compensate for the loss. However, a small clone of such

cells might escape the signal. In addition, there is evidence suggesting that several growth factors function even when secretion is blocked (Browder *et al.*, 1989). If some of the effects of an autocrine regulator are elicited prior to secretion, its loss could potentiate growth. Although these suggestions are highly speculative it is interesting to note that Diaz *et al.* (1989) have detected homozygous deletion of the interferon locus in leukemia cells. This factor is known to exert a strong inhibitory effect on several carcinoma and leukemia/lymphoma cell lines (Chen *et al.*, 1988).

b) Proliferation blocks

Obviously, the genes discussed in the above section could also fit this description. However, there may be a discrete set of tumour suppressor genes that block proliferation but do not stimulate differentiation. This category may include proteins that specifically regulate the function of oncogenes. For example, a large number of oncogene products are protein kinases (eg *src*, *erb*B, *raf*) and so it is possible that some phosphatases may turn out to be tumour suppressors (Hunter, 1989). The deactivation of p21*ras* proteins by GAP is probably not in the same category since GAP is a potential effector molecule for *ras* (section 1.3.3).

Inhibition of oncogene action need not be restricted to post-translational mechanisms. For example, the transcriptional regulation of RSV in rat-1 cells determines whether these cells are transformed or not (Wyke *et al.*, 1989). The role of elevated expression in transformation by several oncogenes (section 1.2.2.2) emphasises the importance of transcriptional down regulation to normal cell growth.

The viral oncogene products E1A, E7 and large T bind, and probably inactivate, the protein encoded by the *RB* gene (DeCaprio *et al.*, 1988; Whyte *et al.*, 1988; Dyson *et al.*, 1989). It is therefore possible that the normal function of this tumour suppressor gene is to block the action of growth promoting proto-oncogenes (and/or to stimulate differentiation), and that this activity is swamped by overexpressed oncogenes or lost by gene deletion.

The p53 gene appears to encode a unique type of tumour suppressor that, like RB, binds to a range of oncogene products. In its wild-type form it suppresses

transformation of rat cells when transfected together with various oncogenes (Finlay *et al.*, 1989). Deletion, mutation and rearrangement of the gene has been detected in several human and animal tumours (Ben-David *et al.*, 1988; Ahuja *et al.*, 1989; Baker *et al.*, 1989; Nigro *et al.*, 1989). Remarkably, the wild-type form of the gene was mistaken for an oncogene until recently, when it was shown that DNA clones used in early studies contained mutations (Hinds *et al.*, 1989). Prior to demonstration of its suppressing properties (Finlay *et al.*, 1989), mutant p53 was known to bind both normal p53 and a heat shock protein, hsc70 (Finlay *et al.*, 1988; Rovinski and Benchimol, 1988). Thus, it has been suggested that the transforming version of p53 acts in a "dominant negative" fashion by binding and inactivating its normal counterpart, and possibly hsc70 (Finlay *et al.*, 1989). This resembles the binding and (presumed) inactivation of the *RB* gene product by various viral oncogenes (see above). Even more intriguing is the fact that one of these, the SV40 large T antigen, binds both RB (DeCaprio *et al.*, 1988) and p53 proteins (Lane and Crawford 1979). Furthermore, the adenovirus oncogene products E1A and E1B behave like two halves of SV40 large T in that E1A binds the RB protein (Whyte *et al.*, 1988) and E1B binds the p53 protein (Sarnow *et al.*, 1982). These findings are evidence for the cooperative effect of disabling two tumour suppressors in the same cell. That p53 and RB may be involved together in non-viral tumours is supported from studies of lung cancer (reviewed by Minna *et al.*, 1989).

Although the direct link between oncogenes and tumour suppressors discussed above lends credibility to the term "anti-oncogene", it remains to be determined whether this is the sole regulatory function of these proteins.

c) Senescence factors

The limited capacity of mammalian cells to divide is a defense against tumour development. However, loss of or insensitivity to the factors that control senescence may facilitate tumorigenesis.

d) DNA repair enzymes

A number of hereditary diseases with predisposition to malignancy are associated with DNA repair defects (Hanawalt and Sarasin, 1986). The genes for DNA repair enzymes can therefore be regarded as tumour suppressor genes in the sense that their loss leads to tumour growth. However, this category also demonstrates the inadequacy of the term "tumour suppressor" since their replacement in transformed cells would not suppress tumorigenicity.

e) Proto-oncogenes

Some studies have found that in tumours with activated *ras* the mutant allele is over-represented (Quintanilla *et al.*, 1986; Taya *et al.*, 1984), over-expressed relative to the normal allele (Capon *et al.*, 1983), or that the normal allele is absent (Taparowsky *et al.*, 1982; Guerrero *et al.*, 1985). Amplification of the mutant signal may be selected (a) because it overcomes a blocking competetive effect of the normal allele, or (b) for some reason totally unrelated to the normal allele. This latter possibility is the view of Paterson *et al.* (1987) who found that a reduction in the level of mutant N-*ras* caused reversion of HT1080 fibrosarcoma cells but microinjection of normal p21*ras* had no effect on the phenotype. That overexpression of normal *ras* is a transforming event (section 1.3.4.2) also suggests that this gene is not a tumour suppressor. However, Spandidos and Wilkie (1988) have reported that normal *ras* can suppress tumorigenicity in NIH3T3 cells and so this field remains controversial. In support of the latter claim, one of the few tumour suppressor genes isolated to date, K-*rev*, encodes a protein of M_R 21K, and shares around 50% homology with *ras* proteins (Kitayama *et al.*, 1989). It is therefore possible that K-*rev* competes for *ras* effectors.

In the cases involving deletion of the wild type allele it is possible that a linked tumour suppressor gene was involved. In this respect it is intriguing to note that tumour suppressor genes have been mapped to human chromosome arms 1p and 11p (Table 2); the location of N-*ras* and H-*ras* genes respectively.

f) Inhibitors of angiogenesis

Angiogenesis, the formation of new blood vessels by endothelial cells, is essential for the growth of tumours beyond a diameter ot 1-2mm (Folkman *et al.*, 1985). Genes encoding the cellular machinery that enables cells to respond to inhibitors of this process could therefore suppress tumour growth. Recently, a glycoprotein of Mr 140000 has been identified in the medium of BHK cells that suppresses neovascularisation (Rastinejad *et al.*, 1989). In the same study it was shown that chemically transformed BHK cells are resistant to the action of this molecule. Reversion of these cells to the normal phenotype and sensitivity to the angiogenesis inhibitor is paralleled by recovery of the ability to suppress transformed cells in somatic hybridisation experiments. Thus transformation and resistance to the inhibitor are linked to loss of a tumour suppressor in these cells.

g) Cell surface interactions

Several categories of molecule located on or outside the cellular membrane are potential tumour suppressors. Sager (1986) and Bernards (1987) have discussed the potential significance of the major histocompatability complex (MHC) class I antigens to the removal of cancer cells by cytotoxic T lymphocytes. Tumour cells may escape a different type of growth restraint by losing the cell surface molecules responsible for attachment to the extracellular matrix (cell adhesion molecules). Reduced levels of cell adhesion molecules have been detected in some transformed cells (Plantefaber and Hynes, 1989) and the Drosophila recessive tumour gene, lethal (2) giant larvae, is probably involved in cell-cell interactions (Lutzelschwab *et al.*, 1987). Furthermore, the recently isolated chromosome 18q tumour suppressor gene that is involved in colorectal cancer, is homologous to neural cell adhesion molecules (Fearon *et al.*, 1990). Disturbance of cell-cell interactions could also occur by degradation of the extracellular matrix. For example, Chen and Chen (1987) have detected increased proteolytic degradation of fibronectin. Thus, molecules that regulate the expression or function of these enzymes may suppress tumour growth and/or metastasis.

1.4.6 Tumour suppressor genes: different classes

It is clear from analysis of tumours in which the *RB* gene is involved that homozygous inactivation of this locus is required for tumorigenesis. This has been broadly accepted as the mechanism by which all tumour suppressor genes contribute to malignancy. However, the role of chromosome dosage in suppression (section 1.4b) suggests that it need not be an all-or-nothing phenomenon. Furthermore, from analysis of the p53 gene it is now obvious that another class of "dominant negative" tumour suppressor genes exists. A third class may include those oncogenes whose wildtype alleles suppress the effects of the activated allele. These alternatives mean that detection of LOH in tumours should not automatically be equated with the retinoblastoma paradigm. In other words, the possibility that LOH has unmasked an activating mutation cannot be discounted until the gene of interest has been isolated.

1.5 The mouse skin carcinogenesis model

Animal model systems have been invaluable in the development of a multistage concept of tumorigenesis (Hecker *et al.*, 1982). Unlike human studies tumours can be reproducibly induced using known carcinogens, and molecular alterations investigated at defined stages following initiation. The activation of oncogenes in various animal models of carcinogenesis has been summarised recently (Guerrero and Pellicer, 1987; Balmain and Brown, 1988). Since the majority of human tumours are epithelial in origin, probably because of exposure to environmental pollutants and UV light, the mouse skin carcinogenesis model is of particular importance in the elucidation of genetic and/or epigenetic events associated with tumour growth.

The two-stage, or initiation-promotion model of carcinogenesis involves the single application of a subthreshold dose (i.e one which will not induce tumour formation by itself) of a complete carcinogen or initiator (an agent which if applied

in a large single dose, or in repeatedly subthreshhold doses will induce tumours). This is followed by repetive applications, to the same area of skin, of a tumour promoter (an agent which induces tumours only if used after application of an initiator) (Slaga, 1983). This protocol results in the appearance of benign papillomas within 10-20 weeks, approximately 10% of which progress to malignant carcinomas.

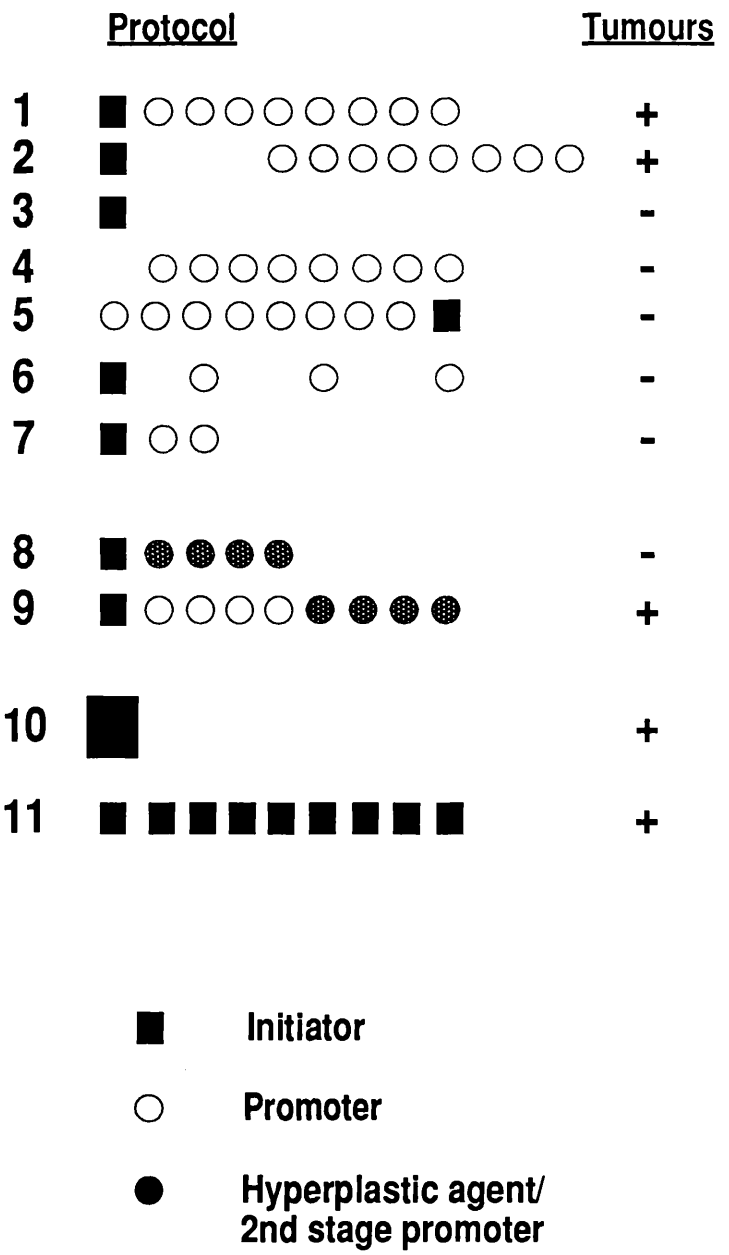
Papillomas have a cauliflower-like structure consisting of several folds joined by one or a few stalks which are linked to the underlying skin. Each fold consists of epithelial projections covering vascular connective stalks. The basic stratified structure of the epidermal component is retained although it tends to be thicker than normal, including the superficial "horny" layer, which is made up of flat anuclear cells packed with keratin.

Macroscopically, carcinomas are firm indurated expanding nodules which often ulcerate. They are characterised by a disorderly proliferation of epithelial cells and can be classified as grade 1-3, with grade 3 showing least differentiation (Kruszewski *et al.*, 1987). The proportion of abnormal cell shapes and bizarre mitotic figures increases with the grade. In the most differentiated tumours so called "horny pearls" are easily identifiable, which appear as pink haematoxylin and eosin stained islands made up of groups of terminally differentiated cells. Another type of poorly differentiated carcinoma is the spindle cell carcinoma. Histologically these can be mistaken for fibrosarcomas, but macroscopically they resemble carcinomas. Evidence of epithelial differentiation can be detected in these tumours, including the presence of desmosomes (structures unique to keratinocytes), and their reaction to keratin antibodies confirms their epidermal origin (Morison *et al.*, 1986).

1.5.1 Factors affecting papilloma and carcinoma production

The requirements for chemically induced tumour formation on mouse skin are summarised in Figure 2. Initiation requires only one application of a carcinogen, and is an irreversible step (protocols 1 and 2). By itself, initiation does not result in the

Figure 2 **Requirements for chemical carcinogenesis in mouse skin**



formation of tumours. Similarly, promotion alone, or prior to initiation, produces no or very few tumours (protocols 4 and 5). If the frequency of promotion is too low no tumours result (protocol 6). This observation suggests that the action of individual exposures is reversible (and therefore epigenetic), or that irreversible changes induced by promotion are only effective if other reversible events are continually induced. The genetic changes resulting from TPA treatment (see below) make this suggestion feasible. Protocol 7 shows that a short period of promotion produces no, or very few, tumours.

Protocols 8 and 9 demonstrate the ability of hyperplastic agents to induce tumours. TPA, and all other tumour promoters induce hyperplasia, but not all hyperplastic agents are tumour promoters (protocol 8). However, these chemicals may complete promotion if initiated skin is first exposed to several applications of promoter (protocol 9). This observation led to the concept of first and second stage promotion. TPA and wounding are first stage (or full) promoters, while retinoyl phorbol acetate (RPA) and mezerein are second stage promoters. RPA is a more powerful second stage promoter, even though mezerein is actually a weak full promoter (Marks *et al.*, 1982).

The induction of tumours by application of a large single dose of carcinogen is known as complete carcinogenesis (Figure 2, protocol 10). Repeated small doses of an initiator will also induce tumour formation (protocol 11).

The total number of tumours induced by initiation/promotion rises with increasing doses of initiator or promoter, and with duration of promoter treatment (Burns *et al.*, 1978; Verma and Boutwell, 1980; Slaga *et al.*, 1982). However, the overall increase in tumour numbers is not proportionate amongst papillomas and carcinomas. For example, in one study using SENCAR mice, a 16-fold increase in TPA raised the induction of papillomas almost 80-fold, but the number of carcinomas less than 40-fold (Ewing *et al.*, 1988). Increasing the amount of DMBA at initiation, or the duration of promoter treatment gave related findings. In an earlier study, using HA/ICR mice it was also found that using more initiator (DMBA), or lengthening

promoter treatment decreased the percentage of papillomas which converted to carcinomas (Burns *et al.*, 1978). Hennings *et al.* (1983) have also observed a drop in malignant conversion with increased promoter treatment. More recently it was observed that although DMBA-initiation followed by bromomethylbenz(a) anthracene (BrMBA)-promotion induces many more papillomas than repeated BrMBA treatment, the carcinoma yield is not similarly affected (Scribner *et al.*, 1983).

One interpretation of these findings is that a subset of papillomas exist which are unlikely to progress to carcinomas, and that a high proportion of DMBA-initiated cells form papillomas in this category. Alternatively, malignant progression may be retarded by the toxicity of a large tumour burden. Thus, increased competition for blood supply and degeneration of animal health could limit tumour progression. Nevertheless, this is probably only partly responsible for the results described above since other evidence has been documented in favour of the existence of subsets of papillomas. For example, it has been known for many years that most papillomas (80-90%) regress if promoter treatment is terminated, and that the remainder persist (Burns *et al.*, 1976). Those that disappear are termed promoter-dependent or conditional, those that remain are promoter-independent or autonomous. Reddy *et al.* (1987) have used an X-linked enzyme polymorphism to analyse biopsies of papillomas taken during promotion and from identical site tumours long after treatment has stopped. They found that many of the biopsies from autonomous papillomas (taken at the end of the experiment) had a different enzyme phenotype from those taken during promoter treatment. Thus promoter-dependent and independent papillomas are not necessarily related.

Molecular evidence for the existence of a subset of papillomas with a greater likelihood of progression has been provided by Brown *et al.* (1990). They have found that the distribution of *ras* gene mutations differs between papillomas and carcinomas initiated with the same carcinogen. Thus it appears that the initiation event influences the probability of progression.

For many years it has been accepted that around 90% of carcinomas arise directly from papillomas (Burns *et al.*, 1978). This assumption has recently been questioned. Reddy *et al.* (1987) used a combination of photography, coordinate measurements and analysis of an X-linked polymorphic enzyme (phosphoglycerate kinase; PGK) to address this problem. Five out of 18 carcinomas exhibited PGK phenotypes discordant with those detected at an earlier biopsy of a papilloma at the same site. The actual number could be twice this since there is a 50% chance that a new tumour will have the same phenotype as a preexisting lesion. In contrast, papillomas induced by repeated initiation morphologically evolve into carcinomas which almost invariably have identical phenotypes (Taguchi *et al.*, 1984; Reddy and Fialkow, 1989). Interestingly, the majority of carcinomas induced in this way do not appear to arise from visible papillomas (Reddy and Fialkow, 1989). The simplest explanation for this is that while initiators efficiently induce mutations which can aid progression, these events are induced rarely, or must occur spontaneously in initiated cells expanded by promoter treatment. Potter (1981) has suggested that the role of promotion is to increase the size of the target cell population available for a second mutation. Hennings *et al.* (1983) have shown that the frequency of this event can be increased by applying mutagens to papillomas induced by an initiation-promotion regime. Although the mutations involved in this process have not been identified (section 1.5.4) they probably differ from those involved in initiation, since chemicals which are good initiators are not necessarily effective progression agents, and vice versa.

1.5.2 Molecular events at initiation

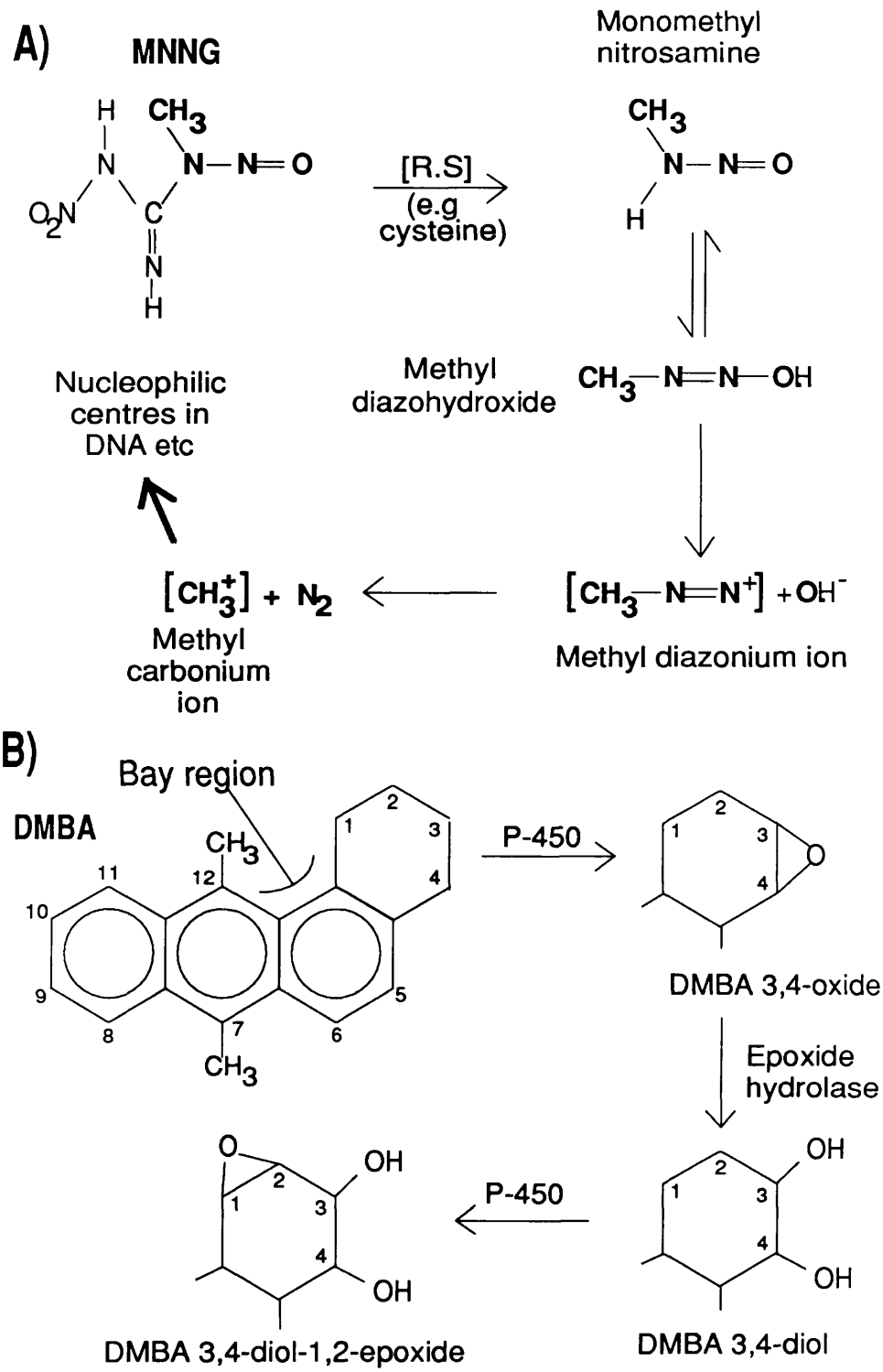
The mutagenic nature of known carcinogens (reviewed by Singer and Kusmierek, 1982) and the irreversible nature of initiation (Van Duuren *et al.*, 1975) suggests that it is a genetic event. Two mutagens which are commonly used as initiators, and were used in the study reported here, are 7, 12 dimethyl

benz(a)anthracene (DMBA) and N-methyl-N'-nitro-N-nitroso guanidine (MNNG). These belong to two distinct chemical classes: DMBA is a polycyclic aromatic hydrocarbon (PAH) and MNNG is an N-nitroso compound.

The chemical action of PAHs has been elucidated mainly from studies on benz(a)pyrene [B(a)P] and its metabolites (reviewed by Conney, 1982). These studies identified the "bay-region" dihydrodiol (or simply diol) epoxide of B(a)P as the ultimate carcinogen. The bay region of DMBA, and its metabolism by P-450s (or monoamine oxidases) and an epoxide hydrolase to the dihydrodiol epoxide, is indicated in Figure 3b. The authenticity of the latter as the ultimate carcinogen has been endorsed by the finding that its putative direct precursor, the 3,4-dihydrodiol (Figure 3b), is more carcinogenic than DMBA itself (Slaga *et al.*, 1979). Both the *syn* and *anti* forms of the ultimate carcinogen bind DNA (Sawicki *et al.*, 1983), but the carcinogenic properties of the chemical are largely due to the *syn* form, which binds almost exclusively to dA residues in DNA (Cheng *et al.*, 1988). This is unusual to DMBA since other PAHs, including B(a)P and methylcholanthrene (MCA), form major adducts with dG residues (Jeffrey, 1985). The affinity which DMBA has for dA residues makes it a better initiator than other PAHs; B(a)P is about 30-fold weaker as an initiator than DMBA (Dipple *et al.*, 1983b).

The N-nitroso compounds, including MNNG, are alkylating agents (for reviews see Singer and Kusmierek, 1982; Margison and O'Connor, 1978). The breakdown of these compounds to ultimate carcinogens occurs in the presence of a nucleophilic reagent (e.g alkali or thiols in cells; Figure 3a), and does not, as in the case of PAHs, require enzymic catalysis. Although alkylation has been observed at all O and N positions in the bases (except the nitrogen attached to the sugar), the primary mutagenic lesion produced by simple methylating agents is the O6-methyl guanine adduct (O6-MeG) (Loveless, 1969 Nature). This species pairs preferentially with thymidine during DNA replication (Toorchen and Topel, 1983), resulting in G:C->A:T transitions. Consistent with this, only G->A lesions have been detected in the H-*ras* gene in mouse skin tumours initiated using MNNG (Brown *et al.*, 1990,

Figure 3 Mechanism of activation of MNNG and DMBA



Bremner and Balmain 1990). G->A mutations have also been detected in mouse carcinomas (Brown *et al.*, 1990) and other animal tumours (reviewed by Balmain and Brown, 1988) which were induced with another simple methylating agent, methylnitrosurea (MNU), although A->T transversions have also been detected in MNU- induced rat tumours (see Balmain and Brown, 1988).

1.5.2.1 Activation of *ras* genes: an early or late event?

The discovery that H-*ras* was activated in papillomas (Balmain *et al.*, 1984) as well as carcinomas (Balmain and Pragnell, 1983) implied that mutation of this gene is an early event. Evidence that it can be the initiation event, and is induced by direct interaction of the carcinogen, was provided by Quintanilla *et al.*(1986). They found that over 90% of DMBA-initiated papillomas and carcinomas had an A:T->T:A transversion in codon 61 of the H-*ras* gene, a mutation consistent with that predicted from the known metabolism and binding of this chemical from studies both *in vitro* (Cheng *et al.*, 1988) and in mouse skin (Dipple *et al.*, 1983a). A correlation between the type of *ras* mutation and the carcinogen was also noted in MNU-induced rat mammary tumours (Sukumar *et al.*, 1983; Zarbl *et al.*, 1985) and in B6C 3F1 mouse hepatomas induced by treatment with the metabolic products of two aromatic amines (Wiseman *et al.*, 1986).

Although these examples support the proposition that activation of *ras* occurs at initiation in the development of several animal tumours, other examples are known in which it is more difficult to correlate the mutagenic activity of the initiating chemical with the mutation detected in the resulting tumours (Bizub *et al.*, 1986, Guerrero *et al.*, 1985; Guerrero and Pellicer, 1987; Balmain and Brown, 1988). Direct evidence that activated *ras* is an initiating event was provided by Brown *et al.*, in 1986. They found that treatment with DMBA could be replaced by application of a retrovirus containing activated H-*ras* to mouse skin. As with chemical initiation, tumours only formed if the skin was treated with TPA. Subsequently, Roop *et al.*

(1986) demonstrated that keratinocytes containing v-H-*ras* could form papillomas when grafted onto the skin of nude mice. These experiments firmly established the correlation between initiation and H-*ras* activation in mouse skin carcinogenesis. The fact that *ras* mutations have been identified in several premalignant human tumours (section 1.3.4.1) illustrates the interpretive value of the mouse model.

1.5.3 Tumour promotion

Initiated cells, whether chemically or virally induced, do not develop into tumours in the absence of promoter treatment (Van Duuren *et al.*, 1975; Brown *et al.*, 1986). Of the large variety of chemicals which are tumour promoters (Slaga, 1983), the strongest are the phorbol esters (e.g TPA). Tobacco smoke condensate and benzoyl peroxide have moderate promoting activity, while some long chain hydrocarbons are weak promoters. From epidemiological and animal model studies several agents have been identified as potential promoters in human carcinogenesis. These include dietary fat, cigarette smoke, asbestos, alcohol and steroid hormones (Pitot, 1983; Kodama and Kodama, 1987).

The outcome of tumour promotion is the proliferation of initiated cells in a target tissue. This may involve partial or complete resistance of initiated cells to promoter-induced differentiation. Thus, accelerated differentiation of normal basal cells might indirectly increase the rate of proliferation of initiated cells. This is supported by studies *in vivo* (Reiners and Slaga, 1983) and *in vitro* (Yuspa *et al.*, 1982) which have shown that tumour promoters induce terminal differentiation in some epidermal basal cells, while others remain in the proliferative pool. Furthermore, Yuspa's group have analysed putative initiated cells *in vitro* and have found resistance to a terminal differentiation stimulus among these (Yuspa and Morgan, 1981, Yuspa *et al.*, 1983 and 1985).

An alternative model is that the tumour promoter acts directly by selectively stimulating proliferation of initiated cells. Finally, enhanced proliferation and abberant differentiation could operate synergistically.

The target cells for initiation are not known. Obvious candidates are the epidermal stem cells. These may be equivalent to so-called "dark staining cells", which are present in large numbers in embryonic epidermis, and decrease throughout adulthood (Klein-Szanto *et al.*, 1980). These are visible in TPA-treated skin and are abundant in papillomas and carcinomas. Initiation of these cells might introduce a differentiation block thus complementing their inherent proliferative capacity. Initiation of suprabasal cells, commited to differentiation, would have to involve reinstatement of their proliferative ability. If cells from different levels of the epidermis can be initiated, this may help to explain the existence of subsets of papillomas with varying abilities to persist in the absence of promoter treatment and to progress to carcinomas (section 1.5.1).

1.5.3.1 Molecular events associated with tumour promotion

The range of cellular and biochemical changes which TPA can induce is so large (Hecker *et al.*, 1982; Slaga, 1983) that it is difficult to identify those which are responsible for this chemical's ability to promote tumour formation. Epidermal hyperplasia (biochemically detectable as increased DNA, RNA and protein synthesis) and ornithine decarboxylase (ODC) activity correlate well with promoter strength, but non-promoters can also induce these effects (Slaga, 1983). Following epidermal cell proliferation and ODC synthesis there is an induction of prostaglandins and polyamines, which also appears to correlate with promotion. Putrescine and prostaglandin E2 and F2 cannot substitute for TPA, but they enhance TPA promotion (Slaga *et al.*, 1982). Furthermore, inhibitors of polyamine and prostaglandin synthesis inhibit tumour promotion. Anti-inflammatory agents can also block promotion, which complements the finding that promoters decrease cellular

levels of superoxide dismutase and catalase; enzymes involved in the control of active oxygen. The relevance of free-radical generation to tumour promotion is further illustrated by tumour promoters such as benzoyl peroxide which, unlike TPA, do not act through protein kinase C. Induction of proteases and reduction of cAMP also appear to be important in promotion by TPA since agents which counteract this inhibit promotion.

The above biochemical findings have been known for several years and have often been reviewed. Some of the more recent discoveries, which can be related to the potential involvement of oncogenes and tumour suppressor genes in tumour promotion, are summarised below.

a) The role of positive and negative growth factors

Enhanced reaction to mitogenic factors (eg through oncogene activation)or refractoriness to differentiation signals (eg through tumour suppressor loss) may both contribute to the expansion of initiated cells. The action of TGF-β may represent one such negative control which initiated keratinocytes elude (Parkinson, 1985; Parkinson and Balmain, 1990). This polypeptide inhibits DNA synthesis in some human (Shipley *et al.*, 1986) and mouse keratinocytes (Coffey *et al.*, 1988). In addition it causes growth arrest and terminal differentiation of bronchial (Masui *et al.*, 1986) and tracheal epithelial cells (Terzaghi, 1989), and is located in differentiating cells of the small intestine epithelium (Barnard *et al.*, 1989). There have also been reports that it stimulates expression of certain keratinocyte differentiation markers (Reiss and Sartorelli, 1987; Mansbridge *et al.*, 1988).

Recently, it was shown that TPA induces high levels of TGF-β mRNA in suprabasal epidermal cells (Akhurst *et al.*, 1988). The block in basal cell DNA synthesis seen 3-9 hours after TPA treatment (Krieg *et al.*, 1974) could therefore be mediated by this growth factor. Complete or partial resistance to this effect could confer a growth advantage on initiated cells. Significantly, loss of responsiveness to the inhibitory effects of TGF-β has been observed in several tumour cell lines (Shipley *et al.*, 1986; Kimchi *et al.*, 1988; Wakefield *et al.*, 1988). Furthermore

resistance to the effects of TGF-β can be conferred on epithelial cells by introduction of activated *ras* (Reddel *et al.*, 1988; Houck *et al.*, 1989). This has important implications for mouse skin carcinogenesis where *ras* activation is frequently the initiating event (section 1.5.2.1; Balmain and Brown, 1988).

Initial inhibition of DNA synthesis by TPA (see above) is followed by a hyperproliferative phase. It is not clear what mediates this response, but it may in part involve TGF-α. This polypeptide is related to EGF, is frequently expressed by tumour cells (Derynck *et al.*, 1987; Derynck, 1988), and induces proliferation and neovascularisation (Schreiber *et al.*, 1986; Barrandon and Green, 1987). TGF-α is synthesised in normal skin and a role in stimulating keratinocyte proliferation after wounding has been suggested (Coffey *et al.*, 1987). That the actions of TGF-α and TGF-β cooperatively stimulate growth of initiated cells is supported by the finding that these two factors can mimic the convertogenic, or first, stage of promotion (Furstenberger *et al.*, 1989). The hyperplastic second stage promoter, RPA, can be used in place of TGF-α in this assay. Furthermore, a papilloma cell line transfected with TGF-α cDNA was found to produce larger papillomas when grafted onto mouse skin than its untransfected parent line (Finzi *et al.*, 1988).

Other growth factors which might play a part in skin tumour formation include the recently identified keratinocyte growth factor (KGF) and the so-called epidermal pentapeptide (EPP). The latter, like TGF-β inhibits epidermal cell growth and stimulates differentiation (Elgjo *et al.*, 1986). This molecule has been shown to enhance skin tumorigenesis in nude mice (Iversen *et al.*, 1989). KGF is related to the fibroblast growth factor (FGF) family (Finch *et al.*, 1989) and is a particularly interesting mitogen; it appears to be specific for epithelial cells (Rubin *et al.*, 1989) and is synthesised by stromal cells derived from epithelial tissues, suggesting a paracrine action (Finch *et al.*, 1989).

b) Genetic events during promotion

A significant amount of evidence now exists in support of the involvement of aneuploidy in the development of papillomas on mouse skin. Overcoming the

difficulties associated with karyotyping solid tumours, Slaga's group have established a correlation between increasing aneuploidy, papilloma age and degree of dysplasia (Conti *et al.*, 1986; Aldaz *et al.*, 1987). A small proportion of early papillomas are as aneuploid as some carcinomas, but whether these are equivalent to promoter-independent tumours (section 1.5.1) is not known. Recently, non-random, sequential trisomy of chromosomes 6 and 7 has been observed during papilloma development (Aldaz *et al.*, 1989). The possible connection between imbalances at the H-*ras* locus (Quintanilla *et al.*, 1986) and chromosome 7 changes is the subject of this thesis.

It is not known whether the progressive aneuploidy observed during mouse skin tumour progression is induced by TPA and/or if it occurs as a consequence of the transformed state. However, TPA has been shown to induce aneuploidy in yeast (Parry *et al.*, 1981), mouse epidermal cells (Dzarlieva and Fusenig, 1982; Petrusevska *et al.*, 1988) and human lymphocytes (Emerit and Cerruti, 1982).

Marczynska *et al.* (1988) have observed pulverisation and other chromosomal aberrations in TPA treated skin fibroblasts from patients with familial polyposis coli (FPC), suggesting that cells which are potentially preneoplastic may be particularly sensitive to TPA-inducible chromosome alterations. Thus, *ras* activation could conceivably be responsible for genetic destabilisation in mouse papilloma cells. However, *ras* activation alone does not appear to induce visible karyotypic abnormalities either in rat embryo fibroblasts (Muschel *et al.*, 1986), or in papillomas formed by grafting HaMSV infected keratinocytes onto athymic mice (Aldaz *et al.*, 1988). In contrast, aneuploidy was observed soon after introduction of v-H-*ras* into human bronchial epithelial cells (Yoakum *et al.*, 1987). Resolution of these discrepencies is only possible through the detection of non-random alterations and eventual identification of the critical genes involved.

c) Gene expression and tumour promotion

The transcription of numerous genes is altered by TPA, several of which have obvious connections with the regulation of growth and differentiation. Induction of

TGF-β has already been mentioned (section 1.5.3.1a; Akhurst *et al.*, 1988). Rapid elevation of c-*fos*, c-*myc* and c-*jun* expression following administration of TPA or growth factors has been observed in various cell types (Greenberg and Ziff, 1984; Skouv *et al.*, 1986; Lamph *et al.*, 1988). Appleby *et al.* (1989) have found that expression of v-*fos* in keratinocytes extends their survival *in vitro* (possibly indefinitely) and, when grafted onto syngeneic recipients, these cells produce an abnormal epithelium (but not tumours).

TPA also stimulates expression of the proteases transin (Matrisian *et al.*, 1986b) and collagenase (Whitman *et al.*, 1986). Induction of plasminogen activator has also been documented (Wigler *et al.*, 1978). Alteration of protease activity could affect tumour invasiveness (Mignatti *et al.*, 1986) and angiogenesis (Montesano *et al.*, 1985). A large number of other genes are also stimualted by TPA (see Angel *et al.*, 1987; Johnson *et al.*, 1987), but their role in promotion is unclear.

Most of these studies have been carried out *in vitro* and so their relevance to tumour promotion *in vivo*, and the mouse skin system in particular, has not been clarified. Relatively few studies have addressed this problem. Stimulation of ODC mRNA synthesis by TPA in mouse skin is well documented (Gilmour *et al.*, 1987), and a transient TPA-induced increase of transin mRNA has been reported (Matrisian *et al.*, 1986b). Expression of the latter metalloproteinase is confined to the basal cells of TPA-treated epidermis (Krieg *et al.*, 1988). Its overexpression has also been observed in carcinomas but not papillomas (Matrisian *et al.*, 1986a; Ostrowski *et al.*, 1989).

TPA-induction of c-*fos* and c-*myc* *in vivo* has also been documented (Rose-John *et al.*, 1988). In this case, sequential transient expression of c-*fos*, c-*myc* and ODC was observed. Two non-promoting hyperplastic agents could not mimic this effect.

Some attempts have been made to isolate new genes that are TPA sensitive. Johnson *et al.* (1987) used differential screening of fibroblast cDNA libraries to isolate two clones. One of these (TPA-S1) is stimulated by TPA, expression of the

other (TPA-R1) is repressed. Melber *et al.* (1986) used the same technique to isolate six clones (pmal1-6) which hybridised stongly to carcinoma RNA, but weakly to RNA from normal epidermis. One of these (pmal-4) was later identified as β-actin (Ostrowski *et al.*, 1989).

The TPA-responsiveness of many genes is mediated by a common promoter element; the AP-1 binding site (Angel *et al.*, 1987), although different TPA-responsive elements (TREs) have been identified (Chiu *et al.*, 1987). The AP-1 trancription factor was was later shown to be identical to the product of the c-*jun* (or *jun*-A) oncogene (Angel *et al.*, 1988). Subsequently, several related studies showed that the c-*fos* protein binds to c-*jun*/AP-1 to stimulate transcription of AP-1 responsive genes (Rauscher *et al.*, 1988; Chui *et al.*, 1988; Sassone-Corsi *et al.*, 1988).

d) Inhibition by normal cells

One of the main barriers preventing the clonal expansion of initiated cells is the inhibitory effect of surrounding normal cells. For example, Harvey sarcoma virus infected keratinocytes do not form tumours if transplanted onto mouse skin with a 4-fold excess of dermal fibroblasts, but form carcinomas if these normal cells are left out (Dotto *et al.*, 1988). The ability of TPA to overcome this effect has been demonstrated *in vitro* with *ras*-transfected C3H 10T1/2 mouse fibroblasts (Hsiao *et al.*, 1984) and rat embryo fibroblasts (Dotto *et al.*, 1985).

1.5.3.2 Mediation of the effects of TPA by PKC

The discovery that the major phorbol ester receptor and protein kinase C (PKC) were the same entity (Neidel *et al.*, 1983) implied that the pleiotropic response effected by TPA may be mediated by this ser/thr kinase. It is activated by diacylglycerol (DAG), one of the second messengers released by phosphoinositide metabolism (section 1.3.4.4, Nishizuka, 1988). DAG interacts with PKC at the same

site as phorbol esters, and may therefore activate this enzyme by a similar mechanism (Sharkey *et al.*, 1984).

It is now known that there are at least seven PKC subspecies (Nishizuka, 1988). The pattern of tissue expression and kinetics of activation are unique for each subspecies, except for the activity of βI and βII, which are indistinguishable. This may explain the diverse effects which TPA has on different cell types, although it is likely that some of the consequences of exposure to TPA are mediated by separate signalling pathways (see below).

The relevance of PKC to growth control is emphasised from studies on its over-expression in fibroblasts (Housey *et al.*, 1988; Persons *et al.*, 1988). Furthermore, a mutant form of PKC has recently been isolated from a murine UV-induced fibrosarcoma cell line which can transform Balb 3T3 fibroblasts (Megidish and Mazurek, 1989).

In addition to its role in stimulating many cellular activities, PKC also effects negative feedback on the biochemical pathway it is part of, and down-regulates several receptor proteins which can activate pathways that do not involve PKC. For example, PKC appears to down-regulate the IP3 signal by phosphorylating, and thus activating, IP3 phosphatase (Connoly *et al.*, 1986). PKC activation is also associated with phosphorylation and inhibition of the EGF, insulin and T-cell growth factor receptors (Davis and Czech, 1985; Takayama *et al.*, 1984; Cantrell *et al.*, 1985). In addition, PKC phosphorylates *ras* proteins (Ballaster *et al.*, 1986), although the function of this event is not known. It does not appear to alter GTPase or GTP binding activities (Ballaster *et al.*, 1986), and so it may interfere with some other property, such as membrane/effector binding (Saikumar, 1988). If this event down-regulates *ras*, it is conceivable that the oncogenic form of p21 is unaffected by it. However, there may be other ways of evading this potential inhibitory influence, including down regulation of PKC itself (see below).

Negative feedback by PKC suggests that the promotional effects of TPA might occur by a series of small intermittent pulses of activity, rather than a continuous

amplification of signal transduction pathways. However sustained stimulation may be possible since, in the long term, TPA induces complete removal of PKC from the cell, possibly through the action of the protease calpain I (Nishizuka, 1988). Significantly, rapid loss of PKC, and maintenance of this state for 3-4 days, has been observed after a single treatment of mouse skin with TPA (Fournier and Murray, 1987). This could maintain the growth factor receptors mentioned above (and possibly *ras*) in a sensitive state. The possible role of TGF-α in skin tumorigenesis (section 1.5.3.1a) is particularly interesting in this respect since it stimulates the EGF receptor which in turn stimulates DNA synthesis via a pathway which is both phosphoinositide and PKC-independent (Wakelam *et al.*, 1986; Lloyd *et al.*, 1989; Morris *et al.*, 1989).

1.5.4 Molecular events during malignant progression of mouse skin tumours

Malignant conversion is associated with invasion, metastasis and progressive loss of tissue organisation. In papillomas expression of keratins specific to basal cells (K5 and K14), the suprabasal layer (K1 and K10), and hyperproliferative skin (K6 and K16) can be detected (Knapp *et al.*, 1987). However, there is a dramatic reduction in expression of K1 and K10, but not the other keratins, in squamous carcinomas (Toftgard *et al.*, 1985) (Roop *et al.*, 1988). These results are consistent with the hyperplastic and altered differentiation state of malignant tumours. Possible molecular events responsible for this process are discussed below.

Double minute chromosomes, indicative of gene amplification, have been detected in approximately 10% of carcinomas, but not in papillomas (Aldaz *et al.*, 1986; Aldaz and Conti, 1989). However the genes involved have not been identified. A 2-10 fold amplification of the mutant H-*ras* gene has been observed in some carcinomas (Quintanilla *et al.*, 1986; Bremner and Balmain 1990; Brown *et al.*, 1990) suggesting that this gene may be involved at both the early and late stages of

tumour progression. There is also evidence implicating the involvement of mutant *ras* genes in the metastasis of mouse fibroblasts (Pozzati *et al.*, 1986; Egan *et al.*, 1987) and hamster glial cells (Fetherston *et al.*, 1989).

Amplification of N-*myc* and c-*neu* is associated with the advanced stages of lung and breast cancer respectively (Brodeur *et al.*, 1984; Slamon *et al.*, 1987). Analysis of the N-*myc* gene in mouse skin carcinomas has not been reported. The *neu* gene does not appear to be amplified (K. Brown, personal communication). However, c-*erb*B amplification has been detected in a cell line derived from a carcinoma of the Syrian hamster cheek pouch induced by repeated DMBA treatment, and its overexpression is associated with development of tumour invasiveness *in vivo* (Wong and Biswas, 1987).

The involvement of other oncogenes in the malignant conversion of mouse skin tumours, all of which cooperate with *ras* in the neoplastic transformation of fibroblasts (section 1.2.3) has been investigated by Yuspa's group. Introduction of v-*fos* into murine papilloma cell lines which contain an activated H-*ras* gene (Strickland *et al.*, 1988) resulted in malignant conversion of these lines, whereas cooperation with E1A or *myc* oncogenes was not observed (Greenhalgh and Yuspa, 1988). However, the resultant carcinomas lacked gamma-glutamyl tranpeptidase activity, which is present in 90% of all chemically induced mouse skin carcinomas (Chiba *et al.*, 1986), and so *fos* activation may not be commonly involved in malignant progression of mouse skin tumours.

Progressive aneuploidy is associated with the development of carcinomas (Aldaz and Conti, 1989). Frequently, they contain near-tetraploid cell populations in both chemically (Conti *et al.*, 1986; Aldaz *et al.*, 1987) and virally initiated tumours (Aldaz *et al.*, 1988). Studies of mouse carcinoma cell lines and malignant keratinocytes (transformed *in vitro*) have also identified near-tetraploidy as a common feature (Pera and Gorman, 1984; Fusenig *et al.*, 1985). In the latter study, under-representation of chromosomes 7 and 14 was detected in 6/9 of the cell lines analysed. Chromosomes 5 and 6 were frequently over-represented (Fusenig *et al.*,

1985). In comparison with karyotypic analysis of papillomas (section 1.5.3.1b; Aldaz *et al.*, 1989) these results suggest maintenance of the chromosome 6 status (over-representation), but reversal of the chromosome 7 status (over-representation –> under-representation) during the conversion to malignancy. However, events *in vivo* and *in vitro* may not be directly comparable.

The potential role of chromosome 7 changes in tumour progression and their effect on the *ras* locus is raised by the findings of Quintanilla *et al.* (1986). It was observed that while DMBA-initiated papillomas contained both mutant and normal H-*ras*, some carcinomas were homozygous for the mutant allele. Thus amplification of mutant *ras*, or loss of the normal allele or a linked suppressor gene were proposed as possible reasons for this event. The latter possibility is particularly intriguing in view of the homology between mouse chromosome 7 and the short arm of human chromosome 11 (Figure 5, section 3.2), which harbours at least two tumour suppressor genes (section 1.4.3a).

It is possible that malignant progression of mouse skin tumours involves the cumulative inactivation of several tumour suppressor genes, as has been suggested from analysis of various human cancers (Table 2, section 1.4.3). Very few candidate genes of this type have been investigated. The association of elevated transin expression with malignant conversion has already been mentioned (section 1.5.3.1c), as has the potential significance of loss of responsiveness to the negative growth regulator TGF-β (section 1.5.3.1a). Matrisian *et al.* (1986a) speculated that loss of a negative growth regulator for transin could mediate deregulation of its transcription.

Presumably, several of the classes of tumour suppressor genes described in section 1.4.5 could be involved in mouse skin tumorigenesis. In the near future animal models should provide a valuable resource for identifying and characterising these genes.

1.5.5 A new application of animal models: the study of tumour-related LOH

Animal model systems have been extensively utilised in the study of oncogene activation and function. However, the benefits of such models, including the ability to analyse specific stages of tumorigenesis and to control tumour-inducing agents, have not been exploited to investigate allele loss in animal tumours. The thesis presented here describes the development of the mouse skin carcinogenesis model for this purpose. Since the (mostly inbred) mice used in skin tumour studies are uniformly homozygous, the application of RFLP analysis to the study of allele loss has not been possible to date. In the work described here, this problem has been overcome by interbreeding different strains of mice to produce F1 hybrids that carry specific RFLP markers. Analysis of these markers in papillomas and carcinomas, induced by a variety of protocols, permits assessment of the contribution of non-random gene loss to tumour development.

Chapter 2

Materials and methods

2.1 Materials

All restriction enzymes were obtained either from Boehringer Manheim, Lewes, East Sussex, or from Pharmacia Ltd., Milton Keynes, Buckinghamshire. Proteinase K, RNase A and the Klenow fragment of *Escherichia coli* (*E. coli*) DNA polymerase were obtained from Boerhinger. Polynucleotide kinase was obtained from Pharmacia. *Taq* polymerase was from Anglian Biotech Ltd., Colchester. The Sequenase™ kit was provided by Cambridge Biosciences, Cambridge.

Deoxynucleotides were supplied by Boerhinger, and mixed hexanucleotides, for random-primed labelling, by Pharmacia.

Radio-isotopes were obtained from Amersham International PLC, Amersham, Buckinghamshire.

DNA size markers (λ/*Hind*III and 1kb ladder), agarose and urea were obtained from Gibco/Bethesda Research Laboratories, Paisley. Sea-Plaque agarose was obtained from ICN Biochemicals Ltd., High Wycombe.

Biogel A-1.5m agarose beads, for separation of labeled DNA probes from unincorporated nucleotides, was supplied by Biorad Ltd., Watford Hertfordshire.

Serum, media and supplements for cell culture were obtained from Gibco/Bethesda Research Laboratories, Paisley.

Bacto-tryptone, Bacto-agar and yeast extract were from DIFCO Laboratories, Detroit, Michigan, USA.

Guanidine thiocyanate was supplied by Fluorochem Ltd., Glossop, Derbyshire.

Phenol was obtained 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 Ltd., Thornliebank, Glasgow, or from Sigma Chemical Co Ltd., Poole,
Dorset.

Plastic-ware for cell culture was supplied by Nunc Intermed, Roskilde, Denmark and by Sterilin Ltd., Feltham, Middlesex.

Nude mice were supplied by Marlan Olac Ltd., Bicester, Oxon.

Biotrace™ and nitrocellulose membranes for nucleic acid blotting was obtained from Gelman Sciences Ltd., Broadmills, Northampton and Sartorius Instruments Ltd, Belmont, Surrey.

2.2 Preparation of DNA and RNA

2.2.1 Preparation of DNA and RNA from mouse tissue and tumours

This was carried out essentially as described by Balmain and Pragnell (1983). Tumour or mouse tissues were frozen in liquid nitrogen, ground into a fine powder and lysed in 5M guanidine thiocyanate, 50mM Tris-HCl (pH 7.0), 50mM EDTA and 5% (v/v) β-mercaptoethanol. This was made to 2% (v/v) sarcosine using 20% (w/v) sarcosine. It was found empirically that an appropriate final volume after lysis was approximately 8mls per 0.1g of tissue. Once lysed, samples were centrifuged (106400g, 36hours, 18°C) through a CsCl/50mM EDTA (pH 7.0) step gradient (CsCl upper layer, η = 3.925; lower layer, η = 4.025). Unused lysate was stored frozen at –20°C. Using this

approach DNA settles between the layers of CsCl while RNA is pelleted.

DNA was removed from the gradient, precipitated with 2 volumes of 70% ethanol, spooled out, washed in 70% ethanol, lyophilised briefly and resuspended in TE; 0.5% (w/v) SDS (1ml per 0.1g of tissue). This was made to 150mM NaCl; 50mM EDTA; 100µg/ml proteinase K and incubated at 37°C for 2 hours. TE buffer was added as necessary to reduce viscosity before further purification. The suspension was extracted twice with an equal volume of phenol/chloroform, and once with an equal volume of 25:1 (v/v) chloroform: isoamyl alcohol. Phase separation was achieved by centrifugation (1600g, 5min, room temperature), the upper aqueous phase being kept, the lower organic phase being discarded. DNA was precipitated by adjusting the solution to 0.3M sodium acetate (using a 3M stock solution) followed by addition of cold (-20°C) absolute ethanol. The DNA was then spooled out, washed in 70% ethanol, lyophilised briefly, resuspended in 100µl TE buffer per 0.1g of tissue, and stored at 4°C.

The RNA was dissolved in 0.6ml of water, precipitated in 3 volumes of 70% ethanol overnight at –20°C. It was then pelleted by centrifugation (16000g, 15min, 4°C) and resuspended in 100µl water per 0.1g of tissue. Precipitation was repeated with 2 volumes of ethanol at –20°C overnight, followed by centrifugation and resuspension as just described. RNA was stored frozen at –20°C.

Nucleic acid concentrations were measured by determining the absorbance at 260nm using the convention that an absorbance of 1 unit is equivalent to a double stranded DNA concentration of 50µg/ml and an RNA concentration of 40µg/ml (Maniatis *et al*, 1982).

2.2.2 Preparation of DNA and RNA from cultured cell lines

Adherent cultured cells grown on 175cm^2 flasks were lysed in 5ml of 5M guanidine thiocyanate, 50mM Tris-HCl (pH 7.0), 50mM EDTA and 5% (v/v) β-mercaptoethanol. This was collected and the flask rinsed with a further 2ml of the same lysis buffer. DNA and RNA were then extracted by treating the lysate exactly as described for tumour and tissue samples.

2.3 Transformation of bacterial cells with DNA

The following bacterial strains were used in transformations:

1. Two *E. coli* K12-derived strains (Yanisch-Perron *et al*, 1985).

   JM83: ara, Δ(lac-proAB), rpsL (=straA), θ80, lacZΔM15

   JM101: thi, SupE, Δ(lac-proAB), [F' traD36, proAB, lacI^{q}ZΔM15]

2. One *E. coli* K-12 x *E. coli* B hybrid (Bolivar & Backman, 1979).

   HB101: F-, hsdS20 (r_B^-, m_B^-), recA13, ara-14, proA2, lacY1, galK2, rpsL20 (Sm^r), xyl-5, mtl-1, supE44, λ$^-$

Preparation of transformation-competent cells was carried out essentially as described by Mandel and Higa (1970).

Fresh overnight cultures were diluted 1:100 in 50ml medium: L-broth (1% w/v bacto-tryptone; 0.5% w/v yeast extract; 1% w/v NaCl) for JM83 and HB101 cells; 2x TY (1.6% w/v Bacto-tryptone; 1% w/v yeast

extract; 0.5% w/v NaCl) for JM101 cells. The culture was grown to an OD_{600} of 0.3-0.4. Cells were pelleted by centrifugation (1000g, 5min, 4°C), resuspended in 1/2 volume of pre-cooled 50mM $CaCl_2$; 10mM Tris-HCl (pH8.0), incubated at 0°C for 30min, then pelleted (1000g, 5min, 4°C) and resuspended in 1/20th of the original volume of ice cold 50mM $CaCl_2$; 10mM Tris-HCl (pH8.0). Cells were used immediately or stored at 4°C for no longer than 24 hours.

100µl of ligation mixture, containing up to 100ng of DNA in TE buffer, was added to 100µl of competent cells, incubated on ice for 30min then heat shocked at 42°C for 2min.

For plasmid transformation, heat-shocked JM83 or HB101 cells were added to 1ml of L-broth; 0.2% glucose and incubated at 37°C (a) for 30min if the culture was to be spread on 9cm Petri dishes containing L-broth; 1.5% w/v agar; 100µg/ml ampicillin, or (b) for 60min if on dishes containing L-broth; 1.5% w/v agar and 15µg/ml tetracyclin. This incubation period allows for the expression of antibiotic resistance genes.

For M13 transfection, heat-shocked JM101 cells were added to a mixture containing 0.2ml of exponentially growing cells; 40µl 2% X-gal (5-bromo-4-chloro-3-indoyl-β-galactoside) in dimethlylformamide; 40µl 100mM IPTG (isopropyl-β-D-thiogalactopyranoside). The mixture was immediately added to 3ml molten H top agar (1% w/v Bacto-tryptone; 0.8% w/v NaCl; 0.8% w/v agar), mixed quickly and poured into a 9cm Petri dish containing solidified H agar (1% w/v Bacto-tryptone; 0.8% w/v NaCl; 1.2% w/v agar).

Plates were incubated inverted, at 37°C overnight.

2.4 Preparation of plasmid DNA

2.4.1 Preparation of plasmid DNA in small amounts

Minipreparation of plasmids was carried out essentially as described by Maniatis *et al*, (1982); the alkaline lysis method.

A single transformed bacterial colony containing the plasmid of interest was inoculated into 5ml L-broth containing either ampicillin at 100μg/ml or tetracyclin at 15μg/ml. The culture was incubated at 37°C overnight with vigorous shaking. Bacterial cells were pelleted from 1.5ml of the overnight culture by microcentrifugation (12000g, 3min, room temperature) and the pellet resuspended in 100μl of solution 1 (50mM glucose; 12.5mM Tris-HCl pH8.0; 10mM EDTA; 2mg/ml lysozome) by vortexing and incubated at room temperature for 5min to allow bacterial lysis. (0.5ml aliquots of the remaining 3.5ml of bacterial culture were mixed with 0.5ml glycerol and stored at –20°C.)

0.2ml freshly-prepared solution 2 (0.2M NaOH; 1% SDS) was added and mixed gently without vortexing followed by incubation on ice for 5min. The solution was neutralised by addition of 0.15ml of ice-cold 5M potassium acetate pH4.8 followed by thorough mixing and incubation on ice for 5min. Precipitated genomic DNA and proteins were removed by microcentrifugation for 5min. The supernatant was extracted once with an equal volume of phenol/ chloroform, plasmid DNA precipitated from the upper aqueous phase with 2 volumes of ethanol at room temperature for 2min and pelleted by microcentrifugation for 5min at room temperature. After rinsing with 70% ethanol the pellet was lyophilised for 5min and resuspended in 50μl TE buffer. The solution was made to 20μg/ml RNase A and incubated at

70°C for 1 hour (in addition to degrading RNA this also inactivates DNases). Plasmid DNA was stored at 4°C.

2.4.2 Preparation of plasmid DNA in large amounts

Bulk preparation of plasmid DNA was carried out using a scaled up version of the minipreparation method described in section 2.4.1.

Bacteria containing the plasmid of interest were taken from storage (section 2.4.1) and 0.1ml inoculated into 10ml of L-broth supplemented with 100μg/ml of ampicillin or 15μg/ml of tetracyclin. Alternatively, a culture was set up by innoculating the growth medium with a single colony of bacteria grown up from the transformation procedure (section 2.3). After overnight growth at 37°C with vigorous shaking, 5ml of the culture was transferred to an additional 500ml of identical medium and incubated overnight again under the same conditions. Bacteria were then pelleted by centrifugation (4000g, 10min 4°C), resuspended in 1/20 volume (25ml) of solution 1 (section 2.4.1), mixed vigorously by pipetting and left at room temperature for 10-15min to allow for bacterial lysis. 1/10 volume (50ml) of freshly prepared solution 2 (section 2.4.1) was then added, mixed gently (so as not to dislodge chromosomal DNA from cell fragments) and left on ice for 10min (or until the solution was clear indicating complete lysis). The solution was then neutralised by additon of 1/13 volume (38ml) of 5M potassium acetate pH4.8, mixed thoroughly and incubated on ice for 10min or longer. Precipitated genomic DNA was then removed by centrifugation (16000g, 10min, 4°C). The supernatant was carefully filtered through gauze and plasmid DNA precipitated by addition of 0.6 volumes of cold (-20°C) isopropanol followed by centrifugation (16000g, 10min, 4°C). The pellet was resuspended in 10mls of TE

buffer plus 1/2 volume of 7.5M ammonium acetate followed by centrifugation (16000g, 5min, 4°C) to remove precipitated protein. Plasmid DNA was precipitated by addition of 2 volumes of absolute ethanol and pelleted by centrifugation (16000g, 10min, 4°C). The pellet was dissolved in 6.8ml of TE buffer. After the additon of 7.2g CsCl and 0.6ml 10mg/ml EtBr the solution was centrifuged (140000g, 40 hours, 20°C). The plasmid band was removed by pipetting and extracted five times with an equal volume of propan-2-ol. Plasmid DNA was precipitated by addition of two volumes of 70% ethanol at –20°C for a maximum of 2 hours (any longer and the CsCl also precipitates), centrifuged (16000g, 20min, 4°C), and the pellet dried and resuspended in 0.25ml TE. Plasmid DNA was stored at 4°C and its concentration measured as described for eukaryotic DNA in section 2.2.1.

2.5 Preparation of bacteriophage λ DNA

Bacteriophage λ DNA was prepared essentially as described by Maniatis *et al*, (1982).

100ml of LAM [L-broth (section 2.3); 20% w/v maltose; 1M $MgSO_4$] was inoculated with a single colony of the bacterial host for the virus, DB102, and incubated at 37°C overnight. Cell concentration was then estimated on the basis that 1 OD_{600} = 8 x 10^8 cells/ml. 10^{10} cells were withdrawn, centrifuged (4000g, 10min, room temperature) and resuspended in 3ml 10mM $MgSO_4$. These cells were then infected with 4 x 10^7 phage (multiplicity of infection = 1:500) at 37°C for 15min. Infected cells were added to 500ml LAM and grown overnight at 37°C. Cells were lysed by addition of 10ml chloroform with shaking for 20min at 37°C. Cell debris was pelleted by centrifugation (7500g, 5min, 4°C)

and the supernatant treated with DNase and RNase (both 1μg/ml) for 1 hour at 37°C. The solution was adjusted to 1M NaCl; 10% (w/v) PEG 6000), left for a minimum of 3.5 hours at 4°C, centrifuged (11000g, 10min, 4°C) and the pellet resuspended in 15ml SM buffer (0.05M Tris-HCl pH7.5; 0.1M NaCl; 8mM $MgSO_4$; 0.01% v/v gelatin). The bacteriophage were then extracted with an equal volume of chloroform, centrifuged (1600g, 15min, 4°C), and the upper phase (containing phage) layered onto a CsCl step gradient previously overlayed with 0.75ml 20% (w/v) sucrose in SM. The CsCl gradient consisted of a top layer of 0.75ml (η = 1.362), a middle layer of 0.75ml (η = 1.381), and a bottom layer of 1ml (η = 1.392); in each case CsCl was dissolved in SM buffer. After centrifugation (80000g, 90min, 4°C) the bacteriophage (which form a bluish band at the interface between the top and middle CsCl layers) were removed using a micropipette and dialysed against 500ml 10mM NaCl; 50mM Tris-HCl pH 8.0; 10mM $MgCl_2$ for 2x 2 hours to remove CsCl.

Dialysed phage solution was made to 20mM EDTA; 50μg/ml proteinase K; 0.5% SDS, mixed thoroughly and incubated for 1 hour at 65°C. The solution was extracted twice with an equal volume of phenol: chloroform (50: 50) and once with chloroform, the aqueous phase being recovered each time after centrifugation (1600g, 5min, room temperature). Phage DNA was precipitated by adjusting the solution to 0.3M ammonium acetate, followed by addition of 2 volumes of absolute ethanol and incubation at –20°C overnight. After microcentrifugation (12000g, 25min, room temperature) DNA was washed in 70% ethanol, microcentrifuged again for 5min, lyophilised and redissolved in TE. The concentration was determined as described in section 2.2.1.

2.6 Restriction enzyme digestion of DNA

2.6.1 Complete digestion

Plasmid DNA was digested with 5-10 units enzyme/μg DNA for 1-3 hours under conditions specified by the supplier. Eukaryotic DNA was digested with 10 units enzyme/μg DNA overnight under conditions specified by the supplier. Digested DNA was precipitated by adjusting the solution to 0.3M sodium acetate followed by addition of two volumes of cold absolute ethanol, incubation at –20°C for 2-16 hours and microcentrifugation (12000g, 25min, room temperature). The pellet was washed in 70% ethanol, microcentrifuged for 5min, after which it was lyophilised briefly and dissolved in TE buffer.

2.6.2 Partial digestion

Partial digestion for restriction mapping of λN1 DNA (section 2.20) was carried out using 0.5μg of λN1 DNA and other constituents as prescribed by the enzyme manufacturers. Five reactions were set up, each with a different concentration of enzyme: 1 unit/μl, 0.2 units/μl, 0.04 units/μl, 0.008 units/μl and 0.0016 units/μl. Digestion was allowed to proceed for 1 hour at 37°C, terminated by heating for 10min at 70°C and the contents of all five reactions combined.

2.7 Agarose gel electrophoresis

Agarose gel electrophoresis of DNA for either analytical or preparative purposes was performed using a flat bed apparatus. Gels were made from 0.8-2.0% w/v agarose dissolved and cast in buffer: 1x

TAE (40mM Tris-HCl pH7.8; 20mM sodium acetate; 1mM EDTA when gels were required for Southern blotting (section 2.10); 1x TBE (90mM Tris-HCl; 90mM boric acid; 2,5mM EDTA, pH8.3) in all other instances. Gels were submerged in the appropriate buffer and wells loaded with DNA samples in TE buffer (3µl/µg of eukaryotic DNA, variable volumes for plasmid DNA) mixed with 1/6 volume of gel-loading dye (0.25% bromophenol blue; 0.25% xylene cyanol; 15% Ficoll type 400 in H2O). TAE gels were run at 2V/cm for 16-20 hours while TBE gels were run at 8-15V/cm for 30-60min. After electrophoresis gels were soaked in ethidium bromide solution (1µg/ml for 10min), washed briefly in water to remove excess ethidium bromide and the DNA visualised by illumination with short wave (312nm) ultra-violet light and photographed through a red filter using Polaroid type 57 high-speed film.

2.8 Isolation of plasmid insert DNA for use as radioactively labelled DNA probes

Electrophoresis of plasmid restriction digests (section 2.6) was carried out through 1xTBE (section 2.7); 0.6-1.5% Sea-Plaque agarose gels. The fragment of interest was excised after staining and photographing the gel (section 2.7). After adjusting the concentration to approximately 3ng/µl DNA inserts were ready for labelling using the random priming method (section 2.9).

2.9 Random priming using Klenow polymerase

Double stranded fragments for probing DNA blots (section 2.11) were labelled using mixed hexadeoxyribonucleotide primers of random sequence essentially as described by Feinberg and Vogelstein (1983 and 1984).

A Sea-Plaque agarose suspension containing 100ng of DNA was boiled at 100°C for 7min then labelled in 50µl of a solution containing 50mM Tris-HCl pH8.0; 5mM $MgCl_2$; 10mM β-mercaptoethanol; 4mM each of dATP, dGTP and dTTP; 0.2M HEPES (N-2-hydroxyethyl-piperazine-N'-2'ethane sulphonic acid) pH6.6; 110µg/ml mixed hexadeoxynucleotides; 0.4mg/ml bovine serum albumin. Labelling was with 1.85x106Bq (1.1x1014Bq/mmol) of [α-32P]dCTP using 5 units Klenow enzyme (labelling grade). Incubation was for 2.5 hours at room temperature.

Labelled probes were generally diluted to 0.3ml with water, denatured by boiling and used immediately. The percentage incorporation of labelled nucleotides was occasionally determined by Cerenkov counting. 1µl of the reaction was spotted onto a 2.5cm disc of DE-81 filter paper, dried and cpm determined using a scintillation counter set to measure 3H. Unincorporated nucleotides were then removed by 3-4 4min washes in 5ml 0.5M Na_2HPO_4. The filter was then washed twice with distilled water, twice in ethanol and once in ether then dried and cpm determined again. Percentage incorporation varied from 45-90%, although in most cases it was around 80%. If incorporation was below 50% unincorporated nucleotides were separated from labelled probe by applying the diluted reaction mixture to a column consisting of a Pasteur pipette filled with Biogel-A 1.5m

equilibrated with 0.1xSSC (20xSSC is 3M NaCl; 0.3M sodium citrate pH7.0); 0.1% SDS. Probe was eluted using this buffer, 0.2ml fractions collected and 2µl aliquots assayed by scintillation counting. Fractions containing labelled probe were pooled and used in hybridisation reactions (section 2.11).

2.10 Southern blot transfer of DNA

DNA fragments were separated by agarose gel electrophoresis (section 2.7) and transferred to Biotrace™ or nitrocellulose membranes by the method of Rigaud *et al*, (1987). Following ethidium bromide staining and photography, DNA was denatured by rinsing the gel twice in 0.5M NaOH; 1.5M NaCl for 20min each, followed by two 30min washes in transfer solution (1M ammonium acetate; 0.02M NaOH). The gel was then transferred to a raised platform covered with a sheet of Whatman 3MM paper soaked in transfer solution, such that the ends of the filter paper extended below the platform into a reservoir of this solution. To ensure that all movement of transfer solution occurred through the gel, it was surrounded with plastic sheets. A sheet of Biotrace™ membrane (pre-soaked in distilled water) was then placed on the gel. The membrane was then covered in 2 sheets of Whatman 3MM paper (pre-soaked in the transfer solution). Finally the contents of a box of tissues was placed on top of the filter paper and compressed with a 1kg weight. Tranfer was allowed to continue overnight after which the membrane was washed briefly in 2xSSC, baked at 80°C for 2.5-3 hours and stored in a sealed plastic bag at 4°C until required.

2.11 Hybridisation of radioactively labelled probes to Southern blots

2.11.1 Single copy probes

Filters were prehybridised at 42°C in a shaking water bath for a minimum of 4 hours in sealed plastic bags containing 20ml of pre-heated 50% formamide; 4x SSPE (20x SSPE is 3.6M NaCl; 200mM NaH_2PO_4 pH7.4; 20mM EDTA pH7.4); 0.5% (w/v) dried milk (Marvel); 1% SDS; 10% (w/v) dextran sulphate; 0.5mg/ml denatured salmon sperm DNA. Radio-labelled probe, made as described in section 2.9, was added after prehybridisation and hybridised overnight at 42°C. Filters were then washed in 1xSSC; 0.1% SDS, then 0.5xSSC; 0.1% SDS, both at room temperature for 15min, and finally, in 0.1XSSC; 0.1% SDS at 55-65°C for 30min, the actual temperature depending on the probe (*fes*, 50°C; H-*ras* and *Cas*-1, 55°C; *Int*-2, *Hbb* and IL3, 65°C). Excess moisture was blotted off the filter with Whattman 3MM paper after which it was wrapped in thin plastic then autoradiographed using Kodak X-OMAT AR and/or X-OMAT S film at –70°C with intensifying screens.

2.11.2 Satellite probes

Prehybridisation of filters that were to be hybridised with a satellite DNA probe was carried out in three stages. All prehybridisation buffers were pre-heated to 65°C before use. First the filter was incubated in a sealed plastic bag in 20ml 1x Denhardt's solution (100x Denhardt's is 2% bovine serum albumin; 2% polyvinylpyrollidone; 2% Ficoll pH8.0) at 65°C for 30min. Second , this solution was poured off and the filter incubated with 20ml filter mix (1x Denhardt's; 1xSSC;

0.1% SDS; 20ng/ml denatured salmon sperm DNA) at 65°C for 30min. Finally, after pouring off this solution, the filter was incubated with 20ml filter mix; 6% PEG 6000 at 65°C for 15min. (The PEG was always added to the filter mix immediately prior to use). Denatured radio-labelled satellite DNA probe was added to the plastic bag and hybridised overnight at 65°C. Filters were washed in 1xSSC; 0.1% SDS at room temperature for 15min, then in 1xSSC; 0.1% SDS at 65°C for 1 hour. Filters were autoradiographed as described above.

2.12 Densitometry/ preflashing film

Quantitative densitometric analysis of autoradiograph film images requires the relationship between the absorbance of the film image and the amount of radioactivity to be proportional (Laskey, 1980). This was achieved by preflashing Kodak X-OMAT S film (through exposure to a flash of light from a photographic flash unit lasting approximately 1msec) so that the absorbance of the film was increased by 0.15 (A_{540}) above the absorbance of unexposed film (Laskey, 1980). The flash unit was covered with yellow/orange paper to reduce light output and the distance from the film required to increase the absorbance by the appropriate amount determined empirically. Densitometry was carried out using a Molecular dynamics 300A laser densitometer.

2.13 Oligonucleotide synthesis

Oligonucleotides were synthesised on an Applied Biosystems 381A Synthesiser using the manufacturers protocols and reagents, and after deprotection, were used without further purification.

2.14 DNA amplificatin by the polymerase chain reaction (PCR)

DNA was amplified as described by Saiki *et al*, (1988). A 0.1ml reaction, containing 1μg of genomic DNA in 67mM Tris-HCl (pH8.8); 16.7mM NH_4SO_4; 6.7mM $MgCl_2$; 10mM β-mercaptoethanol; 6.7μM EDTA; 17μg of BSA; each amplimer at 1μM; each dNTP at 1.5μM; and overlayed by 100μl of paraffin oil, was incubated at 91°C for 7min. After allowing 3min at 45°C to allow amplimer annealing, 2 units of thermostable DNA polymerase from *Thermus aquaticus* (*Taq* polymerase) was added and the extension reaction caried out at 72°C for 2min. Reactions were subsequently subjected to 35 cycles, employing the following conditions: 91°C for 1min 30sec; 45°C for 1min 30sec; 72°C for 2min. Thermal cycling was controlled by a programmable heating block (Perkin Elmer-Cetus).

2.15 5' end-labelling of oligonucleotides

Oligomers, for use as hybridisation probes (section 2.16) and sequencing primers (section 2.18), were 5' end-labelled using (γ-32P)ATP and T4-polynucleotide kinase.

For hybridisation probes, 30ng oligomer was added to 1.5μl 10x kinase buffer (0.5M Tris-HCl pH 7.6; 0.1M $MgCl_2$; 50mM dithiothreitol; 1mM spermidine; 1mM EDTA), 5μl of 1.85x1014Bq/mmol [γ-32P]ATP, 20units of T4 polynucleotide kinase, made to 15μl with water and incubated at 37°C for 40min.

Sequencing primers were labelled by adding 40ng oligomer to 2μl 10x kinase buffer, 2μl 1.85x1014Bq/mmol [γ-32P]ATP, 10 units of

kinase, made to 20µl with water and incubated at 37°C for 40min. The reaction was terminated by addition of 3µl 0.1M EDTA and enzyme inactivated by heating at 70°C for 10min.

2.16 Oligonucleotide dot-blot hybridisations

Amplified DNA (2-5µl) was spotted onto Biotrace™ RP nylon membrane, and the filters dried at 80°C for 3 hours. Filters were prehybridised in 5xSSPE (20x SSPE is 3.6M NaCl; 200mM NaH_2PO_4 pH7.4; 20mM EDTA pH7.4)) containing denatured sonicated salmon sperm DNA (500µg/ml) at 56°C overnight. Radio-labelled oligomer probe (section 2.15) was then added (2ng/ml, specific activity 109 dpm/µg) and the filter hybridised overnight at 56°C. The filters were washed in 2xSSPE; 0.1% SDS for 2x 30min at 20°C, then in 5xSSPE; 0.1% SDS for 2x 30min at selective discriminating temperatures (see Table 3). Filters were autoradiographed by exposure to Kodak X-Omat AR and or X-omat S film at –70°C using intensifying screens.

2.17 Purification of amplified DNA for sequencing

Amplified DNA was separated from unincorporated nucleotides with BRL NACS PREPAC™ mini-columns, using the manufacturers instructions for recovery of doublestranded DNA of less than 1000bp.

Columns were hydrated in 2M NaCl; TE buffer, and equilibrated in low salt buffer (0.2M NaCl; TE buffer). 80-90% of the 100µl sample obtained from amplification (section 2.14) was made to 0.2M NaCl; TE and loaded onto the column. The column was washed through with

A)

Amplimer	Region	Sequence
Ex1A	Codon	CTTGGCTAAGTGTGCTTCTCATT
12B	12/13	CACCTCTATAGTGGGATCATACTCGTC
Ex2A	Codon	CTAAGCCTGTTGTTTTGCAGGAC
Ex2B	61	GCTAGCCATAGGTGGCTCACCTG

B)

	Sequence							Discriminating temperature (°C)
Codon:	9	10	11	12	13	14	15	
Normal	TG	GGC	GCT	GGA	GGC	GTG	GG	67
Mutant				GAA				65
				GTA				65
				GCA				67
				CGA				67
				AGA				65
					GNC			63
					NGC			63

C)

	Sequence							Discriminating temperature (°C)
Codon:	58	59	60	61	62	63	64	
Normal	ACA	GCA	GGT	CAA	GAA	GAG	TA	61
Mutant				AAA				59
				GAA				61
				CGA				62
				CCA				62
				CTA				61
				CAC				62
				CAT				61

Table 3 Sequence of oligomers used as primers for PCR amplification and to detect point mutations in the H-*ras* gene

a) The oligonucleotides were based on the sequence of the mouse c-H-*ras* gene (Brown *et al*, 1988). Ex1A corresponds to the intron sequence immediately 5' to the second exon, while 12B hybridises to the last 27bp of the second exon. Ex2A and Ex2B correspond to intron sequences immediately outwith exon 2. The letter A signifies the 5'-amplimer and B the 3'-amplimer.

b) Sequence of oligomers used to detect point mutations at codons 12 and 13.

c) Sequence of oligomers used to detect point mutations at codon 61.

Oligomers containing thymidine at the first base of either codon 12 or codon 61 were omitted since these would detect nonsense codons. Also omitted were probes for mutations in the third base of codons 12 or 13, as no change in amino-acid would occur. Probes 13A and 13B were each mixtures of oligomers, with N representing either A, C or T.

3-5ml low salt buffer, and DNA eluted using 0.2ml high salt buffer (1M NaCl; TE). The amount of DNA present was quantified approximately by spotting 2µl of each sample onto a piece of plastic wrap, along with a series of DNA spots of equal volume (2µl) but various amounts (1, 2, 10, 20 and 40ng). To each spot an equal volume of TE containing 2µg/ml of ethidium bromide was added and mixed. The spots were photographed as described for agarose gels in section 2.7 and the amount of DNA in the samples estimated by comparison with the standards. Using this approach it was estimated that approximately 0.5µg of DNA was recovered from the columns. DNA was precipitated by adjusting the samples to 0.01M $MgCl_2$ followed by addition of 2 volumes of cold ethanol and incubation at –20°C overnight. DNA pellets were microcentrifuged (12000g, 25min, room temperature), washed in 70% ethanol, microcentrifuged again for 5min, lyophilised and redissolved in 10µl TE.

2.18 Sequencing of amplified DNA

5µl of end-labelled primer (2ng/µl; section 2.15) was annealed with 5µl purified amplified 150bp DNA fragment (50ng/µl; section 2.17) in a total volume of 12µl of a 10mM Tris-HCl pH8.5; 10mM $MgCl_2$ solution by heating at 100°C for 3min and cooling immediately on ice. 2µl of Sequenase™ (0.5units/µl) was then added to the annealed DNA. Sequencing reactions were then initiated by adding 3.5µl of this solution to each of four tubes containing 2µl of the following mixtures of deoxyribonucleotides (dNTPs) and dideoxyribonucleotide (ddNTP):
Tube A 80µM dATP; 80µM dTTP; 80µM dGTP; 80µM dCTP; 8µM ddATP; 50mM NaCl

Tube T 80μM dATP; 80μM dTTP; 80μM dGTP; 80μM dCTP; 8μM ddTTP; 50mM NaCl

Tube G 80μM dATP; 80μM dTTP; 80μM dGTP; 80μM dCTP; 8μM ddGTP; 50mM NaCl

Tube C 80μM dATP; 80μM dTTP; 80μM dGTP; 80μM dCTP; 8μM ddCTP; 50mM NaCl

After incubation at 37°C for 5min the reactions were terminated by addition of 4μl of stop solution (95% formamide; 20mM EDTA; 0.05% w/v Bromophenol Blue; 0.05% w/v Xylene cyanol). Samples were denatured by heating at 80-90°C for 5min and run on an 8% denaturing polyacrylamide gel (section 2.19).

2.19 Denaturing polyacrylamide gel electrophoresis

Polyacrylamide gels for running sequenced DNA samples (section 2.18) were made by mixing 50ml of 8% w/v acrylamide; 0.4% w/v bisacrylamide; 8M urea; 1x TBE pH8.3 with 200μl of 10% ammonium persulphate and 25μl of TEMED (N,N,N',N'-tetra-methylethylenediamine). Immediately after mixing these reagents, gels were cast by pouring between two glass plates separated by 0.4mm plastic spacers, and a well-former inserted. Once solidified the gel was placed on a vertical apparatus with each end submerged in a reservoir of 1x TBE buffer. Gels were run at 40W for 2 hours after which they were transferred to Whatman 3MM paper for drying. Autoradiography was carried out as described in section 2.11.1.

2.20 Restriction mapping of λN1 by hybridisation with cos ends

The λN1 clone was mapped by hybridisation of radio-labelled oligonucleotides (complementary to the cos ends of bacteriophage λ) to partial digests of λN1 essentially as described by Rackwitz *et al*, (1984). Following hybridisation, gel electrophoresis, and autoradiography the restriction map was read from the ladder of partial digestion products.

Cos-L (5'-dAGGTCGCCGCCC-3') and Cos-R (5'-dGGGCGGCGACCT-3') oligonucleotides were supplied by K. Brown and 5' end-labelled as described for oligomer hybridisation probes in section 2.15 0.12pmol of radio-labelled cos-L or cos-R was mixed with 0.15μg of partially digested λN1 DNA (section 2.6.2) and NaCl and water added to obtain 10μl of 100mM NaCl. The mixture was incubated for 4min at 68°C and immediately transferred to a 45°C waterbath for 30min. 5μl of gel-loading buffer (section 2.7) was added and the mixture loaded onto a 0.5% agarose gel. Electrophoresis was carried out at 1.5V/cm for 36 hours in 1x TAE. The gel was dried onto Whatman DE-81 cellulose paper and autoradiographed as described in section 2.11.1.

2.21 Eukaryotic cell culture

2.21.1 Explants from primary tumours

Tumour explants were initially grown in Special Liquid Medium (SLM) supplemented with 20% v/v foetal bovine serum (FBS); 4mM glutamine; 5μg/ml penicillin; 100μg/ml streptomycin in 5% CO_2/95%

air at 37°C. The concentration of FBS was reduced to 10% once the tumour cell line was established (passage level 2-3). Explants were photographed, using a Leitz Wetzler Diavert microscope with polaroid land attachment at 60x and 120x magnification in phase. Photographs were taken at the initial stage of growth and just before storage in liquid nitrogen and/or lysis for preparation of DNA.

All cells were passaged by first washing with PBS (phosphate buffered saline: 0.14M NaCl; 27mM KCl; 10mM Na_2HPO_4; 15mM K_2HPO_4), followed by removal of cells with trypsin solution (0.025% w/v in citrate buffer, pH 7.8). Trypsin was inactivated by addition of a 5-10 fold volume of culture medium, the mixture transferred to a new flask and medium added to the appropriate volume. If required, an aliquot of trypsinised cells was removed for determination of cell number using a Coulter counter.

One explant, XMSC1, was grown using feeder cells (grown and irradiated by K. Parkinson) at 1.5 x 10^4cells/cm^2.

2.21.2 Tumour growth in nude mice

As a test for tumo rigenicity and as a purification step, some cell lines were grown in nude mice. Cells were trypsinised, counted, centrifuged (1000g, 5min, room temperature), and redissolved in SLM at 2 x 10^7cells/ml. 4-6 week old nude mice were then injected subcutaneously with 2 x 10^6 cells at two points. If required, explants were grown from nude mouse tumours as described in section 2.21.1.

2.21.3 Virus preparation

HaMSV was isolated from non-producer NIH 3T3 cells after rescue with Friend murine leukemia virus (obtained from I.B. Pragnell).

Friend helper virus was obtained from SC1 cell lines (clones 643/22 F and 643/22 N) infected with Friend murine leukemia virus (from I.B. Pragnell and W. Ostertag) (Ostertag *et al*, 1980). Virus-containing supernatants were concentrated as described by Ostertag *et al*, (1980).

2.22 Ploidy determination

Ploidy of cell lines was determined by flow cytometry using the Becton-Dickinson FACS II. Cells were trypsinised, diluted with SLM; 10% FBS, centrifuged (250g, 5min, room temperature) and resuspended vigorously in 10ml cold PBS. Centrifugation and resuspension were repeated, and, after centrifuging for a third time, cells were fixed by resuspending vigorously in 5ml cold 70% methanol. 5 x 10^6 cells were pelleted by centrifugation as above and resuspended in staining solution (20μg/ml chromomycin A3; 7.4μM $MgCl_2$) to 1 x 10^6cells/ml. The chromophore was excited using a wavelength of 458nm.

Mouse keratinocytes were used as a standard in ploidy determination. Mouse dorsal skin was shaved and treated with a depilatory agent. 48 hours later mice were sacrificed, the dorsal epidermis scraped off and placed in a beaker of distilled water (1ml/mouse skin) at 55°C for 30sec then cooled on ice. The sample was then homogenised (4 strokes maximium speed), centrifuged (900g, 10min, room temperature) and resuspended in 0.18M Tris-HCl (pH7.5), 1mg/ml RNase at 37°C for 1 hour. Cells were then centrifuged, (400g, 5min, room temperature) and resuspended in staining solution.

2.23 Animals

2.23.1 Sources

BALB/c and C57BL/6J mice were obtained from Olac. NIH/Swiss mice were originally obtained from Olac and have been bred in the Beatson for 8 years. SENCAR mice were obtained from A. Kinsella at the Paterson laboratories. NMRI mice were a gift from the School of Tropical Medicine, Liverpool. 129 and TFH mice were a gift from J. Peters at the MRC Radiobiology Unit, Harwell. *Mus spretus* x CBA/J hybrids were a gift from G. Bulfield at the AFRC, Edinburgh.

2.23.2 Tumour induction

8-12 week old mice were used for tumour induction in every case. The dorsal skin was shaved and the mice initiated 24 hours later. Promotion was started 1 week after this, except in the virus initiated group where promotion was started 24 hour after initiation. Protocols used were as follows:

1. Initiation: 25µg DMBA in 200µl acetone.
   Promotion: 10^{-4}M TPA in 200µl acetone twice weekly.
2. Initiation: 25µg DMBA in 200µl acetone.
   Promotion: 25µg DMBA in 200µl acetone twice weekly ; this dose irritated the skin and was therefore reduced 4-fold 4 weeks after initiation.
3. Initiation: 600µg MNNG in 200µl acetone.
   Promotion: 10^{-4}M TPA in 200µl acetone twice weekly.
4. Initiation: 600µg MNNG in 200µl acetone.
   Promotion: 600µg MNNG in 200µl acetone twice weekly; this dose irritated the skin and was therefore reduced

4-fold 3 weeks after initiation.

5. Initiation: Mice were treated with 10^{-4}M TPA in 200µl acetone, then 24 hours later mice were ether-anesthetised and treated with 100µl of HaMSV viral supernatant (0.2-1 x 10^6 focus forming units/ml) containing polybrene (2µg/ml) by scarification using a 25 gauge needle attached to a 1ml syringe.

   Promotion: 10^{-4}M TPA in 200µl acetone twice weekly.

6. Initiation: 25µg DMBA in 200µl acetone.

   Promotion:25µg DMBA in 200µl acetone once a week and 10^{-4}M TPA in 200µl acetone once a week.

7. Initiation: 25µg DMBA in 200µl acetone.

   Promotion: 25µg DMBA in 200µl acetone twice weekly. The DMBA concentration was raised 4-fold 5 months after initiation since no tumours had developed on the CBA x *Mus spretus* mice that received this treatment.

2.24 Histology

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

Chapter 3

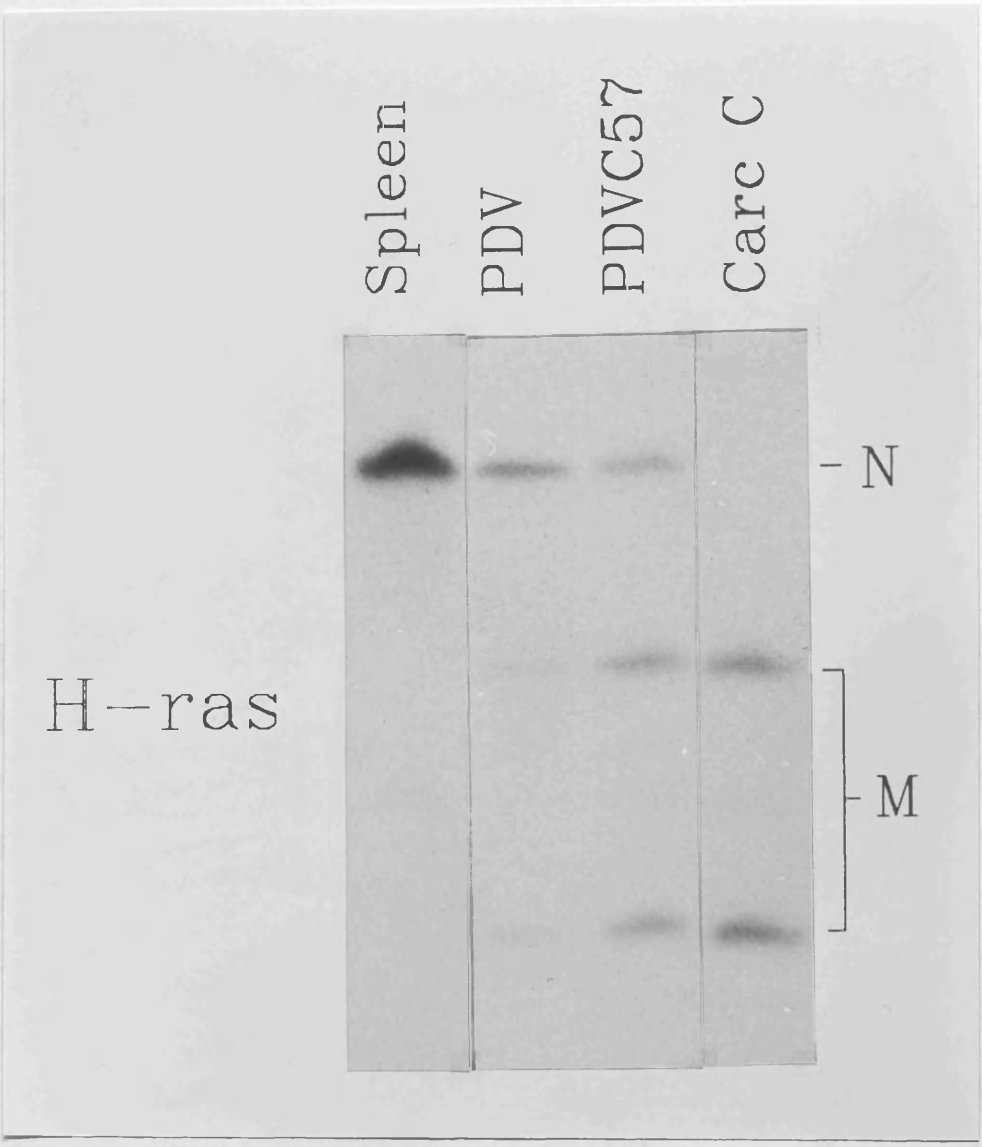
Results

3.1 Loss of normal *ras* genes in skin carcinomas in inbred mice

Initiation of skin carcinogenesis by treatment with 7,12-dimethyl benz[a]anthracene (DMBA) and promotion with TPA leads to formation of tumours with H-*ras* genes activated by an A:T->T:A transversion at codon 61 (Quintanilla *et al.*, 1986; Bizub *et al* 1986). This mutation introduces a new restriction site for the enzyme *Xba*1, which therefore constitutes a tumour-specific RFLP. Quintanilla *et al.* (1986) showed that the mutation appears to be heterozygous in most premalignant papillomas, but can be homozygous or amplified in some carcinomas. Analysis of primary tumours can be complicated by the presence of contaminating stromal cells, leading to the presence of faint bands corresponding to normal H-*ras* alleles on Southern blots. Complete loss of the normal allele in carcinomas has been confirmed by investigating early passage cell lines derived from carcinomas. Figure 4 shows that the cell line carc C, derived from a primary carcinoma induced in an NIH mouse, has totally lost the normal *ras* allele. Similar results were obtained in at least 30-40% of carcinomas from NIH mice (P. Burns, personal communication). Loss of normal H-*ras* genes is therefore a relatively frequent event in this mouse strain.

Figure 4 also shows analysis of a cell line (PDV) transformed *in vitro* by DMBA treatment (Fusenig *et al.*, 1985) and a derivative line (PDVC57) obtained by transplantation in a syngeneic host. PDV cells are known to contain 3 copies of mouse chromosome 7 (Fusenig *et al.*, 1985). It can be seen that 2 of the chromosome 7 homologues carry normal H-*ras* genes whereas 1 exhibits the codon 61 RFLP. The approximate 2:1 ratio of normal to mutant alleles was confirmed by densitometric scanning of amplified PDV DNA probed with oligomers which distinguish the normal and mutant alleles (A. Buchmann, personal communication). The PDVC57 line, on the other hand, displays an excess of mutant H-*ras* alleles, in accordance with its more aggressive tumorigenic properties. Full details of the derivation and

Figure 4 Loss of heterozygosity at the H-*ras* locus



Varying ratio of mutant:normal H-*ras* genes in two transformed keratinocyte cell lines (PDV and PDVC57), and complete loss of the normal allele in a cell line (Carc C) grown from a DMBA/TPA carcinoma obtained from an NIH mouse. DNA samples were digested with *Xba*I.

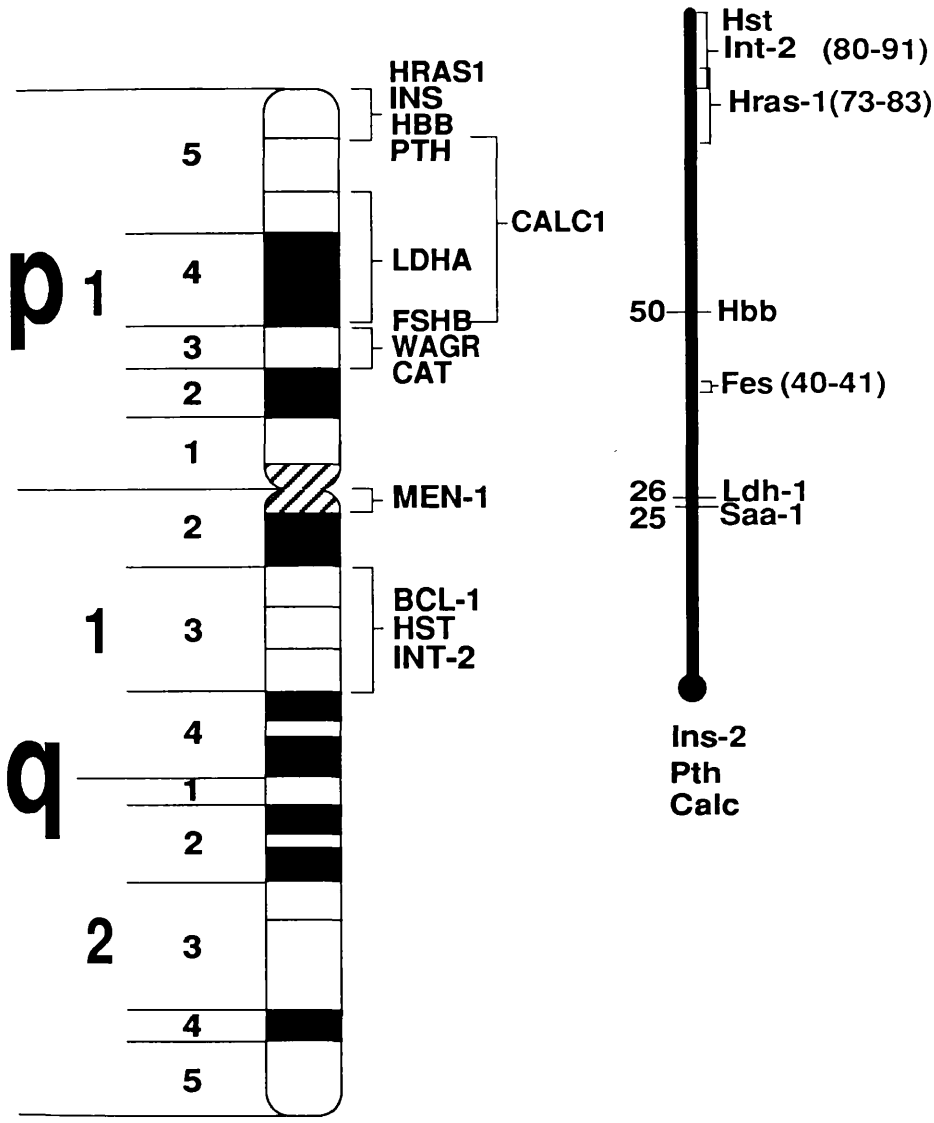
properties of these cell lines will be reported elsewhere (Quintanilla *et al.*, in preparation), but they serve as useful controls for the RFLP analysis described below.

3.2 The use of hybrid mice to analyse mechanisms of allele loss

The above results showed that the normal *ras* allele is lost in some tumours carrying the codon 61 RFLP. At least three possible explanations can be envisaged for this event. First, increase in the expression level of the mutant H-*ras* gene may give tumour cells a growth advantage. Second, the normal allele may exert a "tumour suppressive" influence, such that its loss contributes directly to progression of neoplasia. Finally, the gene may be lost by virtue of linkage to a true tumour suppressor locus on the same chromosome. It is noteworthy that mouse chromosome 7 is syntenic with a group of genes on the short arm of human chromosome 11 (Figure 5). This group includes several loci close to H-*ras* at human 11p15, a region which frequently shows loss of heterozygosity both in Wilms' tumours (Mannens *et al.*, 1988) and in a variety of other neoplasias (Koufos *et al.*, 1985; Scrable *et al.*, 1987).

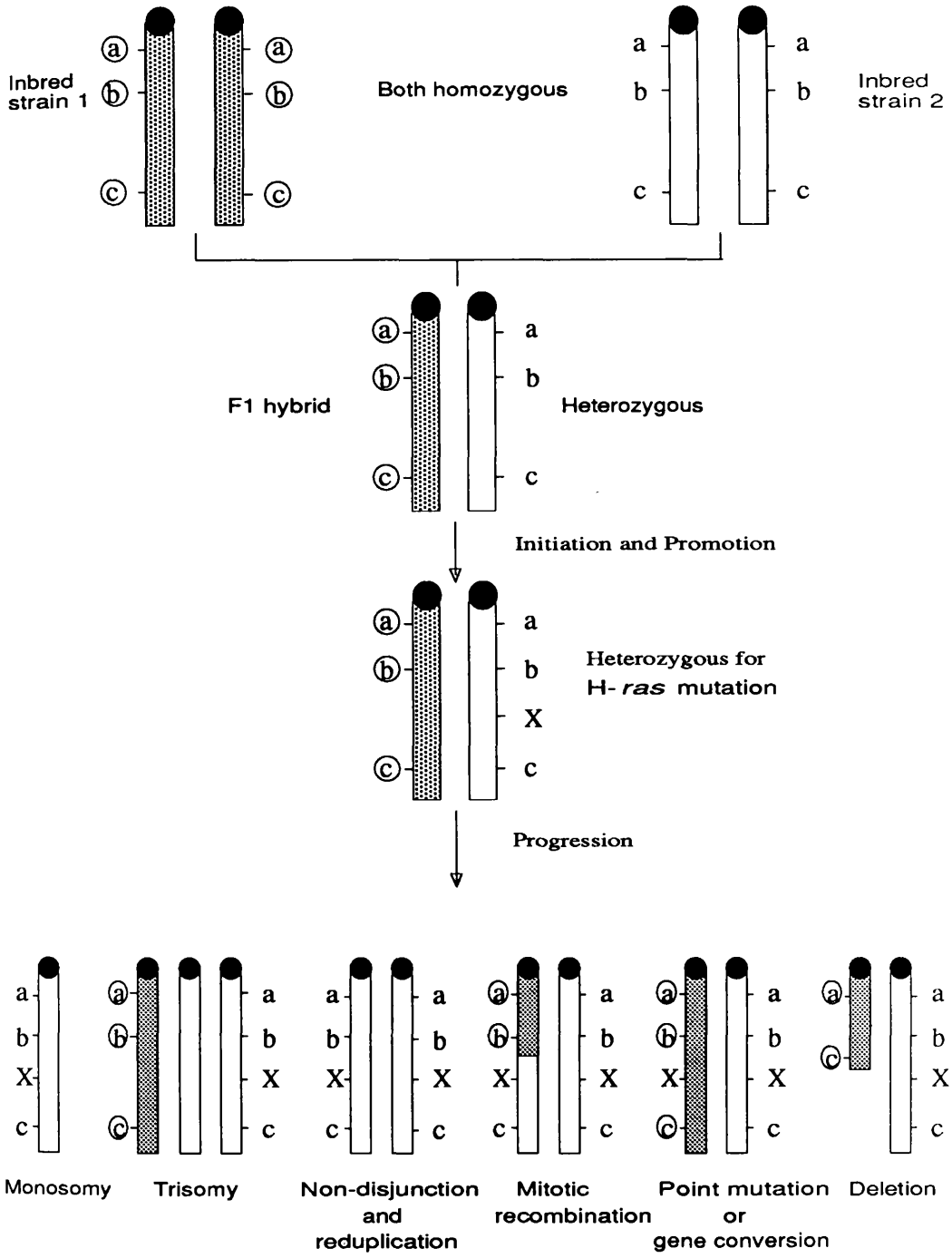
Tumours in F1 hybrid mice offered the possibility of determining whether loss of chromosome 7 alleles is always associated with the presence of a mutant H-*ras* gene, or, as might be expected if a tumour suppressor gene is involved, occurs also in tumours in which *ras* activation is undetectable. The strategy used is outlined in Figure 6.

Figure 5 Comparison of human chromosome 11 and mouse chromosome 7



The maps are an update of those described by Searle et al., (1989). The mouse centromere is represented by a black dot. Distances in centimorgans from the mouse centromere are shown on the left. Those in brackets on the right are approximate and are based on data kindly provided by T. Glaser. Loci known to be on mouse chromosome 7 but otherwise unlocated are listed below the chromosome.

Figure 6 The F1 hybrid tumour model



Interbreeding inbred strains of mice generates F1 hybrids polymorphic at theoretical loci a, b and c. Following H-*ras* activation, the mechanism responsible for loss or under-representation of the normal allele can be determined by tumour RFLP analysis. X = mutant H-*ras* allele.

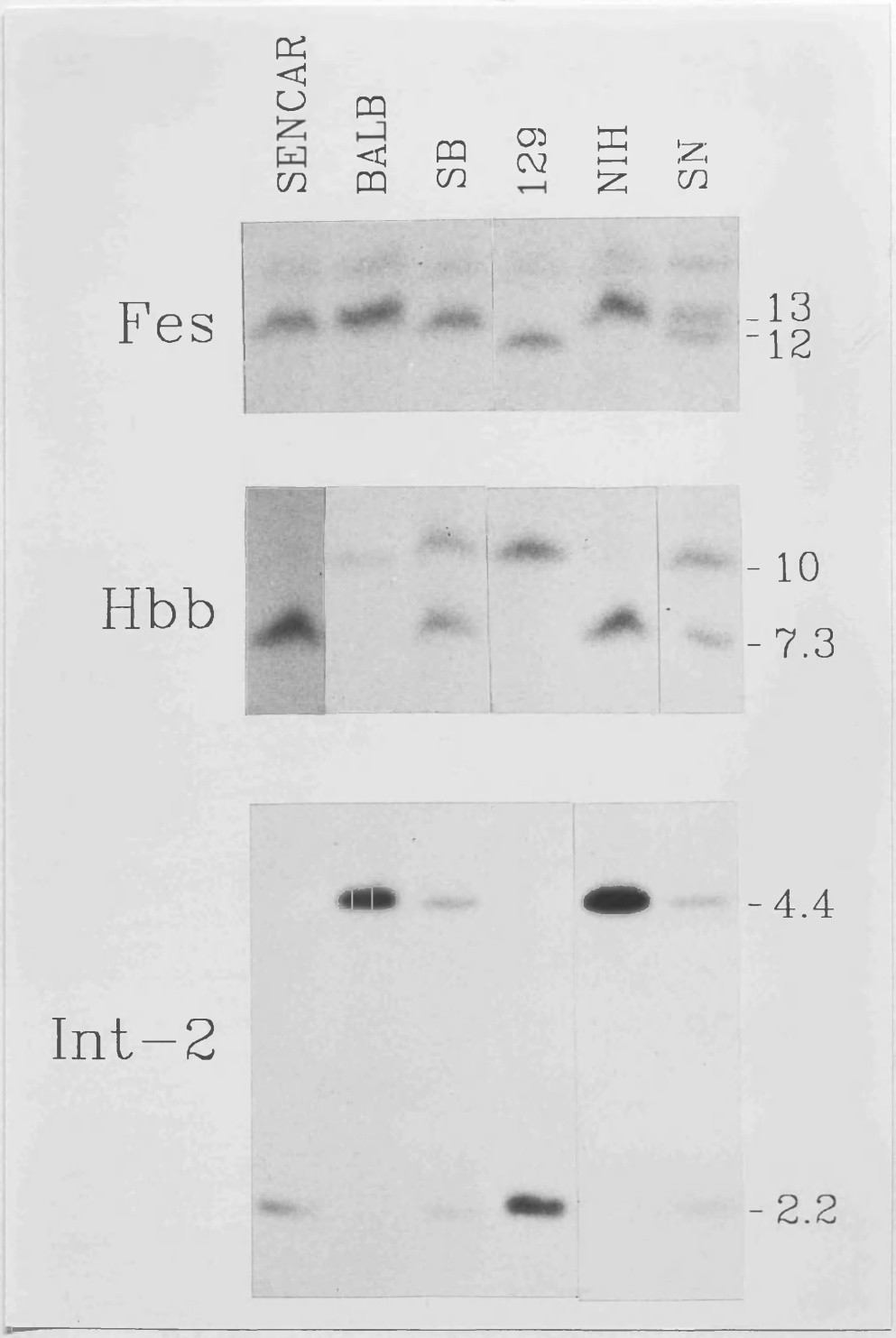
3.3 Detection of RFLPs and choice of mouse hybrids

Seven *Mus musculus* strains were screened for chromosome 7 RFLPs using probes for serum amyloid A (*Saa*-1), *fes*, H-*ras*, β-haemoglobin (*Hbb*), calcitonin (*Calc*) and *Int*-2 genes. The location of these genes is shown in Figure 5. The strains used were: BALB/c, C57BL/6J, NIH/Swiss, NMRI, SENCAR, 129 and TFH. Much of the previous work on mouse skin carcinogenesis has been carried out using some of these strains (Slaga, 1984). *Mus spretus* mice were also screened for polymorphisms since these mice have diverged significantly from *Mus musculus*, thus increasing the chance of finding RFLPs.

Based on preliminary RFLP data, two *Mus musculus* hybrids were set up for tumour induction: 129 x NIH (SN) and SENCAR x BALB/c (SB). The chromosome 7 RFLPs which were used to analyse tumours from these hybrids are shown in Figure 7. The *fes*/*Eco*RI and *Int*-2/*Pst*I RFLPs have been reported elsewhere (Blatt *et al.*, 1984; Silver and Buckler, 1986), but the *Hbb*/*Xba*I RFLP has not. *Mus spretus* x CBA (MSC) hybrids were also used for tumour induction and are discussed in more detail in section 3.4.4.

A complete summary of the chromosome 7 RFLP search is given in Table 4. No RFLPs were found in *Mus musculus* strains at H-*ras*, *Calc* or *Saa*-1, despite a previous report of a *Hind*III RFLP for the latter (Blatt *et al.*, 1984). Probes for the H-*ras* locus included the gene itself and two upstream fragments p6 and p11. These were previously subcloned from the λ-clone containing mouse H-*ras* into pUC8 (by G. Moffat). These were characterised along with other *Bam*HI and *Hind*III subclones. Their position in the original λ-clone containing the H-*ras* gene was mapped by analysis of partial digests of the clone hybridised to labelled cos L and R probes and run on an agarose gel (Figure 8). The absence of polymorphisms upstream of H-*ras* suggests that this region is highly conserved and may contain important regulatory sequences and/or another gene.

Figure 7 Chromosome 7 RFLPs used to analyse tumours



SN mice are polymorphic for *fes*, *Hbb* and *Int*-2. SB hybrids are only polymorphic for *Hbb* and *Int*-2. The probe used is indicated at the left of each panel (For details of clones see Table 4). Fragment sizes are indicated to the right in kb. Genomic DNA was digested with *Eco*RI for *fes* analysis (0.7% agarose gel), *Xba*I for *Hbb* and H-*ras* analyses (1% agarose gel), and *Pst*I for *Int*-2 analysis (1% agarose gel). BALB = BALB/c; SB = SENCAR x BALB/c hybrids; NIH = NIH/Swiss; SN = 129 x NIH/Swiss hybrids.

Table 4 Chromosome 7 RFLPs

* = RFLP *s = *spretus* RFLP ND = not determined

... = pattern as in mouse to left Sizes are in Kb

1. Locus: H-*ras*

Probe: 300bp insert of BS9 (Ellis *et al.*, 1980)

Enzyme	*Mus musculus* BALB	C57	NIH	NMRI	SENCAR	TFH	129	*Mus spretus*
Bam HI	3.4	...	...	...	...	...	ND	...
Bcl I	9.0	...	...	...	...	...	ND	...
Bgl I	1.2	...	...	...	...	...	ND	...
*s *Bgl* II	11.5	...	...	...	...	...	ND	10
*s *Eco* RI	23	...	...	...	...	...	...	22
*s *Hinc* II	9.0	...	...	...	...	...	ND	10
Hind III	4.7, 2.8	...	...	...	...	...	ND	...
Kpn I	6.5	...	...	...	...	...	ND	...
Msp I	0.8	...	...	...	...	...	ND	...
Pst I	2.0	...	...	...	...	...	ND	ND
Pvu II	1.1	...	...	...	...	...	ND	...
Sac I	6.0, 0.7	...	...	...	...	...	ND	...
*s *Sin* I	0.6	...	...	...	...	...	ND	1.3
Taq I	1.6	...	...	...	...	...	ND	...
*s *Xba* I	12	...	...	...	...	...	...	13

(CBA *Xba* I band also determined: 12Kb)

2. Locus: H-*ras*

Probe: 1.8Kb *Bam* HI insert of p6 (Figure 8)

Enzyme	*Mus musculus* BALB	C57	NIH	NMRI	SENCAR	TFH	129	*Mus spretus*
Bam HI	1.8	...	...	...	...	...	ND	ND
Bcl I	9.0	...	...	...	...	...	ND	...
Bgl I	4.0	...	...	...	...	...	ND	...
Bgl II	2.5	...	...	...	...	...	ND	ND
*s *Eco* RI	23	...	...	...	...	...	ND	22
Hinc II	5.0	...	...	...	...	...	ND	ND
Hind III	2.8, 0.7	...	...	...	...	...	ND	ND
Msp I	0.9, 0.6, 0.4	...	...	...	...	...	ND	ND
Pst I	2.0	...	...	...	...	...	ND	ND
Sac I	5.0	...	...	...	...	...	ND	ND
Taq I	2.4	...	...	...	...	...	ND	ND
*s *Xba* I	12	...	...	...	...	...	...	13

3. Locus: H-*ras*
Probe: 3.5Kb*Bam*HI insert of p11 (Figure 8)

Enzyme	*Mus musculus* BALB	C57	NIH	NMRI	SENCAR	TFH	129	*Mus spretus*
*Bam*HI	3.5	...	...	...	...	...	ND	ND
*s *Bcl* I	8.0	...	...	...	...	...	ND	4.7
Bgl II	1.5, 1.3, 0.7	...	...	...	...	...	ND	...
*Eco*RI	24	...	...	...	ND	ND	ND	ND
*Hinc*II	4.5, 3.2, 2.3	...	...	...	...	...	ND	ND
*Hind*III	14, 1.6, 1.1	...	...	...	(+1.8)	(+1.8)	ND	...
Msp I	2.2, 1.8, 0.5, 0.3	...	...	...	...	...	ND	...
Pst I	1.9, 0.9, 0.85, 0.65	...	...	...	...	...	ND	ND
Pvu II	2.4, 0.8	...	...	...	...	...	ND	...
Sin I	0.8, 0.65, 0.55	...	...	...	(+3)	...	ND	...
Taq I	2.4, 1.4, 1.1, 0.8	...	...	...	...	...	ND	No 2.4
Xba I	7.3, 3.2, 2.0, 1.0	ND	...	ND	(a)	...	(a)	ND

(a) 129 mice lack the 3.2kb band. This band was present in SENCAR and BALB DNA, but absent in SB hybrid DNA. This may have been a methylation effect. The same explanation may apply to the extra *Hind* III and *Sin* I p11 bands seen in some strains.

4. Locus: *Fes*
Probe: 400bp *Bam* HI insert of pBRFO4 (Shibuya *et al.*, 1982)

		Mus musculus							
	Enzyme	BALB	C57	NIH	CBA	SENCAR	TFH	129	*Mus spretus*
*	*Eco* RI	13	12	13	12	13	12	12	13
*s	*Xba* I	15, 6.0	ND	ND	...	...	ND	ND	14, 6.0

The enzymes *Bgl* II, *Hind* III, *Msp* I, *Pst* I and *Pvu* II were non-polymorphic in the strains used by Blatt *et al.*, (1984), and were not checked in this study. (NMRI: ND)

5. Locus: *Calc*
Probe: 600bp *Pst* I insert of pHT-B3 (Allison *et al.*, 1981)

		Mus musculus						
	Enzyme	BALB	C57	NIH	NMRI	SENCAR	TFH	*Mus spretus*
	Bam HI	18	...	...	...	...	...	...
	Bcl I	6.7	...	...	...	(+9.5)	...	ND
	Bgl I	25	...	...	...	...	...	ND
	Bgl II	10, 3.7	...	...	...	...	...	...
	Eco RI	7.0	...	...	...	...	...	ND
	Hinc II	3.8	...	...	...	...	...	...
*s	*Hind* III	8.0	...	...	...	...	...	9.5
	Msp I	6.0	...	...	...	...	...	ND
	Pst I	2.9	...	...	...	...	...	ND
	Pvu II	11	...	...	...	...	...	...
	Sac I	11, 9.5	...	...	...	...	...	ND
*s	*Sin* I	2.1	...	...	...	...	...	3.5
	Taq I	9.0	...	...	...	...	...	ND

(129: ND)

6. Locus: *Int*-2
Probe: 2.3Kb *Bgl* II fragment cut from pU418 (Dickson *et al.*, 1984).

		Mus musculus						
	Enzyme	BALB	NIH	CBA	SENCAR	TFH	129	*Mus spretus*
	Eco RI	ND	22.	ND.	ND.	ND.	...	...
*	*Pst* I	4.4	4.4	2.2	2.2	2.2	2.2	2.2
	Xba I	22, 6.0	...	ND	...	...	...	...
*s	*Kpn* I	ND	ND	22	ND	ND	ND	9.0

(NMRI and C57: ND)

7. Locus: *Hbb*
Probe: 1.1Kb *Hind*III insert of pTK1H1-1 (Gilmour *et al.*, 1984)

Enzyme	*Mus musculus* BALB	C57	NIH	NMRI	SENCAR	TFH	129	*Mus spretus*
* *Bam*HI	5.0	12	12	12	12	5.0	ND	ND
*Bcl*I	7.0	...	...	...	...	...	ND	ND
Bgl II	5.0	...	...	...	...	...	ND	ND
* *Eco*RI	7.0	9.4	9.4	7.0	7.0	7.0	7.0	9.4
*Hinc*II	1.2	...	...	...	...	...	ND	ND
*Hind*III	1.7	...	...	...	...	...	ND	ND
*Msp*I	3.6	...	...	...	...	...	ND	ND
* *Pst*I	2.2, 0.8	2.2, 0.7	2.2, 0.7	2.2, 0.8	2.2, 0.7	2.2, 0.8	ND	ND
* *Sac*I	4.2	6.7	6.7	4.2	6.7	4.2	ND	6.7
* *Taq*I	4.0, 3.5	7.5	7.5	4.0, 3.5	7.5	4.0, 3.5	ND	ND
* *Xba*I	10	ND	7.5	ND	7.5	10	10	7.5

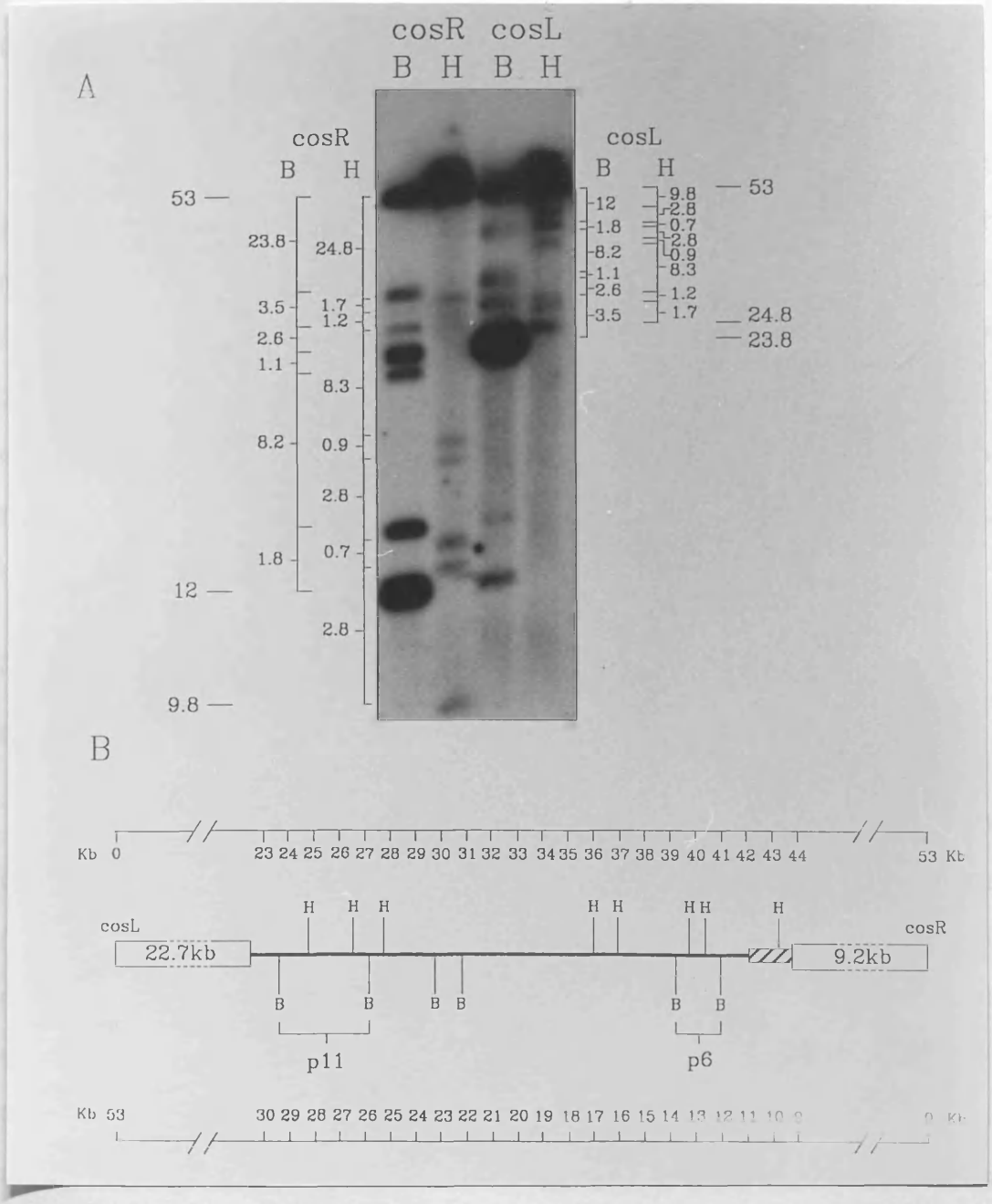
(CBA *Xba* I band also determined: 10Kb)

8. Locus: *Saa*-1
Probe: 500bp *Pst* I insert of pRS48 (Taylor and Rowe *et al.*, 1984)

Enzyme	*Mus musculus* BALB	C57	NIH	NMRI	SENCAR	TFH	129
Kpn I	25, 20, 9.0	...	...	...	...	...	ND
Pst I	3.5,	ND	...	ND	...	...	ND
Pvu II	6.4	ND	...	ND	...	...	...
Xba I	12	ND	...	ND	...	...	ND

(*Mus spretus* : ND)

Figure 8 Restriction map of λNI



A. Mapping of λNI by hybridisation of radiolabelled cosR and cosL oligomers to *Bam*HI and *Hind*III partial digests of λNI. Two batches of samples were run in a 0.5% agarose gel, one for 24hours and the other for 36hours. The autoradiograph shown is of the batch run for 36hours. Distances between restriction sites are shown in kb at the sides. The size of undigested λNI (53kb) and the smallest *Bam*HI and *Hind*III restriction fragments for the cosL and cosR probes are also indicated. Marker DNAs were λ/*Nar*I, λ/*Nde*I and λ/*Sal*I. B = *Bam*HI; H = *Hind*III.

B. Restriction map of λNI. The map was derived by combining data obtained in A with knowledge of the restriction maps of p6, p11, the 3' region of λNI and the sizes of *Bam*HI and *Hind*III fragments obtained by digesting λNI with these enzymes. The latter two pieces of information were obtained by F. Fee and M. Ramsden. The scales above and below the map permit comparison with the autoradiograph in A. The location of the 1.8kb and 3.5kb p6 and p11 probes respectively is indicated. The H-*ras* gene is represented by a hatched box; only the 5' non-coding exon and the two coding exons are present in this clone. The λ Charon 30 arms are represented as open boxes.

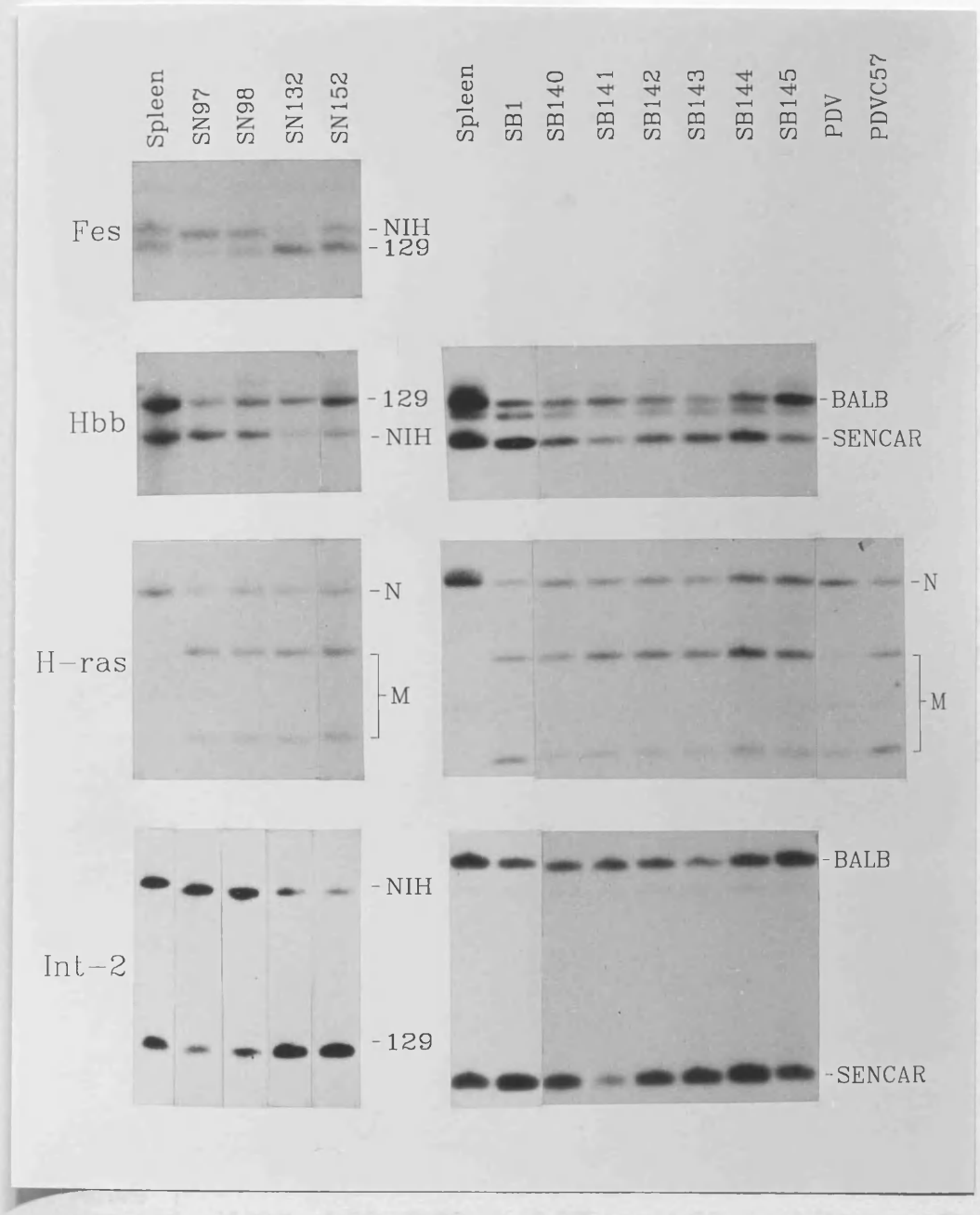
3.4 Carcinomas with activated H-*ras* also have gross chromosome 7 Changes

Carcinomas were induced in SN and SB F1 hybrid mice by DMBA initiation and twice weekly TPA promotion. All carcinomas obtained were analysed for mutation of H-*ras* and/or loss of alleles on chromosome 7. An A:T->T:A transversion at the second nucleotide of codon 61 indicated by the *XbaI* RFLP described above was detected in all 16 of the carcinomas and in two lymph node metastases (Figures 9, 11 and 12, H-*ras* row). Analysis of chromosome 7 RFLPs showed that, in all of these tumours, non-disjunction, mitotic recombination/deletion, or both had occurred.

3.4.1 Over-representation of the mutant H-*ras* allele occurs most commonly by non-disjunction

Figure 9 displays the carcinomas in which non-disjunction was detected. In these tumours the normal 12kb H-*ras* allele was fainter than the mutant 8+4kb allele (Figure 9). The intensity of the 8kb and 4kb bands added together gives the intensity of the mutant allele. The relative under-representation of the normal allele was confirmed by densitometric scanning in comparison with the control cell lines PDV and PDVC57, which exhibit ratios of normal to mutant alleles of 2:1 and 1:2 respectively. It was deduced that the unequal ratio of normal:mutant H-*ras* in the primary carcinomas (Figure 9) was most probably due to gross chromosomal imbalance, since unequal ratios of alleles were detected at all other polymorphic loci on mouse chromosome 7. For example, in SN97 (Figure 9), the 129 allele was fainter at *fes*, *Hbb* and *Int*-2. Since these loci represent markers both proximal and distal to the H-*ras* gene, it can be assumed that the weak normal H-*ras* band was also on the 129 chromosome. In SB143 (Figure 9) the BALB/c allele was weaker at *Hbb* and

Figure 9 Non disjunction in DMBA/TPA carcinomas



Chromosome 7 RFLP analysis of 4 SN and 7 SB DMBA/TPA carcinomas. SN97 and 98 were obtained from the same mouse, as were SB141-145. Other tumours were each derived from separate mice. The spleen samples were derived from SN and SB hybrids as appropriate. The order of the genes on chromosome 7 is as shown i.e. *fes*, *Hbb*, H-*ras*, *Int*-2 (Figure 5). For the background to the cell lines PDV and PDVC57 see text. To avoid duplication the control samples from only one gel are shown. Variation in the hybridisation pattern from gel to gel can occur because of differences in probe length; the pattern is reasonably consistent within each gel. N = normal 12kb H-*ras* allele, M = mutant 8+4kb H-*ras* allele. Other details as in Figure 7.

Table 5 Allele ratios in DMBA/TPA tumours in which non-disjunction was detected

Tumour Grade:		1-2	1-2	2	2	1	2-3	-
% contamination:		10	20	10	10	50	10	-
Tumour:		**SN97**	**SN98**	**SN132**	**SN152**	**SN133**	**MSC1**	**XMSC1**
Most intense allele:		NIH M	NIH M	129 M	129 M	129 N	spretus M	spretus M
Allele ratios	**Fes**	3.07	1.74	3.84	1.78	ND	1.61	1.19
	Hbb	3.13	1.75	3.20	2.18	1.46	2.15	2.36
	H-ras	2.48	1.16	2.59	1.75	1.77	1.5	2.79
	Int-2	3.37	1.52	3.90	4.2	ND	2.79	5.04

Tumour Grade:		3	1-2	2	1-2	1	1-2	1
% contamination:		10	20	<10	20	20	10	10
Tumour:		**SB1**	**SB140**	**SB141**	**SB142**	**SB143**	**SB144**	**SB145**
Most intense allele:		SENC M	SENC M	BALB M	SENC M	SENC M	SENC M	BALB M
Allele ratios	**Hbb**	3.04	1.72	1.94	1.70	2.8	2.20	2.12
	H-ras	2.46	1.31	1.76	1.65	2.85	2.11	1.9
	Int-2	2.71	1.91	1.93	1.77	3.25	2.04	1.6

Tumour Grade:		-	Spindle	Spindle	-	2	1
% contamination:		-	-	-	-	20	30
Tumour:		**X1**	**SB1NMa**	**SB1NMd**	**X1NMa+d**	**SB136**	**SB137**
Most intense allele:		— As for SB1, i.e SENCAR— and M				BALB N	BALB N
Allele ratios	**Hbb**	3.59	3.55	4.74	3.91	1.63	1.64
	H-ras	4.60	3.39	3.67	4.23	1.74	1.9
	Int-2	2.87	4.4	4.31	4.06	1.94	1.64

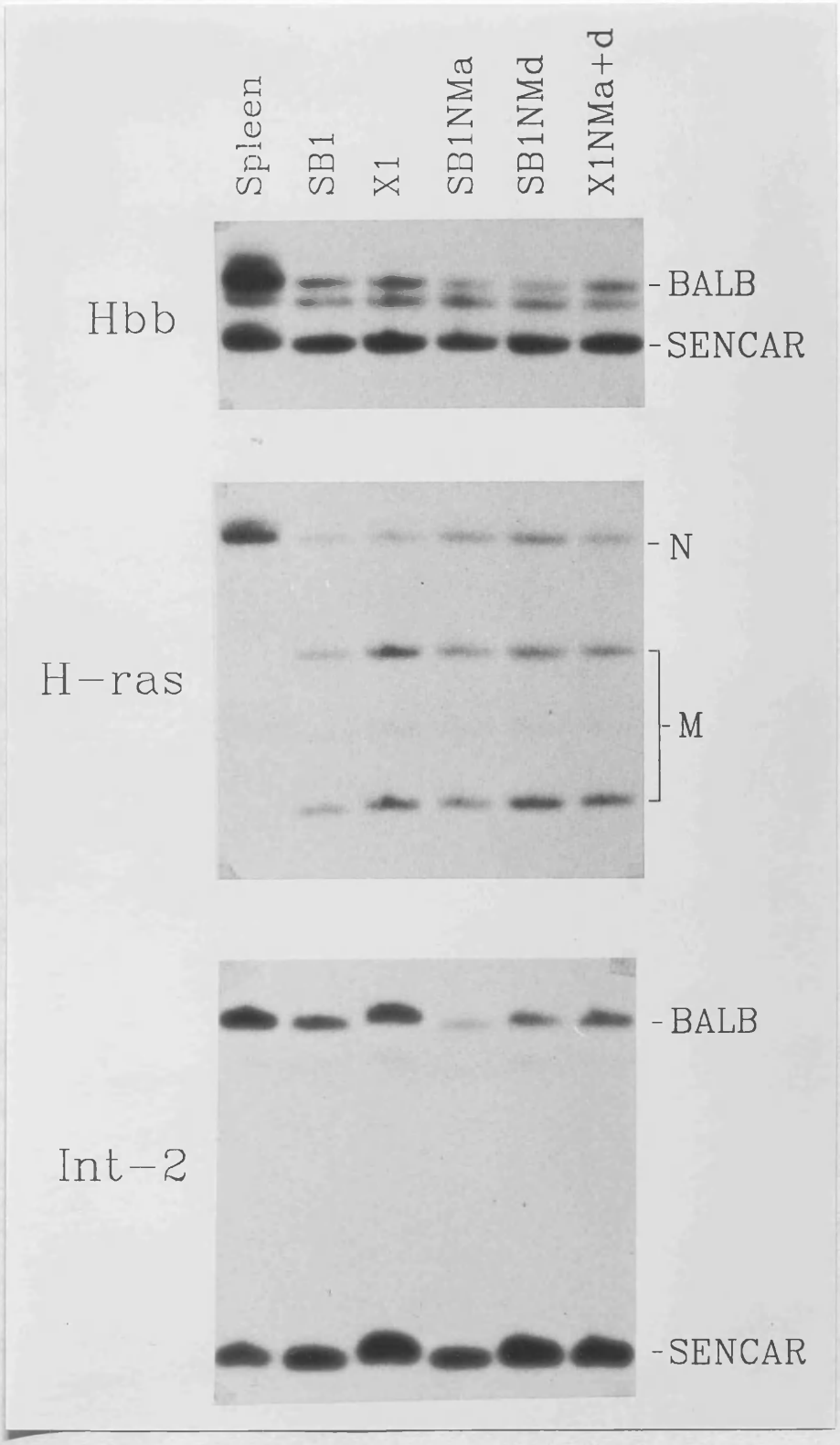
Ratios were determined by densitometry. Tumour ratios are adjusted to compensate for the ratio seen in normal tissue. To simplify matters, only the larger number in the ratio is shown; the most intense allele is indicated at the top of each column. For example, in SN97 the ratio at *fes* is 3.07:1 in favour of the NIH allele, while at H-*ras* the mutant allele (M) is 2.48x as intense as the normal allele. The allele ratio at H-*ras* was calculated using PDV as a control for the ratio of mutant:normal; these values were similar to those calculated using PDVC57 as the control. Tumour grade and normal cell contamination were determined by histological analysis. ND = not determined due to insufficient DNA.

Int-2. Presumably, the faint normal H-*ras* allele was also on the BALB/c chromosome.

In the tumours in which non-disjunction had taken place, it was important to know whether the chromosome carrying the normal H-*ras* allele was completely lost or under-represented. Clarifying this problem is complicated by the presence of contaminating normal cells in tumours, and by the fact that mouse skin carcinomas are frequently near- or hypertetraploid (section 1.5.4, Pera and Gorman, 1984, Fusenig *et al.*, 1985, Aldaz *et al.*, 1987). Thus, a ratio of 3 copies of a chromosome to 1 of its homologue might be mistaken for complete loss of an under-represented chromosome, with the presence of the fainter allele being wrongly attributed to contaminating normal tissue. Densitometric analysis of the tumours in Figure 9 showed that the allele ratios were in fact close to 2 or 3:1 (Table 5). Histological sections of most of these tumours suggested contamination levels of 10-20% or less (Table 5). At these levels of contamination, much larger allele ratios would be expected if complete loss had occurred. For example, in a tumour which had lost the chromosome carrying the normal allele and duplicated the chromosome carrying the mutant allele, 20% contamination would reduce the 2:0 ratio at H-*ras* to 4:1, and 9:1 at other loci (Table 16D, section 3.13.2). These ratios are clearly much higher than those seen in the tumours discussed above. It is therefore likely that the chromosome carrying the normal H-*ras* allele was not lost but under-represented in these tumours.

This matter was investigated further in the tumour SB1 by using early passage cell lines and transplanted tumours from this primary carcinoma in an attempt to remove contaminating normal cells. The original cell line (X1) obtained from SB1 was injected into nude mice and the resulting carcinomas (SB1NMa and SB1NMB) were further propagated in culture (XNM1a+d). Analysis of these samples showed that the normal H-*ras* allele and the faint BALB/c alleles at *Hbb* and *Int*-2 were still present after all of these culturing and transplantation steps (Figure 10). Hence, it would appear extremely unlikely that the signal due to the normal allele derives from contaminating normal cells which have survived transplantation and explanting,

Figure 10 Persistence of the weaker BALB/c alleles during purification of carcinoma SB1



Chromosome 7 RFLP analysis of the carcinoma SB1 (Figure 9), a cell line derived from SB1 (X1), nude mouse tumours derived from this cell line (SB1NMa and SB1NMd) and a cell line derived from these tumours (X1NMa+d), The spleen is from the SB hybrid that had the SB1 carcinoma. Other details as in Figures 7 and 9.

particularly since explants of tumours which had genuinely lost the normal H-*ras* gene showed complete loss of the 12kb band at early passage levels (Figure 10). Further evidence that under-representation, rather than complete loss, of the chromosome carrying the normal H-*ras* allele occurs commonly in DMBA/TPA tumours was provided by analysis of a cell line grown from an MSC hybrid mouse carcinoma (section 3.4.4).

3.4.2 Some carcinomas have an apparent over-representation of the normal H-*ras* allele

From the above discussion it is clear that the normal H-*ras* allele is lost or under-represented in the vast majority of DMBA/TPA carcinomas. However, preliminary analysis of three more tumours induced in this way (SN133, SB136 and SB137) suggested a different pattern. In these cases, an imbalance was detected at all the polymorphic markers flanking the H-*ras* locus, consistent with non-disjunction (Figure 11), At the H-*ras* locus it appeared that the mutant allele was under- rather than over-represented, implying that non-disjunction had resulted in duplication of the chromosome carrying the normal allele. As discussed below, a more detailed analysis suggested that this interpretation was erroneous.

Normal cell contamination has a differential effect on allele ratios at polymorphic loci compared with the normal : mutant H-*ras* ratio. This is discussed in detail in section 3.13.2 and predicted effects given in Table 16. The phenomenon can be summarised as follows. Contaminating stromal cells contribute two normal H-*ras* alleles but no mutant allele, so that contamination above 10% dramatically reduces (and can reverse) any imbalance in tumours in favour of the mutant allele. In contrast, normal cells contribute only one of each allele at polymorphic loci (eg *fes*), and so the diluting effect on a tumour-related imbalance is much less pronounced (see Table 16, section 3.13). As with other carcinomas in this study, SN133, SB136 and SB137 were subjected to histological analysis in order that the level of normal

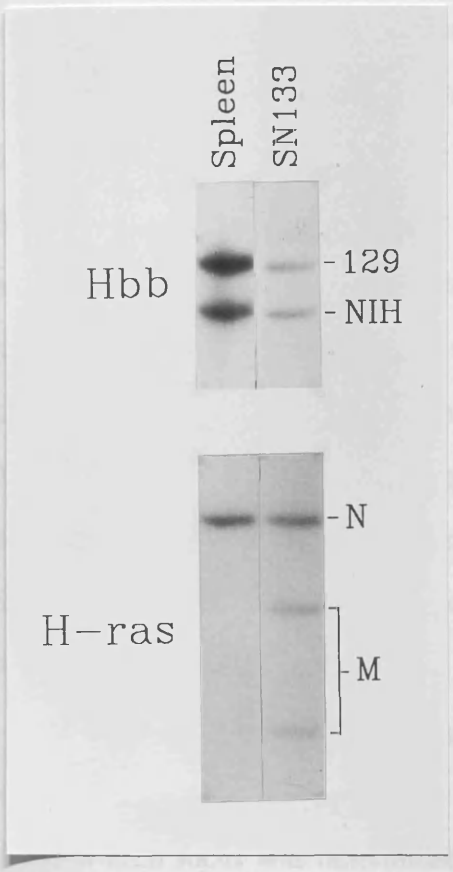
cell contamination might be assessed. In conjunction with densitometric measurement of allele ratios a more accurate interpretation of the chromosome 7 status in these tumours was possible and is discussed below.

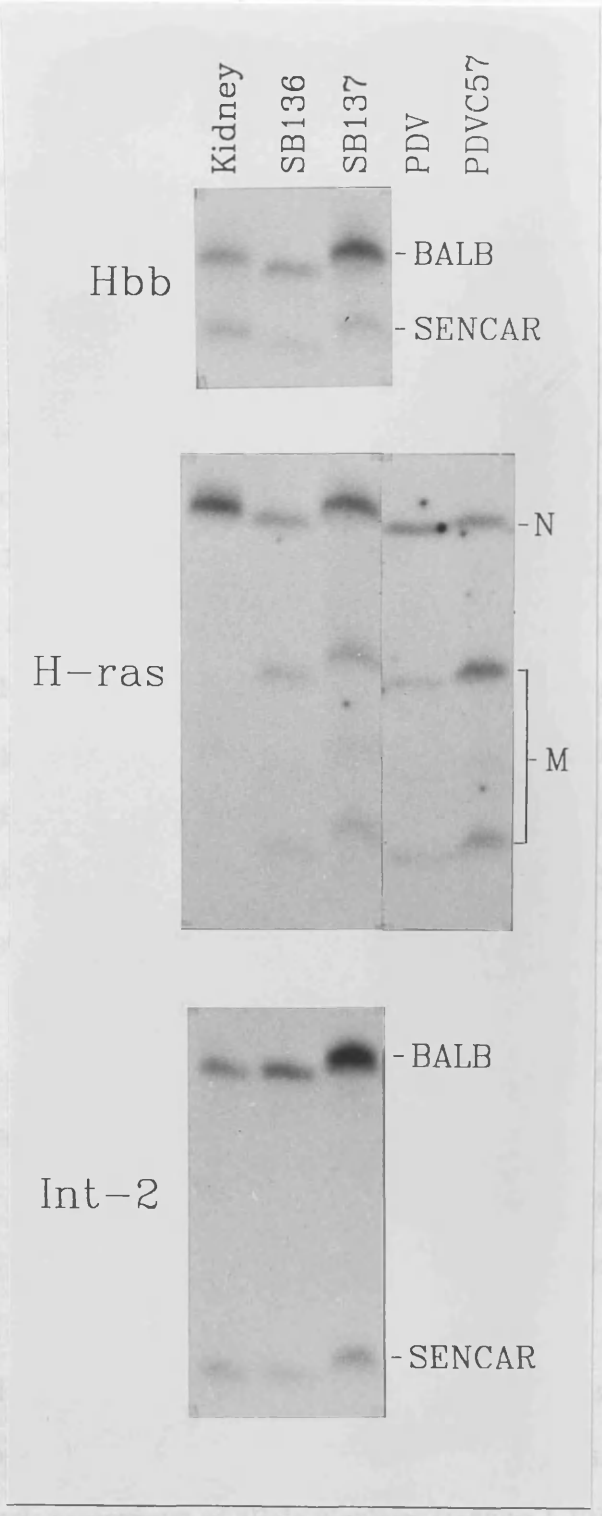
In SN133 (Figure 11) the apparent excess of normal H-*ras* over mutant H-*ras* and the slight imbalance of alleles at *Hbb* in favour of the NIH allele can be attributed to the high level (50%) of normal cell contamination in this tumour (Figure 11 and Table 5). This level of contamination would distort a 2:1 ratio in favour of the mutant H-*ras* allele such that the apparent ratio would be 1.5 : 1 in favour of the normal gene (Table 16). A ratio of two NIH alleles to one 129 allele at the *Hbb* locus would not be reversed by such contamination, but would be reduced to 1.5 : 1 (Table 16). This is the approximate pattern seen in SN133 (Figure 11; insufficient DNA was available to assess the allele ratios at *Int*-2 and *fes*). Densitometry gave allele ratios of 1.77:1 normal to mutant H-*ras* and 1.46 : 1 at *Hbb*, close to the predicted values. Hence, SN133 is probably also in the category of tumours like SN97 (Figure 9) which have an excess of the chromosome carrying the mutant H-*ras* gene.

A similar interpretation may also be applied to tumours SB136 and 137 (Figure 11). Again the apparent over-representation of the normal H-*ras* allele and the reduced imbalance of alleles at *Hbb* and *Int*-2 in favour of the BALB/c allele can be attributed to a high level of normal cell contamination. These tumours did not appear to be as contaminated as SN133 with normal cells (Table 5), but it may be that the histology section was not representative of the whole tumour in these cases. It is very unlikely that there really was a ratio of normal:mutant H-*ras* of 2:1 in these tumours since contamination levels of 20-30% would increase this ratio to around 3:1, which was clearly not the case (Figure 11).

Although less dramatic, the effect of contaminating normal cells can be seen in some of the carcinomas discussed in section 3.4.1 (Figure 9); in other words, the imbalance of alleles is not as great at H-*ras* as at other loci. The presence of large amounts of normal cells in only a few carcinomas differed from the pattern observed in papillomas where contamination appeared to be uniformly high (section 3.8).

Figure 11 Carcinomas with an apparent over-representation of normal H-*ras*





Chromosome 7 RFLP analysis of 1 SN and 2 SB DMBA/TPA carcinomas. The spleen and kidney were from SN and SB hybrids respectively. Insufficient DNA was obtained from SN133 to permit analysis of *fes* and *Int*-2 loci. Other details as in Figures 7 and 9.

3.4.3 Mitotic recombination or deletion in carcinomas and lymph-node metastases

While imbalance at all polymorphic loci, indicative of non-disjunction, was detected in most DMBA/TPA carcinomas, loss of heterozygosity at only one or two chromosome 7 loci, indicative of mitotic recombination or deletion, was detected in two carcinomas induced in this way; SN158 and 184 (Figure 12). In SN184 the normal H-*ras* and 129 *Int*-2 alleles were extremely faint. The ratio of the more intense to the fainter allele was greater than 20:1 in each case. It is therefore likely that the faint bands were due to the low level of contamination (<5%) of SN184 by normal cells (Table 6), and that the 129 H-*ras* and *Int*-2 alleles have been lost by mitotic recombination or deletion. In addition, since the NIH alleles in SN184 were approximately twice as intense as the 129 alleles at *fes* and *Hbb* (Figure 12) it appears that non-disjunction resulting in an increase in copy number of the NIH chromosome may also have occurred. This could have taken place before or after mitotic recombination, as is illustrated in Figure 13A (the loss of the H-*ras* and *Int*-2 129 alleles is assumed to have been caused by mitotic recombination for the purpose of this Figure).

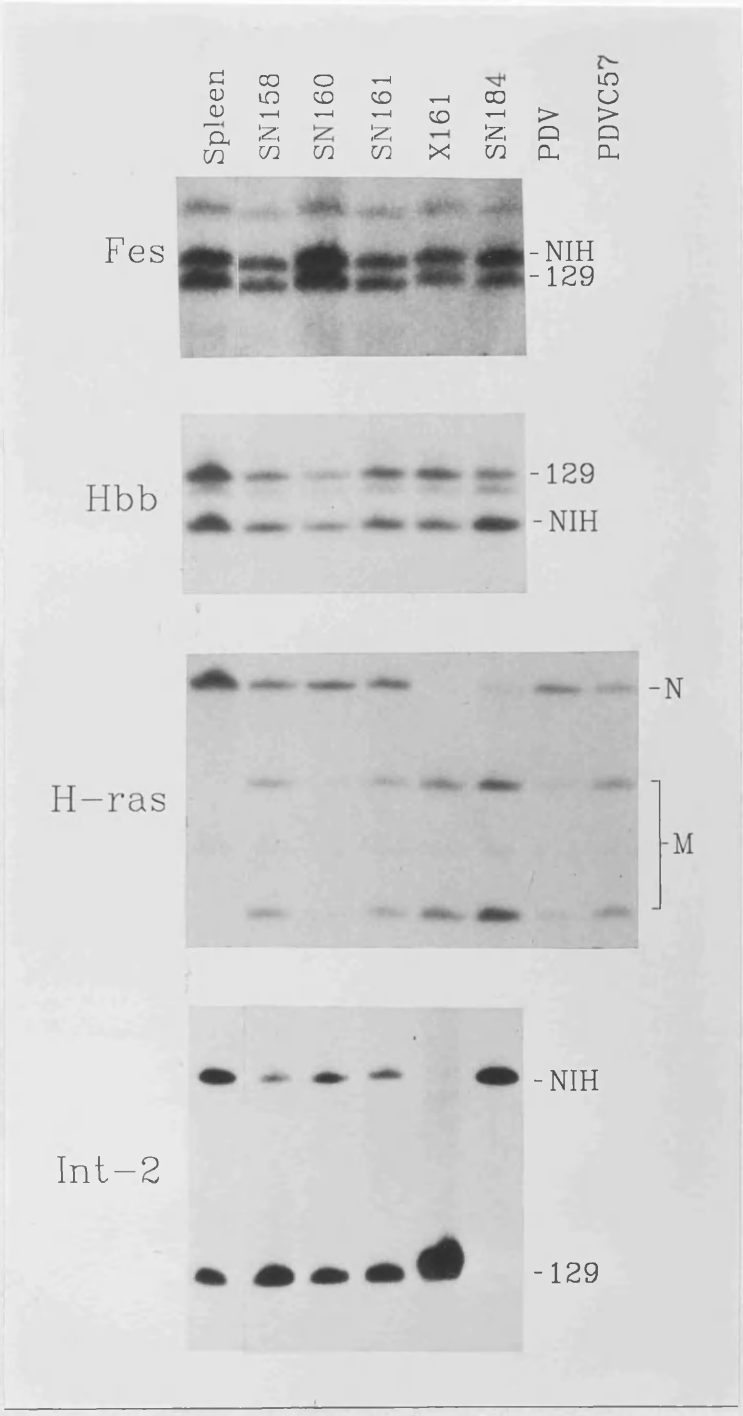
Distinguishing between mitotic recombination and deletion should be possible using densitometry. In this study, the absolute number of tumour compared to normal alleles at each locus was determined after normalising for DNA loading. This was possible after rehybridisation of blots with a probe containing an IL3 cDNA and an unidentified cDNA (see section 3.13.1).

In the case of SN184, the total number of *Hbb*, H-*ras*, and *Int*-2 alleles was similar, making mitotic recombination the most likely interpretation of the results. However, the variability of densitometric assessment of allele numbers (section 1.13) meant that interpretation could not be conclusive. Similar problems have been noted previously (Naylor *et al.*, 1987).

Tumour SN158 was heterozygous at *fes*, *Hbb* and also for the H-*ras* mutation, but the NIH *Int*-2 allele was weaker than the 129 allele (Figure 12). Histological analysis showed that approximately 10-15% of this tumour consisted of contaminating normal cells. Densitometry revealed that the ratio of 129 to NIH alleles at *Int*-2 was approximately 5:1 (Table 6). If the faint band was due to contaminating normal tissue, this could represent loss of the NIH *Int*-2 allele by deletion or mitotic recombination. The faint band could, however, represent one remaining *Int*-2 NIH allele after a mitotic recombination in a tetraploid cell, resulting in three copies of the 129 *Int*-2 allele to one of the NIH allele. After normalising for DNA loading, the number of alleles in SN158 ranged from 1.35-2.24 at the *Int*-2 locus and 1.2-2.0 at the *Hbb* locus, depending on which band was used for normalisation of DNA loading differences. The similarity of these figures suggests that there are equal numbers of these alleles and therefore that mitotic recombination had occurred. However, for the reasons described in section 3.13 a conclusive interpretation is not possible. Nevertheless, irrespective of which interpretation is correct, the loss of heterozygosity distal to H-*ras* is potentially very significant since this supports the idea that a gene on mouse chromosome 7 other than H-*ras* is involved in carcinoma development.

SN158 came from a mouse which also had two lymph node metastases, SN160 and 161. It is possible that SN160 and 161 were derived from SN158, since all these tumours were grade 3 carcinomas (Table 6) and there were no other carcinomas on the mouse from which SN158 was obtained. Histological examination showed that both metastases were highly contaminated with lymphocytes (Table 6) making interpretation of band intensities in the original tumour difficult. However, a cell line grown from SN161 (X161) revealed that this tumour had completely lost the normal H-*ras* and NIH *Int*-2 alleles (Figure 12). This could have resulted from mitotic recombination distal to *Hbb* (Figure 13B). Total allele numbers were similar at all chromosome 7 loci in SN161, consistent with this mechanism. However, the same pattern would result from activation of the remaining normal H-*ras* by a second point

Figure 12 Mitotic recombination or deletion in DMBA/TPA carcinomas and metastases



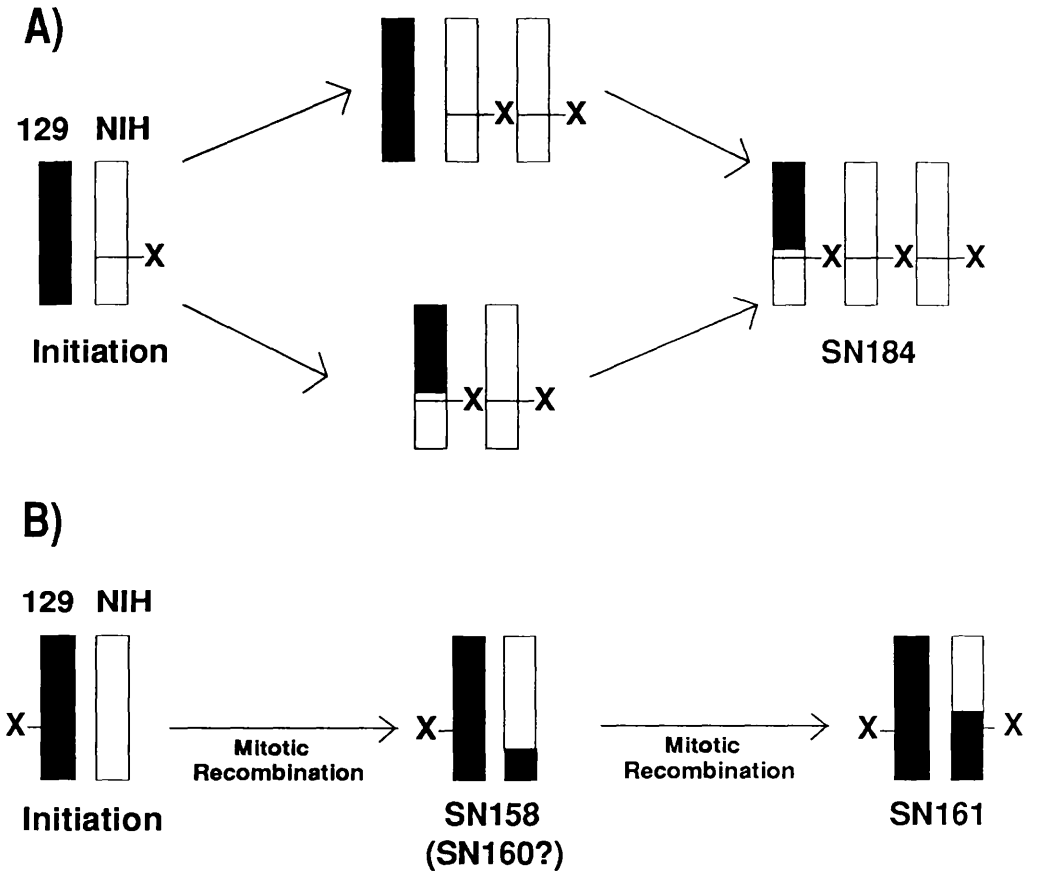
Chromosome 7 RFLP analysis of 2 SN carcinomas (SN158 and 160), 2 SN lymph node metastases (SN160 and 161), and a cell line derived from SN161 (X161). Tumours were derived by DMBA-initiation and TPA-promotion. The metastases came from the same mouse as SN158. SN184 was from a separate mouse. The spleen was from an SN hybrid. Other details as in Figures 7 and 9.

Table 6 Allele ratios in tumours in which mitotic recombination or deletion had occurred

Tumour Grade:		2-3	3	3	-	2	?
% contamination:		10	50	50	-	<5	?
	Tumour:	**SN158**	**SN160**	**SN161**	**X161**	**SN184**	**SN80**
Allele ratios	**Fes**	1.00	1.22 (129)	1.11 (129)	1.17 (NIH)	1.63 (NIH)	1.14 (NIH)
	Hbb	1.22 (NIH)	1.14 (NIH)	1.10 (NIH)	1.10 (NIH)	2.35 (NIH)	1.11 (NIH)
	H-ras	1.11 (N)	7.6 (N)	1.12 (N)	NA	23.4 (M)	3.29 (N)
	Int-2	4.79 (129)	2.80 (129)	3.73 (129)	NA	26.3 (NIH)	2.55 (129)

Only the larger value in the ratio is shown; the parental origin of the more dense allele is indicated below the ratio, except for the H-*ras* locus, in which case the normal (N) or mutant (M) allele is indicated. ? = no histology available. NA = not applicable. Other details as in Table 5.

Figure 13 Possible mechanisms explaining allele ratios observed in SN184, 158, 160 and 161



A) Alternative routes to the chromosome 7 status of SN184. After activation of one H-*ras* allele at initiation (represented by "X"), trisomy may have followed or preceded mitotic recombination.

B) Possible chromosome 7 status of SN158, 160 and 161.

mutation or gene conversion. In addition, the interpretation may be oversimplified given that analysis of X161 by flow cytometry (section 3.12) revealed that this cell line was highly aneuploid. The higher normal:mutant H-*ras* ratio in SN160 compared with SN161 (Figure 12), even though the tumours were similarly contaminated with normal cells (Table 6), suggests that the H-*ras* mutation is still heterozygous in the former (Figure 13B).

3.4.4 The use of *Mus spretus* x *Mus musculus* hybrids to analyse tumour-related LOH

The relatedness of *Mus musculus* strains of mice limits the number of available RFLPs which can be used to analyse LOH in tumour DNA. This problem can be overcome by interbreeding *M. musculus* with a strain which has diverged significantly; *M. spretus*. This point is clearly demonstrated by the results summarised in Tables 4 (section 3.3) and 13 (section 3.9). All of the DNA probes which were hybridised to *spretus* DNA detected polymorphisms. In total, around 50 different probe and enzyme combinations were tested and almost half detected variant *spretus* fragments. This has important implications for the fine mapping of limited LOH on chromosome 7 and for the assessment of changes on other chromosomes in mouse tumours. In addition, the presence of an *Xba*I H-*ras* polymorphism in *spretus/musculus* hybrids (Table 4) permits direct identification of the parental origin of the mutant allele in tumours which possess the codon 61 mutation recognised by this enzyme. This ability to distinguish mutant and normal H-*ras* alleles also reduces the complexity of interpreting allele ratios at this locus in tumours highly contaminated with normal cells (c.f. section 3.4.2).

In this study, tumours were analysed from *Mus spretus* x CBA (MSC) hybrids. Carcinomas were induced by treating once a week with DMBA and once a week with TPA. These hybrids proved relatively resistant to tumour induction, and only small numbers of tumours were obtained even using this more rigorous protocol. Of

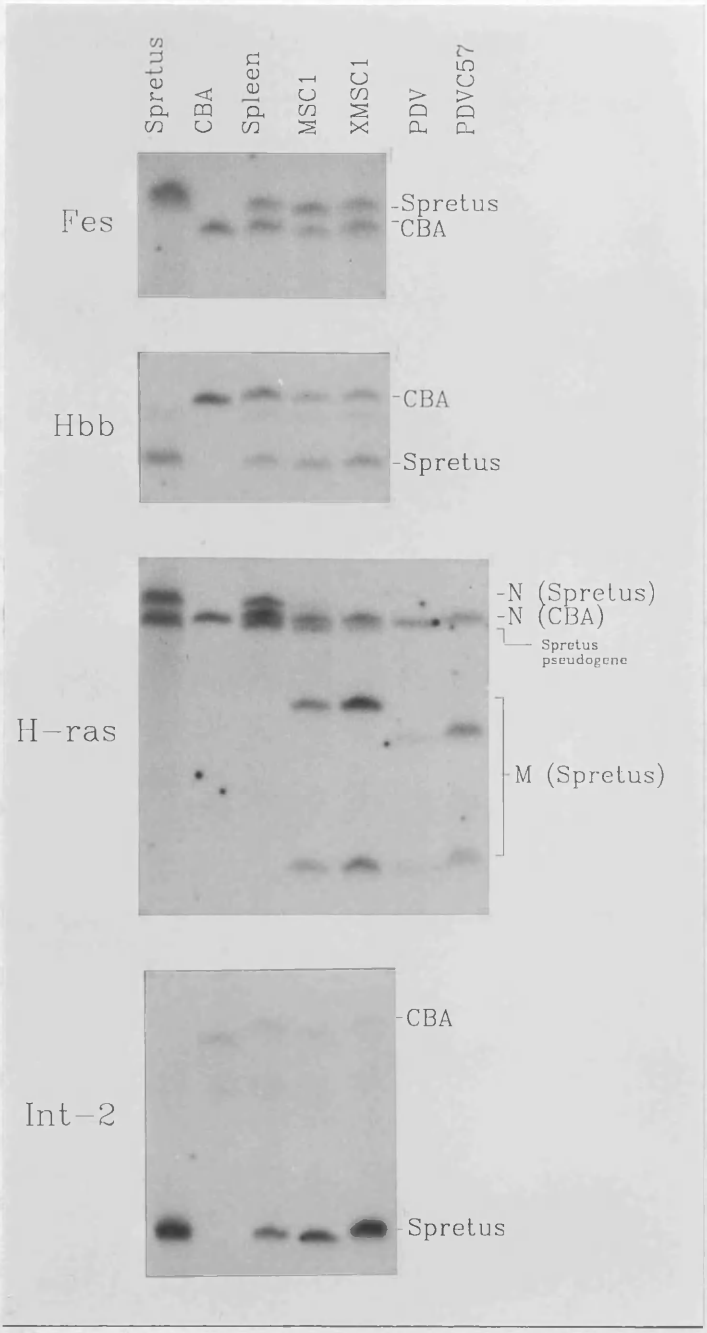
four carcinomas, and cell lines derived from three of these, the chromosome 7 analysis on one tumour (MSC1) and its cell line (XMSC1) is reported here.

Figure 14 shows that, as with most DMBA-initiated carcinomas, MSC1 was positive for the codon 61 A:T->T:A mutation which introduces a new *Xba*I restriction site. (Note also that *spretus* mice have a pseudogene which hybridises to the H-*ras* probe almost as strongly as the functional gene. The *spretus* pseudogene *Xba*I fragment is just under 12kb and should not be confused with the CBA H-*ras* allele in MSC hybrids). In the case of MSC1, it is clear that the *spretus* (13kb) H-*ras* allele has been mutated and that the remaining normal (12kb) allele is derived from the CBA parent. Analysis of RFLPs at other chromosome 7 loci in this tumour demonstrated that the *spretus* chromosome was over-represented (Figure 14; Table 5). This is another example of duplication of the chromosome carrying the mutant H-*ras* allele by non-disjunction.

Confirmation that the chromosome carrying the normal H-*ras* allele was under-represented and not lost in MSC1 was obtained by analysis of XMSC1 (Figure 14). (This complements the work described in section 3.4.1 on cell lines derived from the carcinoma SB1). In XMSC1, although the pattern of imbalances resembled the original tumour at *Int*-2, H-*ras* and *Hbb*, the allele ratio at the *fes* locus was similar to the ratio observed in spleen. This suggests that in the cell line, one of the two *spretus* *fes* alleles was lost, possibly by deletion. The *spretus* pseudogene also appears to have been affected by this event since it is fainter in the cell line than in the original tumour (Figure 14). This agrees with the mapping of the pseudogene close to the *fes* locus (T. Glaser, personal communication). The detection of these losses in a cell line, but not the original tumour, implies that they were unrelated to tumour development.

The allele ratios at the *Int*-2 locus in MSC1 and XMSC1 were higher than at other chromosome 7 loci (Table 5). This may have been due to poor transfer of the large (20kb) CBA *Kpn*I band (Figure 14) rather than a second genetic event at this locus.

Figure 14 Direct identification of the mutant H-*ras* allele in a *spretus*/*musculus* carcinoma



Chromosome 7 RFLP analysis of an MSC carcinoma induced by DMBA-initiation and combined DMBA/TPA promotion. XMSC1 is a cell line derived from MSC1. The spleen was from the same MSC hybrid as the tumour.The *spretus* and CBA normal (N) H-*ras* bands, *spretus* mutant (M) H-*ras* band, and the *spretus* pseudogene are indicated. Restriction digests are as in Figure 7, except for *Int*-2 analysis in which DNA was digested by *Kpn*I. The CBA *Int*-2 band is large (20kb) and transfers poorly. MSC = *Mus spretus* x CBA hybrid. Other details as in Figures 7 and 9.

As described above, one of the major advantages of MSC F1 hybrids is that the normal *spretus* and CBA H-*ras* alleles can be distinguished (Figure 14). Thus, direct proof can be obtained concerning which H-*ras* allele is mutated in tumours. This has important implications for the assessment of tumour clonality. If mouse skin carcinomas were polyclonal (derived from two or more initiated cells) then a proportion of these should have both mutant *spretus* and CBA H-*ras* alleles (the exact proportion of tumours like this would depend on the extent of polyclonality). If, on the other hand, these tumours were monoclonal (derived from a single initiated cell) then the presence of both activated alleles would only be found if the second allele had also been mutated.

In MSC1, for example, a mutant *spretus* allele was detected (Figure 14). In the analysis of several other MSC carcinomas and papillomas, no tumours were found in which both *spretus* and CBA mutant H-*ras* alleles were present (P. Burns, R.Bremner and A. Balmain, in preparation). This evidence confirms previous assumptions based on indirect evidence that skin tumours are monoclonal in origin.

3.4.5 Summary of the analysis of DMBA/TPA carcinomas and metastases.

The analysis of 17 DMBA/TPA carcinomas and two metastases, revealed that 100% of these were positive for both mutant H-*ras* and subsequent gross chromosome 7 changes. In 15/17 carcinomas non-disjunction was detected, while in the other two mitotic recombination appeared the most likely explanation for the observed LOH.

3.5 Carcinomas with no activated H-*ras* have no chromosome 7 changes.

The above results demonstrate that over-representation of mutant H-*ras* in DMBA/TPA tumours is caused by gross chromosome 7 changes. However, what they do not address is whether similar alterations play a part in the growth of skin tumours that lack activated H-*ras*. Evidence that chromosome 7 changes are not involved in such tumours is presented below.

3.5.1 Analysis of tumours induced by MNNG-initiation and TPA-promotion

To determine whether chromosome 7 changes were limited to carcinomas with activated H-*ras* genes, tumours were induced in SN hybrids by initiation with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and promotion with TPA. In this laboratory it has been found that only about 10-20% of carcinomas induced in this way have an H-*ras* mutation; a G:C->A:T transition at the second nucleotide of codon 12 (Brown *et al.*, 1990).

Figure 15 shows the results obtained from one papilloma (SN112) and four carcinomas (SN115, 165, 171 and 176). Only SN165 and SN176 had H-*ras* mutations. SN165 was shown, by use of the polymerase chain reaction and hybridisation to oligonucleotides, to have a G:C->A:T transition at the second nucleotide of codon 12 (Figure 16). None of the other possible codon 12 or 13 mutations were detected in SN165 or the other MNNG/TPA tumours (Figure 16 and Table 8). However, SN176 did have the *Xba*I RFLP found in all the DMBA/TPA carcinomas (Figure 15). This is unlikely to have been caused by MNNG but may be a rare example of a spontaneous activation event (c.f Pelling *et al.*, 1988).

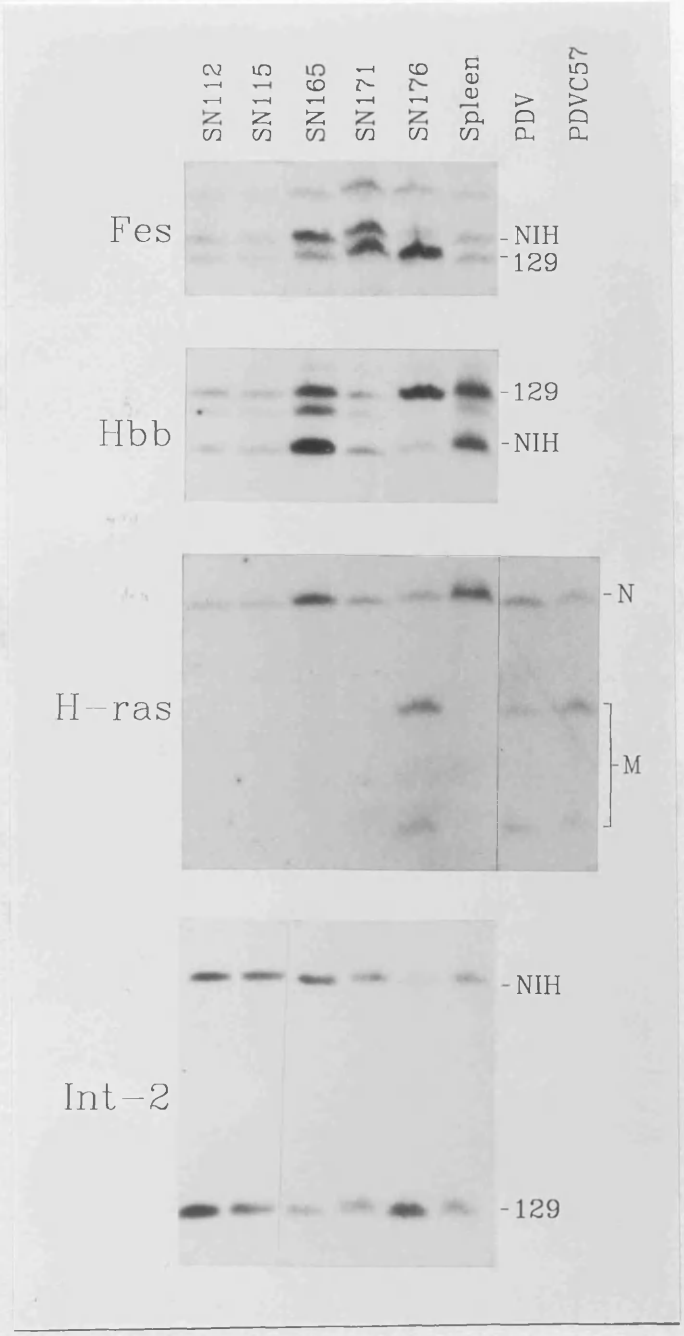
When analysed for imbalances at the *fes*, *Hbb* and *Int*-2 RFLPs, only those tumours with activated H-*ras* (i.e SN165 & SN176) were positive (Figure 15). In

SN165 all the 129 alleles were weaker than the NIH alleles, while in SN176 the NIH alleles were uniformly faint (Figure 15). The approximate allele ratios in SN165 and SN176 were 2:1 and 3 or 4:1 respectively (Table 7). The actual ratio in SN165 was probably higher, since a large amount of normal cells were present in this tumour (Table 7). It is possible that the chromosome carrying the normal H-*ras* allele has been lost in SN165 and 176, but without the benefit of cell line analysis (c.f. sections 3.4.1 and 3.4.4) this cannot be proved.

In order to confirm any codon 12 or 13 mutations detected by hybridisation to oligomers, and to detect any additional mutations, amplified DNAs from MNNG/TPA tumours were analysed by direct sequencing. No mutations were detected using this approach including, surprisingly, the codon 12 mutation detected in SN165 by hybridisation to oligomers (Figure 17). One explanation for this is that oligomer hybridisation is more sensitive than direct sequencing. The former permits detection of a mutation if it is present in 10% of the cells (Farr *et al.*, 1988). Bar-Eli *et al.* (1989) quote a similar figure for direct sequencing, but from the analysis of SN165, which was estimated to contain around 50% normal cells (Table 6), this approach appears to be less sensitive than oligomer hybridisation. One approach to resolving this would be to clone the amplified SN165 DNA into M13 and sequence several clones. This should give a reliable indication of the proportion of mutant:normal H-*ras* sequences assuming that the PCR reaction amplifies mutant and normal alleles equally. Equal amplification of all alleles should occur as predicted ratios have been observed for amplification of H-*ras* exon 2 in the cell lines PDV and PDVC57 (A. Buchmann, pers. communication).

Thus, MNNG/TPA carcinomas that were positive for activated H-*ras* also had chromosome 7 changes, but those that did not have activated H-*ras* did not exhibit any allelic imbalance involving this chromosome. In one of the *ras*-positive tumours an unambiguous increase in the copy number of the chromosome carrying the mutant H-*ras* allele was detected, while in the other case it was not clear whether the mutant allele was on the over- or under-represented chromosome.

Figure 15 Only MNNG/TPA tumours with an H-*ras* mutation have chromosome 7 changes



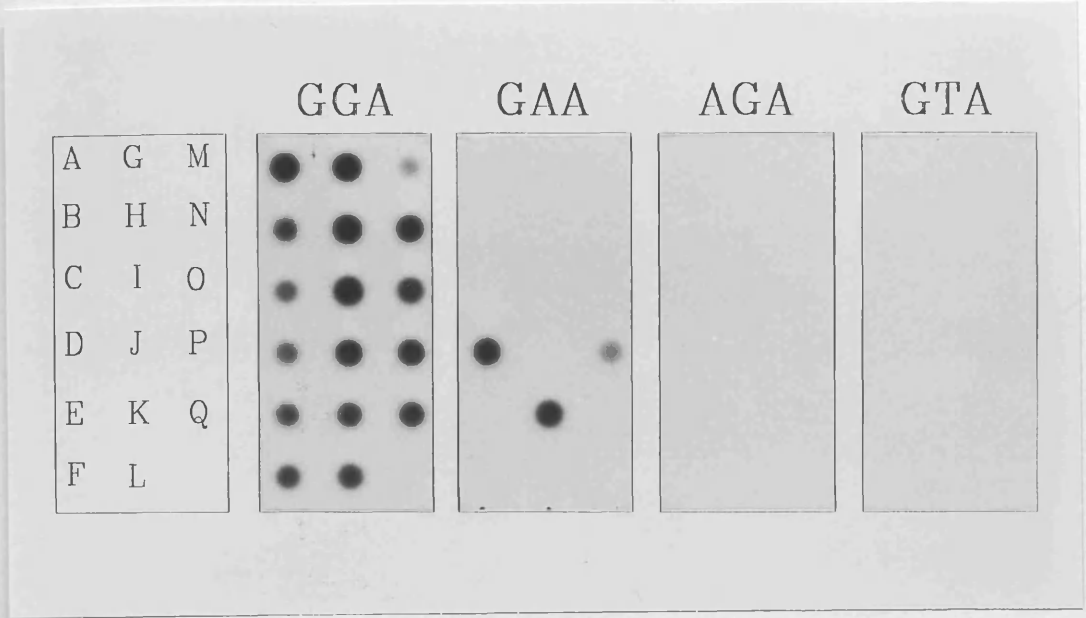
Chromosome 7 RFLP analysis of an SN papilloma (SN 112) and 4 SN carcinomas derived by MNNG-initiation and TPA-promotion. All tumours were from separate mice. The spleen sample was from an SN hybrid. SN165 was shown to have a codon 12 H-*ras* mutation (Figure 16). Other details as in Figures 7 and 9.

Table 7 Chromosome 7 allele ratios in MNNG/TPA tumours

Tumour Grade:		Pap.	1-2	1	1	2-3
% contamination:		?	50	50	5	10
	Tumour:	**SN112**	**SN115**	**SN165**	**SN171**	**SN176**
Allele ratios	**Fes**	1.24 (NIH)	1.18 (NIH)	1.71 (NIH)	1.11 (NIH)	3.14 (129)
	Hbb	1.27 (129)	1.06 (NIH)	1.82 (NIH)	1.19 (129)	3.94 (129)
	H-ras	NA	NA	NA	NA	3.15 (M)
	Int-2	1.26 (NIH)	1.13 (NIH)	1.61 (NIH)	1.16 (NIH)	3.54 (129)

The Table is in the same format as Table 6.

Figure 16 Codon 12 H-*ras* mutations in MNNG initiated tumours



MNNG-initiated tumour samples were amplified with exon 1 amplimers and probed with oligomers specific for the normal codon 12 sequence (GGA) and mutant sequences (GAA, AGA and GTA). Grid letters correspond to the following amplified DNA samples:
MNNG/TPA tumours:A) SN spleen; B) SN112; C) SN115; D) SN165; E) SN171; F) SN176;
MNNG/MNNG tumours: G) SN73; H)X73; I)SN76; J)X76; K)SN88; L) X88;M) SN spleen; N) SN103; O) X103; P) SN110; Q)X110.
These tumours lacked all other possible codon 12 and 13 mutations; in addition, MNNG/MNNG tumours lacked any codon 61 mutations (Table 8). The sequence of the amplimers and hybridisation oligomers is given in Table 3, section 2.16.

		Mutation					
		Codon 12 (GGA)		Codon 13 (GGC)	Codon 61 (CAA)		
Induction protocol	Tumour	GAA	AGA CGA GTA GCA	NGC GNC	CTA	CAT	GAA AAA CGA CCA CAC
M+T	SN112	-	-	-	(-)	ND	ND
	SN115	-	-	-	(-)	ND	ND
	SN165	+	-	-	(-)	ND	ND
	SN171	-	-	-	(-)	ND	ND
	SN176	-	-	-	(+)	ND	ND
M+M	SN73	-	-	-	-	-	-
	X73	-	-	-	(-)	ND	ND
	SN76	-	-	-	-	-	-
	X76	-	-	-	(-)	ND	ND
	SN88	+	-	-	-	-	-
	X88	-	-	-	(-)	ND	ND
	SN103	-	-	-	-	-	-
	X103	-	-	-	(-)	ND	ND
	SN110	+	-	-	-	-	-
	X110	-	-	-	(-)	ND	ND
D+D	SN30	ND	ND	ND	+	-	-
	SN31	ND	ND	ND	+	-	-
	SN33	ND	ND	ND	+	-	-
	X34	-	-	-	-	-	-
	SN40	-	-	-	-	-	-
	X40a	-	-	-	-	-	-
	X40b	-	-	-	-	-	-
	SN47	ND	ND	ND	+	-	-
	X47	ND	ND	ND	+	-	-
	SN49	-	-	-	-	-	-
	X49	-	-	-	-	-	-
D+T pap's	SN136	-	-	-	-	+	-
	SN144	-	-	-	-	+?	-
v-*ras* +T	SN182	-	-	-	-	-	-
	SN186	-	-	-	-	-	-
	SN197	-	-	-	-	-	-

Table 8 Summary of mutations identified by hybridisation of oligomers to amplified tumour DNAs

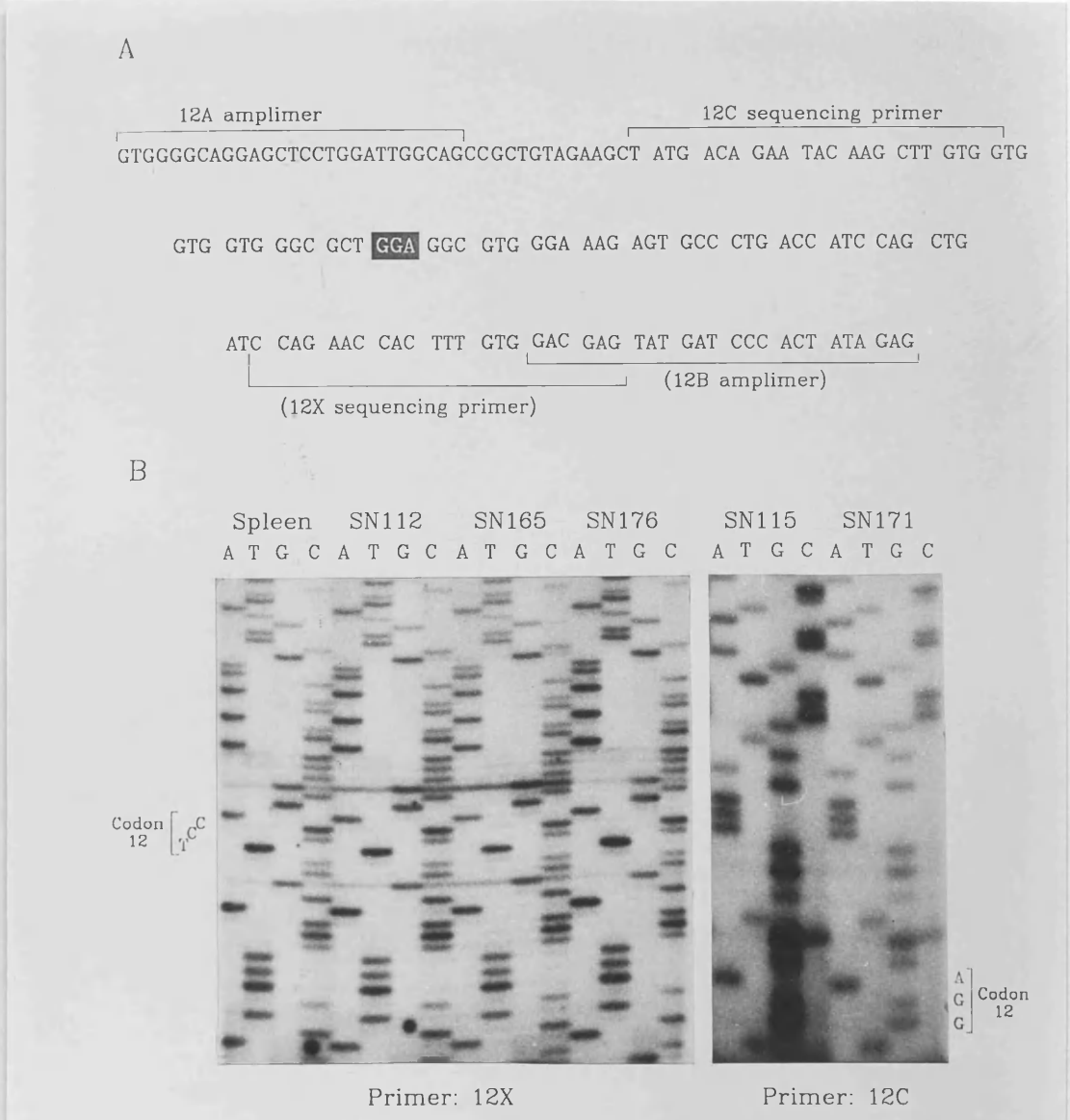
All tumours are carcinomas except: SN112, 136 and 144 = papillomas
SN182=mammary adenocarcinoma

The normal sequence of each codon is indicated in parentheses. The base substitution analysed is in bold. Full sequence of hybridisation oligomers is given in Table 3.

M+T: MNNG-initiation, TPA-promotion
M+M: " , MNNG-promotion
D+D: DMBA-initiation, DMBA-promotion
D+T: " , TPA-promotion
v-*ras*+T: v-*ras*-initiation, "

ND: not determined
+: mutation detected
-: no mutation
+?: possible CAT mutation
(-) or (+): analysis by *Xba*-1 only.

Figure 17 Direct sequencing of amplified MNNG/TPA tumour DNAs



A. Position and sequence of amplimers and sequencing primers. The region of H-*ras* around codon 12 (highlighted by the black box) was amplified using the 12A and 12B amplimers and sequenced using the 5' end-labelled primer 12C or 12X. The 12B and 12X oligomers are shown in parenthesis to indicate that the actual sequence is complementary to the one shown. Nucleotides shown in triplets represent the coding region of this fragment of mouse H-*ras*. The sequence was derived by Brown *et al.* (1988).

B. Direct sequencing of amplified MNNG/TPA tumour DNAs; SN112 was a papilloma, the 4 other tumours were carcinomas, the spleen was from an SN hybrid. SN112, 165 and 176 were sequenced after annealing to 5' end-labelled primer 12X; the primer used to sequence SN115 and 171 was 12C. The codon 12 sequence is indicated.

The absence of chromosome changes in tumours lacking activated H-*ras* also applies to carcinomas described in section 3.6 which were induced by protocols other than the classical initiation/promotion regime.

3.5.2 A DMBA/TPA lymphoma lacks both activated H-*ras* and chromosome 7 changes

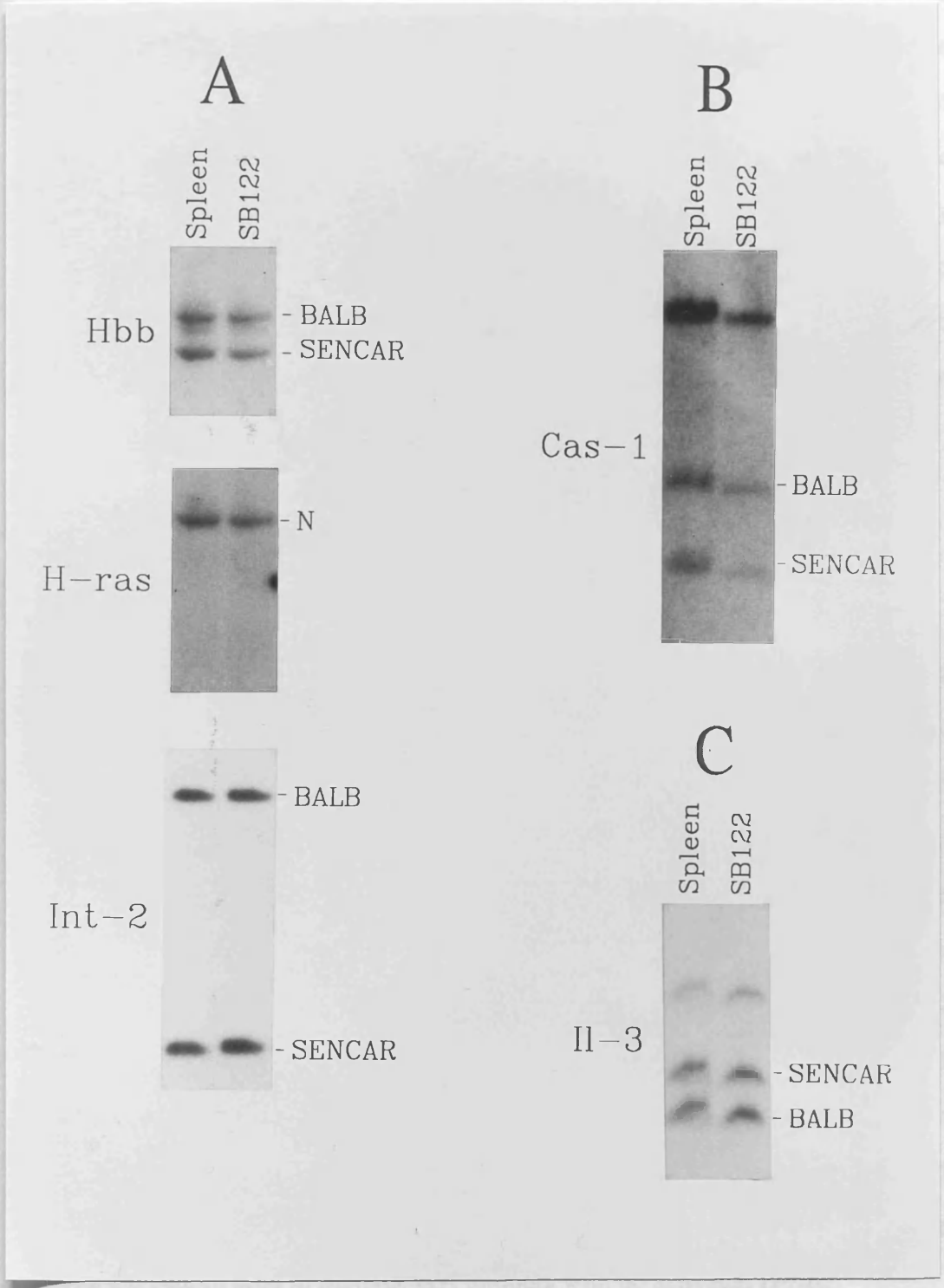
Occasionally, non-epithelial tumours are detected in mice whose skin has been treated with chemical carcinogens and tumour promoters. One such tumour (SB122), a lymphoma isolated from a SB hybrid treated with DMBA and TPA, was analysed for an activated H-*ras* gene and chromosome changes. The *ras* mutation detectable by *Xba*I analysis was not observed in this tumour, nor were any chromosome 7 changes (Figure 18). In addition, no alterations were detected at polymorphic loci on chromosome 2 and 11 (Figure 18; see section 3.9 for details of these RFLPs).

Mouse tumours of haematopoietic origin frequently possess activated N- and/or K-*ras* genes (Guerrero *et al.*, 1985; Diamond *et al.*, 1988). Furthermore loss of the corresponding normal *ras* allele has been reported in some thymic lymphomas (Guerrero *et al.*, 1985; Diamond *et al.*, 1988). It would therefore be appropriate to check SB122 for other *ras* gene mutations, and to analyse polymorphisms on mouse chromosomes 3 and 6; the locations of the N- and K-*ras* gene respectively.

3.6 Tumours induced by repeated carcinogen treatment show a more heterogeneous pattern of genetic changes

Although the initiation/promotion regime is the best studied mouse skin carcinogenesis protocol, it is possible to induce mouse skin tumours in other ways. One of these involves the repeated application of chemicals which can act as initiators (Figure 2, section 1.5.1). It is likely that, as with initiation/promotion

Figure 18 A DMBA/TPA derived lymphoma lacks both an H-*ras* mutation and any detectable chromosome 2,7 or 11 changes



RFLP analysis of an SB lymphoma (SB122) derived from a mouse in which the skin was initiated using DMBA and promoted using TPA. RFLPs analysed were on chromosomes (A) 7 (B) 2, and (C) 11. The tumour lacks the codon 61 H-*ras* mutation detectable by the *Xba*I RFLP. The spleen sample was obtained from the same mouse as tumour SB122. Details of the *Cas*-1 and IL3 RFLPs are given in Figure 29. Other details as in Figures 7 and 9.

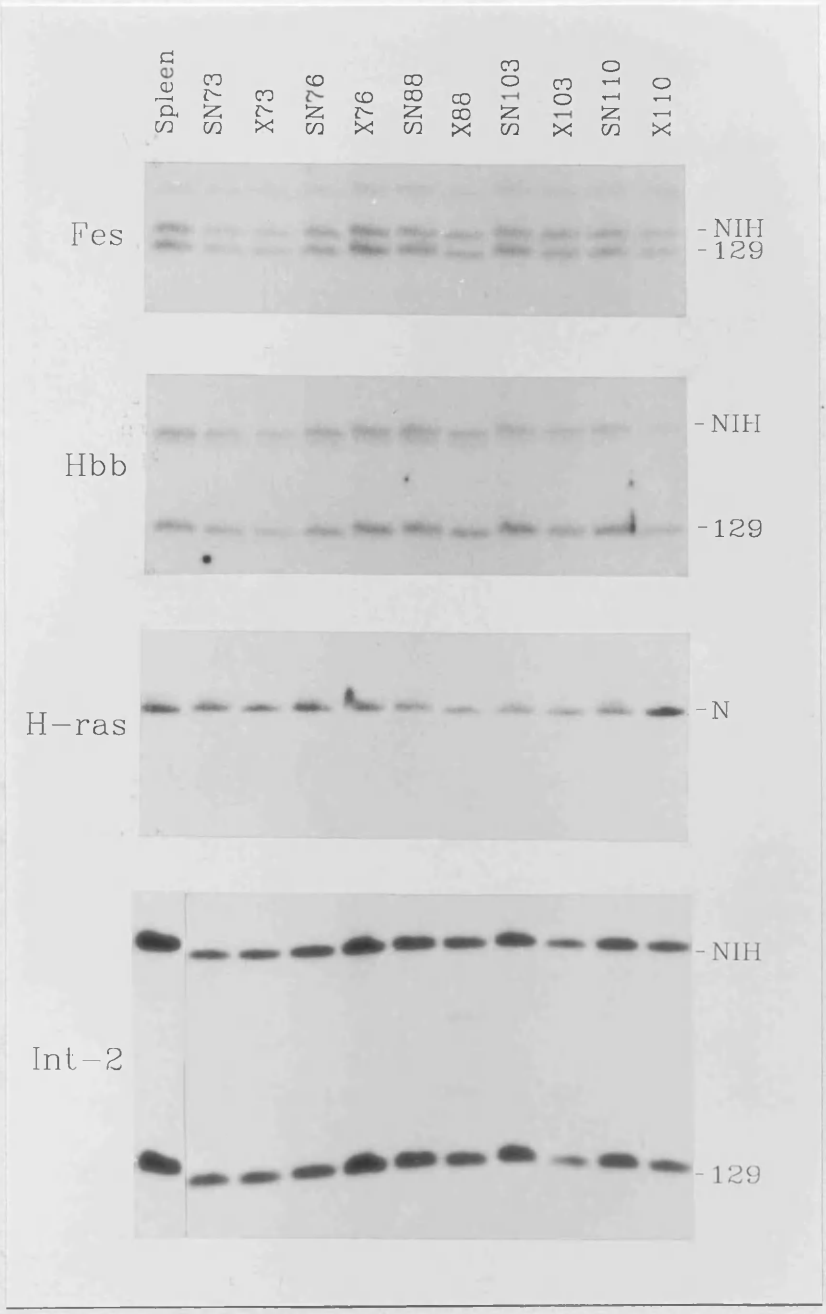
(section 1.5), repeated exposure to initiators is responsible for the induction of some human tumours. Although the latter method produces more mouse skin carcinomas than initiation/promotion (eg Reddy and Fialkow, 1983), little is known about the molecular basis for this difference. F1 hybrid mice therefore offered the possibility of comparing the type of gross chromosomal changes associated with each of these approaches. The results described below include analysis of carcinomas induced by repeated MNNG treatment (section 3.6.1) , and others induced by multiple applications of DMBA (section 3.6.2). In addition, an attempt to determine the molecular changes in tumours induced by a combined initiation/promotion/initiation protocol is described in section 3.6.3.

3.6.1 Repeated MNNG treatment does not produce carcinomas with chromosome 7 changes, even if they have activated H-*ras*

Hennings *et al.* (1983) have shown that papillomas progress more frequently if they are treated with mutagens such as DMBA and MNNG rather than TPA. This suggests that these chemicals induce the same, or an equivalent event that occurs spontaneously in TPA promoted tumours. In this study five carcinomas (SN73, 76, 88, 103 & 110) , and cell lines grown from all of these (prefixed "X"), were studied which had been obtained from SN hybrids treated twice weekly with MNNG. These tumours were analysed for codon 12 and 13 H-*ras* mutations by hybridisation of oligomers to amplified DNAs, and for the codon 61 mutation detectable by *Xba*I digestion. Three of the carcinomas (SN73, 76 and 103) and the cell lines grown from them, were negative in this search (Figures 16 and 19; Table 8). In addition they did not have any detectable chromosome 7 changes (Figure 19, Table 9). These therefore resemble the pattern seen in MNNG/TPA tumours with no activated H-*ras*.

The two other MNNG/MNNG carcinomas, SN88 and SN110 had a G:C->A:T transition at the second nucleotide of codon 12 (Figure 16). Unfortunately, the cell lines grown from explants of these tumours did not have this mutation (Figure 16),

Figure 19 Carcinomas induced by repeated MNNG treatment lack chromosome 7 changes



Chromosome 7 RFLP analysis of 5 SN MNNG/MNNG carcinomas. Cell lines were grown from explants of all 5 tumours; these have the same number as the original tumour but are prefixed "X". However, at least 2 of these (X88 and X110) are known not to be derived from the original tumour since they lack the codon 12 H-*ras* mutation detected in the tumour (Figure 16). All the tumours lack the codon 61 H-*ras* mutation detectable by the *Xba*I RFLP. SN73 and 76 were from the same mouse, other tumours were from separate mice. The spleen was from an SN hybrid. The digest for the *Hbb* probe was *Eco*RI (band sizes in Table 4). Other details as in Figures 7 and 9.

Table 9 Chromosome 7 allele ratios in MNNG/MNNG tumours

Tumour Grade:		1	-	1-2	-	1-2	-
% contamination:		10	-	50	-	<10	-
	Tumour:	**SN73**	**X73**	**SN76**	**X76**	**SN88**	**X88**
Allele ratios	**Fes**	1.2 (129)	1.47 (NIH)	1.16 (NIH)	1.21 (129)	1.02 (129)	1.13 (NIH)
	Hbb	1.06 (NIH)	1.13 (NIH)	1.03 (NIH)	1.11 (129)	1.00	1.24 (129)
	Int-2	1.32 (129)	1.3 (129)	1.1 (129)	1.18 (129)	1.17 (129)	1.23 (129)

Tumour Grade:	Spindle	-	1	-
% contamination:	<10	-	50	-
Tumour:	**SN103**	**X103**	**SN110**	**X110**
Allele ratios **Fes**	1.23 (129)	1.41 (129)	1.05 (NIH)	1.15 (129)
Hbb	1.01 (129)	1.35 (129)	1.23 (129)	1.16 (NIH)
Int-2	1.2 (129)	1.09 (NIH)	1.14 (129)	1.06 (129)

The Table is in the same format as Table 6. Spindle = spindle cell carcinoma.

implying that they were derived from contaminating normal cells or tumour cells of another origin. Unlike all of the DMBA/TPA and MNNG/TPA carcinomas which had activated H-*ras*, these tumours did not exhibit any chromosome 7 changes (Figure 19). Note that although there is a suggestion of imbalance at the *Hbb* locus in SN103 and 110 compared with the spleen DNA, the cell lines, which at least in the case of SN110 was not derived from the tumour, also have this very slight difference. The absence of changes at these loci however does not exclude the possibility that changes at the H-*ras* locus (e.g gene duplication) had occurred in these tumours. There are no controls available that allow for quantitation of the number of codon 12 H-*ras* mutant alleles compared to normal alleles, and so the ratio of mutant:normal in tumours with this H-*ras* mutation cannot be assessed.

3.6.2 Increase in mutant H-*ras* copy number by various mechanisms in tumours induced by repeated DMBA treatment

The comparison of carcinomas produced by repeated initiation with those produced by initiation followed by TPA promotion, was extended to the analysis of tumours induced by twice weekly treatment with DMBA (DMBA/DMBA carcinomas). Eight carcinomas and cell lines grown from four of these were analysed in total. Four of the DMBA/DMBA carcinomas (SN33, 30, 31 and 47) were positive for the *Xba*I RFLP signifying an A:T->TA transversion at the second nucleotide of codon 61 of H-*ras* (Figure 20). No other codon 61 mutations were detected (Figure 21 and Table 8) and in the four tumours negative for the *Xba*I RFLP no codon 12 or 13 mutations were detected (Table 8).

As with DMBA/TPA and MNNG/TPA tumours, chromosome 7 changes were only found in DMBA/DMBA carcinomas which also had an activated H-*ras* gene. SN31, for example, displayed an excess of the mutant H-*ras* and an excess of *fes*, *Hbb* and *Int*-2 NIH alleles (Figure 20). The allele ratios at *fes*, *Hbb* and H-*ras* suggested that over-representation of the NIH chromosome carrying the mutant

H-*ras* allele had occurred, reminiscent of several DMBA/TPA carcinomas (section 3.4). These ratios were below 2:1, possibly because of normal cell contamination. At the *Int*-2 locus the imbalance in favour of the NIH allele was around 8:1 (Table 10). This large imbalance suggests that after or before non-disjunction, deletion or mitotic recombination resulted in total loss of the 129 allele, with contaminating normal cells responsible for the remaining hybridisation signal. The number of NIH *Int*-2 alleles was calculated to be 2.37-3.17 (depending on which IL3 band was used to normalise for DNA loading), resembling the total number of alleles at *fes* and *Hbb* (which ranged from 2.92-3.81). This makes mitotic recombination the most likely explanation for the allele ratios observed in this tumour (Figure 22A). The fact that after normalising for DNA amounts, the NIH *Int*-2 allele was approximately 3x as intense as the same allele in the neighbouring lane (SN30, Figure 20) also supports this conclusion.

The ratio of mutant:normal H-*ras* in SN47 was close to 1:1.5 (Table 10). However, the normal cell contamination of this tumour was high (Table 10) and the true ratio was probably 2:1 (c.f. Table 16B). This conclusion was supported by analysis of a cell line (X47) grown from SN47. In X47 the excess of mutant H-*ras* was unambiguous (Figure 20), with the ratio of mutant:normal being approximately 2:1 (Table 10). An excess of the 129 alleles at *fes* and *Hbb* was also detected (Figure 20). In the original tumour this imbalance was diluted by the presence of contaminating normal cells (Figure 20). In contrast to these imbalances, the ratio of alleles at *Int*-2 in both the tumour and cell line was similar to that seen in the control spleen (Figure 20, Table 10). One explanation for these observations is that, as in many of the DMBA/TPA and both of the MNNG/TPA carcinomas with activated H-*ras*, nondisjunction occurred resulting in duplication of the chromosome carrying the mutant H-*ras* gene, but that subsequently one of the *Int*-2 NIH alleles was lost by deletion (Figure 22B). Densitometric analysis of SN47 was consistent with this interpretation. The total number of *Int*-2 alleles was 0.66-1.90 (depending on the

which IL3 band was used to normalise for DNA loading), while at other loci there were more alleles, eg 1.98-2.99 at *fes*.

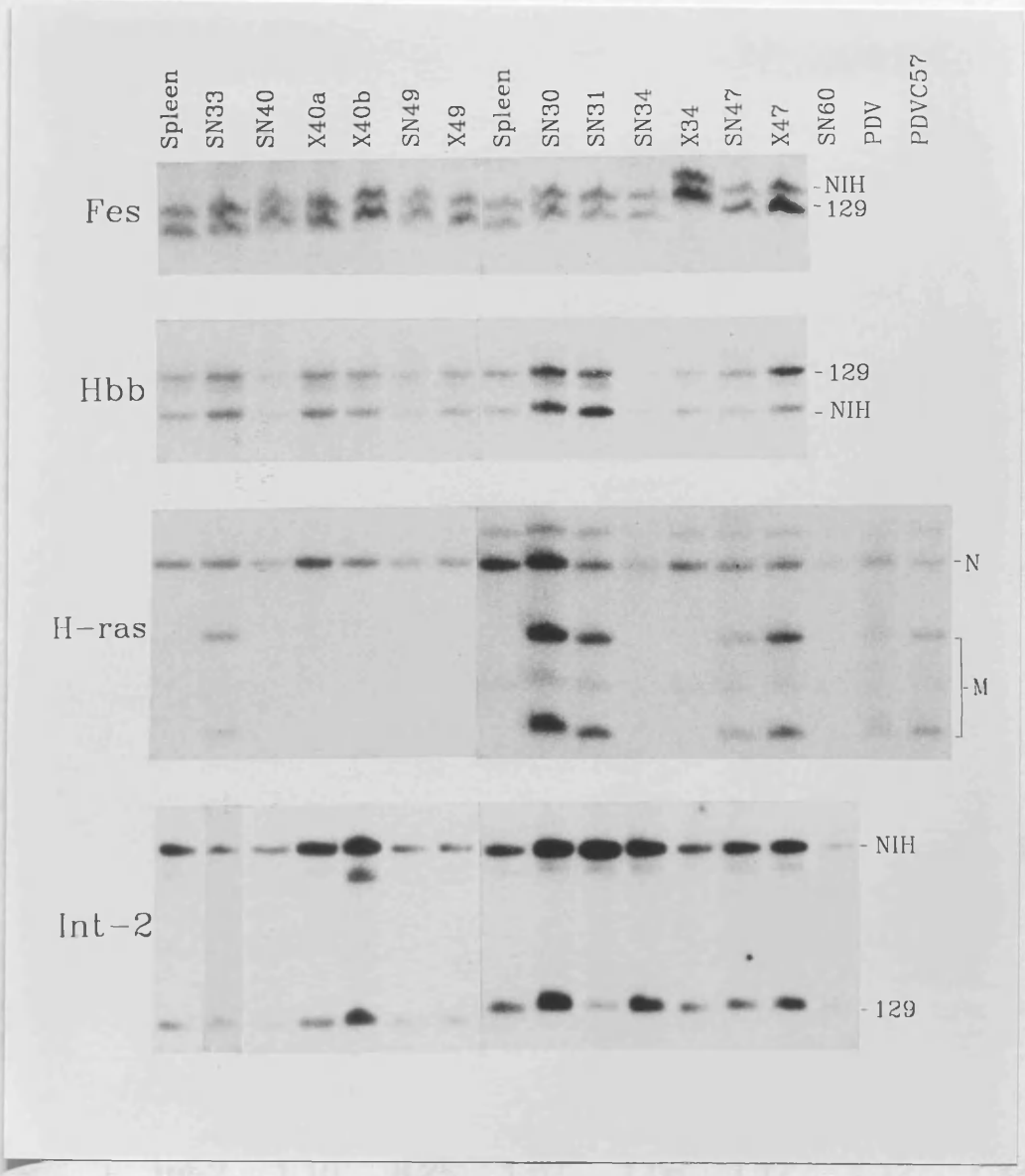
A third pattern was observed in SN30. As with SN31 and 47 an excess of mutant H-*ras* was detected, but unlike these other tumours, no clear imbalance was found at *fes*, *Hbb* or *Int*-2 (Figure 20, Table 10). Thus, duplication of the mutant H-*ras* gene may have occurred, leaving the allele ratios at *fes*, *Hbb* and *Int*-2 unchanged (Figure 22C).

In the other DMBA/DMBA carcinoma which had an H-*ras* mutation, SN33, there appeared to be a slight imbalance in favour of the NIH alleles (Figure 20, Table 10). The ratios are close to those predicted for a tumour 50% contaminated with normal cells in which the chromosome carrying the mutant H-*ras* allele has been duplicated (Table 16B). Histological analysis suggested only 20% contamination, but it is possible that a different section of the tumour contained more normal cells. Over-representation of the NIH chromosome would place this tumour in the same category as the large number of DMBA/TPA carcinomas in which non-disjunction was detected (section 3.4).

In view of the problems associated with densitometric analysis of aneuploid tumours which are contaminated with normal cells (section 1.13; Naylor *et al.*, 1987) other interpretations of the allele ratios detected in the above tumours should not be excluded. However, irrespective of the exact nature of these changes, it remains clear that H-*ras* activation is frequently associated with subsequent alterations involving chromosome 7.

The remaining four DMBA/DMBA carcinomas, which were negative for codon 12, 13 and 61 H-*ras* mutations (SN40, 49, 34 and 60), did not appear to have significantly different allele ratios at any of the polymorphic chromosome 7 loci compared to spleen controls (Figure 20; Table 10). These therefore resemble the MNNG/TPA and MNNG/MNNG tumours which were also negative for H-*ras* mutations and chromosome 7 changes.

Figure 20 RFLP analysis of tumours induced by repeated DMBA treatment



Chromosome 7 RFLP analysis of 8 DMBA/DMBA carcinomas. Cell lines grown from 4 of these were also analysed; these have the same number as the original tumour but are prefixed "X". Two cell lines were derived from tumour SN40 (X40a and b). Insufficient DNA was obtained from tumour SN60 to carry out a full analysis. SN30 and 31 were from the same mouse, other tumours were from separate mice. Spleens were from SN hybrids. Note that, due to variation in the labelled probe size, the *Int*-2 spleen samples display slightly different band ratios; for this reason the tumour band ratios in each panel should only be compared with the spleen from the same panel. Other details as in Figure 7 and 9.

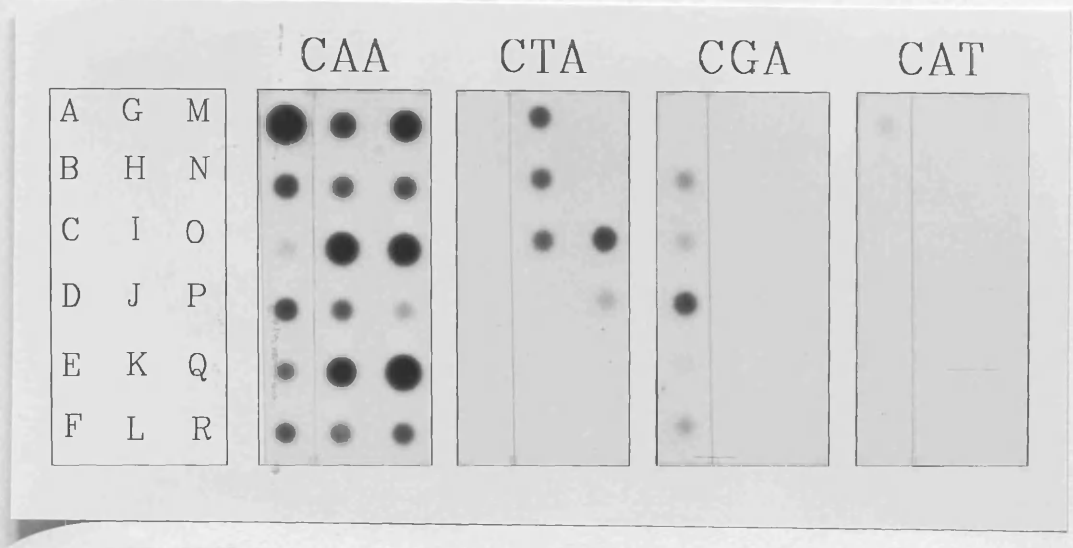
Table 10 Chromosome 7 allele ratios in DMBA/DMBA tumours

Tumour Grade:		2	Spindle	-	-	Spindle	-	
% contamination:		20	10	-	-	10	-	
	Tumour:	**SN33**	**SN40**	**X40a**	**X40b**	**SN49**	**X49**	
Allele ratios	**Fes**	1.47 (NIH)	1.13 (129)	1.16 (129)	1.31 (129)	1.21 (129)	1.26 (129)	
	Hbb	1.23 (NIH)	1.28 (NIH)	1.15 (NIH)	1.12 (NIH)	1.39 (129)	1.18 (129)	
	H-ras	1.57 (N)	NA	NA	NA	NA	NA	
	Int-2	1.29 (NIH)	1.22 (NIH)	1.35 (129)	1.85 (129)	1.10 (129)	1.2 (129)	

Tumour Grade:		1-2	3	2-3	-	1	-	?
% contamination:		20-30	20	20	-	50%	-	?
	Tumour:	**SN30**	**SN31**	**SN34**	**X34**	**SN47**	**X47**	**SN60**
Allele ratios	**Fes**	1.22 (129)	1.64 (NIH)	1.17 (129)	1.43 (129)	1.60 (129)	2.13 (129)	ND
	Hbb	1.03 (NIH)	1.57 (NIH)	1.18 (NIH)	1.14 (NIH)	1.74 (129)	1.81 (129)	ND
	H-ras	1.63 (M)	1.67 (M)	NA	NA	1.47 (N)	1.82 (M)	NA
	Int-2	1.10 (129)	8.25 (NIH)	1.02 (129)	1.06 (129)	1.17 (NIH)	1.12 (129)	1.32 (129)

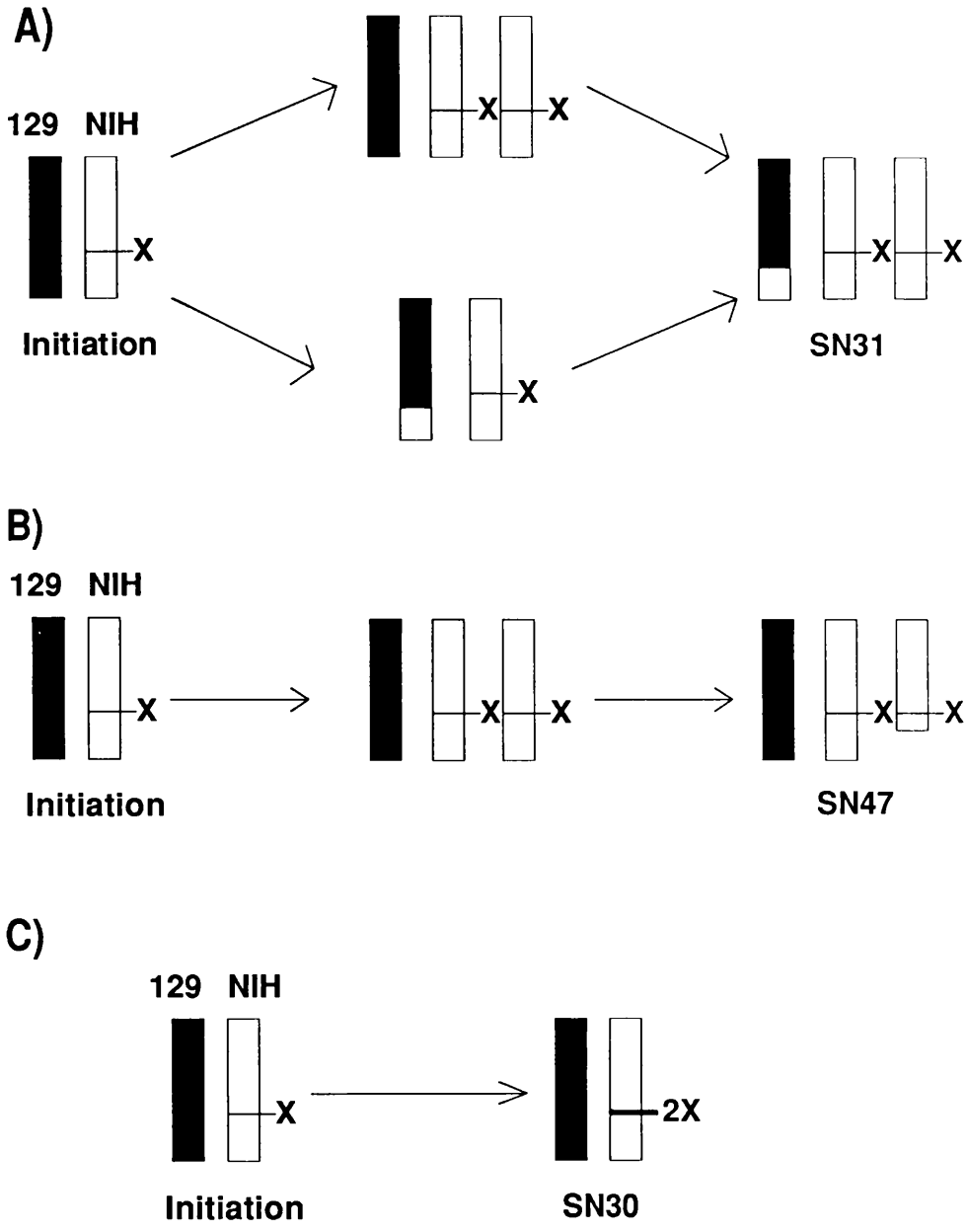
The Table is presented in the same format as Table 6.

Figure 21 Absence of alternative codon 61 mutations in DMBA/DMBA carcinomas



DMBA/DMBA carcinoma samples were amplified with exon 2 amplimers and probed with oligomers specific for the normal codon 61 sequence (CAA) and mutant sequences (CTA, CGA and CAT). Grid letters correspond to the following amplified DNA samples: A) Control for CAT mutation; B-F) Controls for CGA mutation; G) SN30; H) SN31; I) SN33; J) X34; K) SN40; L) X40a; M) SN spleen; N) X40b; O) SN47; P) X47; Q) SN49; R) X49. Tumours negative for codon 61 mutations were also negative for codon 12 and 13 H-*ras* mutations (Table 8). No DNA was available to analyse SN34 or SN60. The sequence of amplimers and hybridisation oligomers is given in Table 3, section 2.16.

Figure 22 Possible mechanisms explaining allele ratios in DMBA/DMBA tumours with activated H-*ras*



A) Alternative routes to the chromosome 7 status of SN31. Mutant H-*ras* is represented by "X". Trisomy may have preceded or followed mitotic recombination.

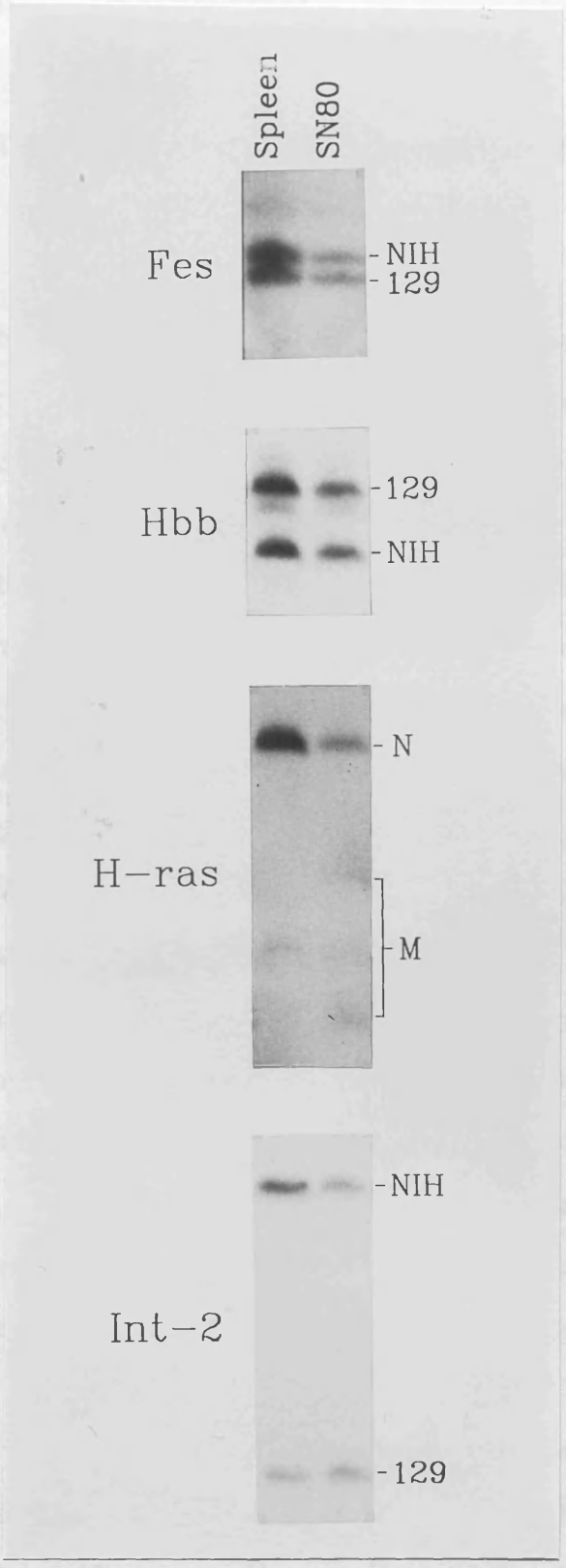
B) Non-disjunction followed by deletion in SN47.

C) Duplication of mutant H- *ras* in SN30 (represented by "2X").

3.6.3 Imbalance at *Int*-2 in a carcinoma obtained by treatment of DMBA/TPA papillomas with MNNG

The frequency of malignant progression of papillomas is known to increase if they are treated with mutagens such as MNNG (Hennings *et al.*, 1983). It was therefore of interest to see if chromosome 7 changes were involved in the progression of these tumours. Four 129xNIH hybrid mice, each with around a dozen papillomas that had been initiated with DMBA and promoted with TPA, were selected for this experiment. TPA treatment was replaced with application of MNNG or benzoyl peroxide 6 months after initiation. Treatment with benzoyl peroxide was apparently very toxic as papillomas disappeared and the epidermis became inflamed. However, in the two mice that were treated with MNNG one carcinoma was obtained 9 months after initiation with DMBA, and two more 11 months after initiation. Only the former was analysed. As expected, this tumour (SN80) was positive for the DMBA inducible *Xba*I RFLP indicative of an A:T->T:A transversion at the second nucleotide of codon 61 of H-*ras* (Figure 23). The ratio of mutant:normal H-*ras* in SN80 was close to 1:3 (Table 6). Analysis of the *fes*, *Hbb* and *Int*-2 RFLPs suggested that while there was no imbalance at loci proximal to H-*ras*, at the *Int*-2 locus there was a 2.55:1 imbalance in favour of the 129 allele (Table 6). These ratios are close to those expected for a tumour 50% contaminated with normal cells in which mitotic recombination distal to H-*ras* has occurred (Table 16E, section 1.13.2). The number of 129 *Int*-2 alleles in SN80 was calculated to be 1.15-1.71, depending on which IL3 band was used to normalise for DNA amounts. This is close to the expected value (1.5) for a tumour of the type just described. The total number of alleles at other (balanced) loci were, as expected, close to 2.0. Thus, mitotic recombination seems the most likely explanation for the allele ratios detected in this tumour, although the lack of histological analysis (Table 6) and the problems associated with densitometric analysis (section 1.13) mean that this conclusion is only tentative. Nevertheless, this represents another tumour in which an imbalance distal to H-*ras* was detected, and

Figure 23 Imbalance at *Int*-2 in a DMBA/TPA/MNNG carcinoma



Chromosome 7 RFLP analysis of an SN carcinoma derived by repeated MNNG treatment of DMBA/TPA papillomas. The spleen was from an SN hybrid. Other details as in Figures 7 and 9.

supports the possibility that a gene other than H-*ras* in this region of chromosome 7 is involved in mouse skin tumorigenesis.

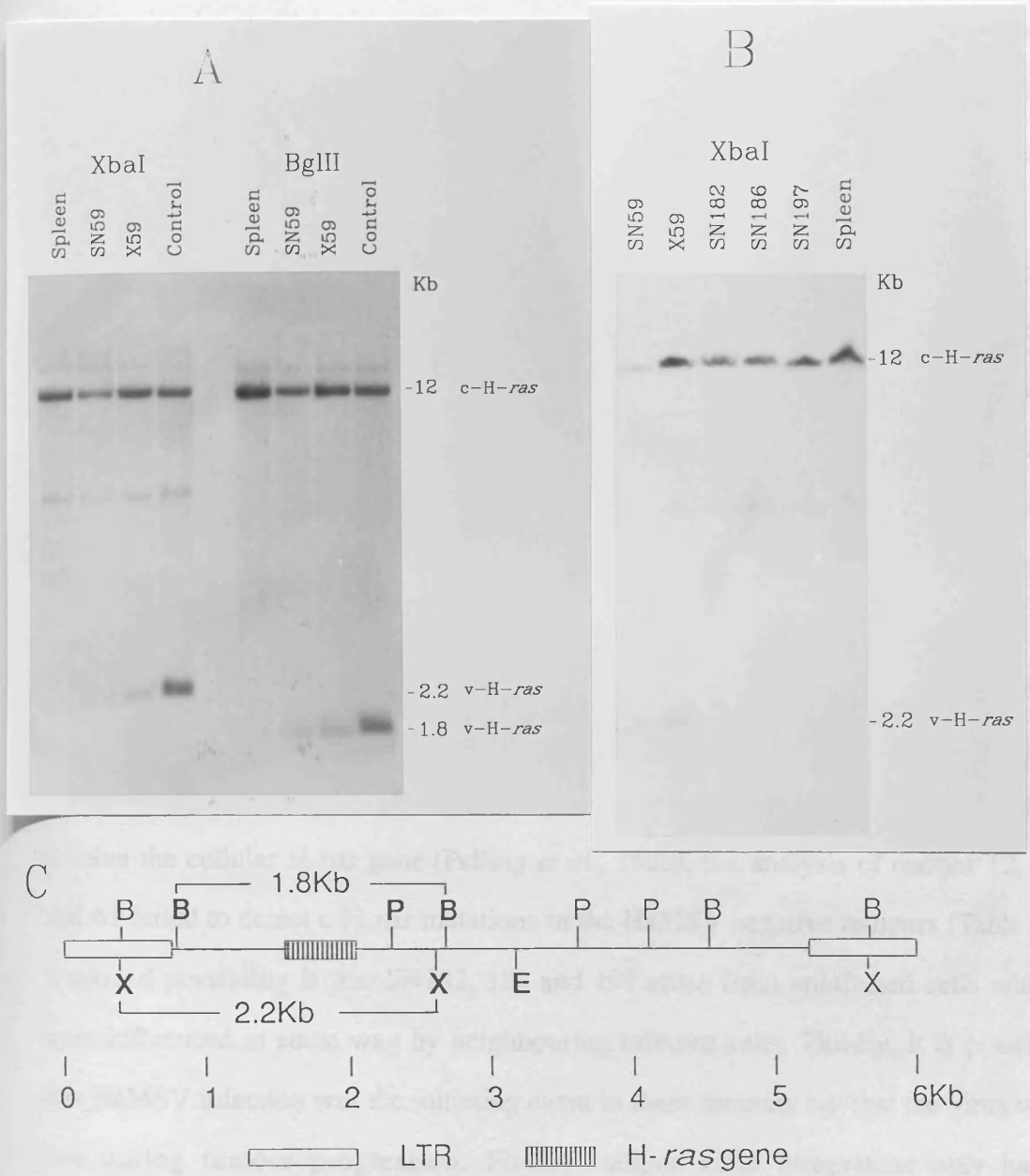
3.7 Chromosome 7 analysis in tumours initiated with v-H-*ras*

Part of the evidence supporting the conclusion that activation of H-*ras* is the initiating event in DMBA/TPA carcinomas was the discovery that treatment of skin with DMBA can be replaced by application of Harvey murine sarcoma virus (HaMSV), which contains an activated version of H-*ras*, to scarified skin (Brown *et al.*, 1986). Subsequent treatment with TPA produces papillomas some of which progress to carcinomas (Brown *et al.*, 1986). The chromosome 7 status of tumours induced in this way was investigated using the F1 hybrid mouse model. Although the integration sites of the virus were not determined, it is probable that they involved loci on chromosomes other than 7. It was considered therefore, that these tumours would provide evidence for or against a role for changes involving this chromosome other than an increase in the ratio of mutant:normal H-*ras* alleles.

Four v-*ras*/TPA tumours were analysed. Three of these (SN59, 186 and 197) were carcinomas, while the other tumour (SN182), which was dissected from beneath the epidermis, was found to be a mammary adenocarcinoma (Table 11). A cell line (X59), grown from an explant of SN59, was also analysed.

The presence of HaMSV in SN59 and X59 was confirmed by hybridisation of a v-H-*ras* probe to *Xba*I and *Bgl*II digests of these tumours. The fragment sizes predicted from the restriction map of HaMSV (Figure 24C) were obtained (Figure 24A). *Pst*I and *Eco*RI were used to determine the number of viral integration events in SN59. These enzymes cut on one side of the v-H-*ras* gene (Figure 24C), so the fragment which hybridises to a v-H-*ras* probe varies depending on the site of viral integration. Only one band was detected in SN59 indicating one viral integration

Figure 24 Analysis of v-H-*ras* initiated tumours for presence of virus



Southern analyses of v-H-*ras*/TPA carcinomas with BS9 probe (Table 4)

(A) The 2.2kb *Xba*I band and 1.8kb *Bgl*II band are the expectedfragment sizes for these enzymes (see restriction map in C). X59 is a cell line derived from carcinoma SN59. The control is a cell line previously derived from a v-H-*ras* induced carcinoma. The spleen was from an SN hybrid. The 12kb fragment is the cellular H-*ras* gene.

(B) SN182 was a mammary adenocarcinoma. SN186 and 197 were carcinomas. All 3 tumours were negative for HaMSV.

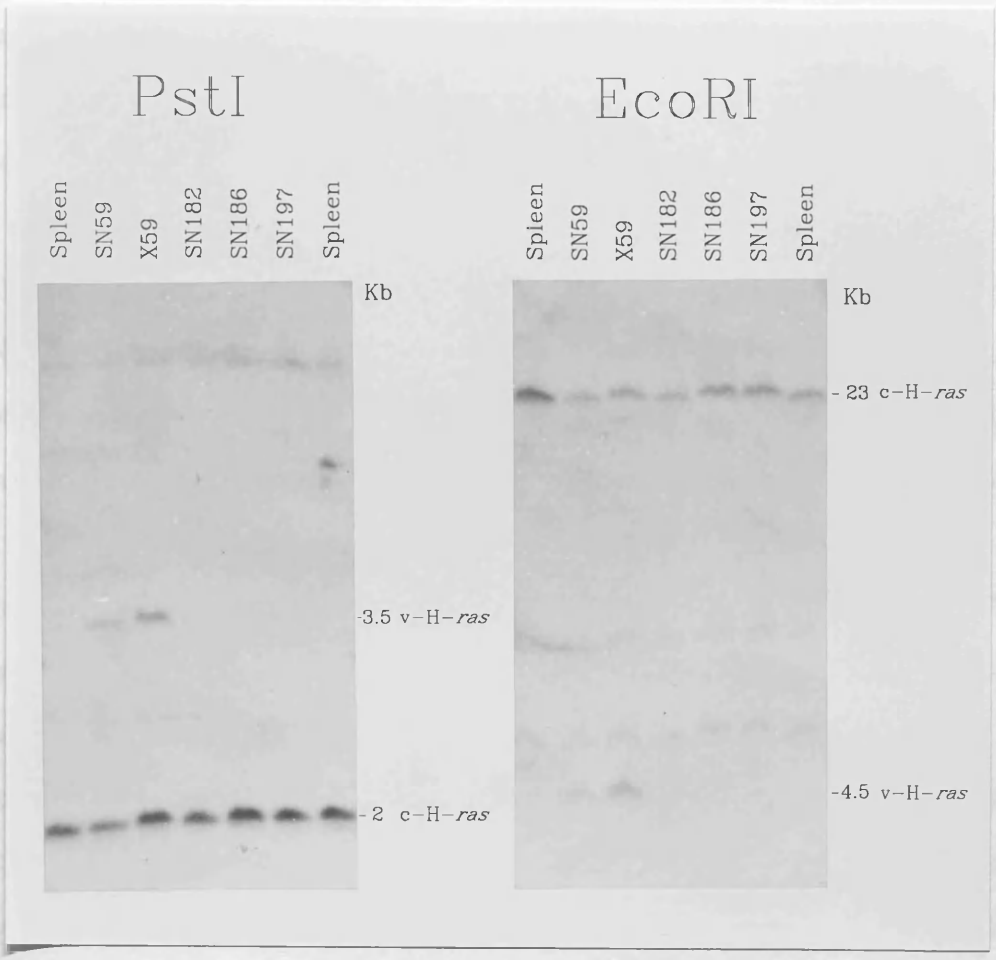
(C) Restriction map of HaMSV (Ellis *et al.*, 1980; Goldfarb and Weinberg, 1981). Restriction sites in bold indicate those responsible for the bands detected in A, B and Figure 25. B: *Bgl*II; E: *Eco*RI; P: *Pst*I; X: *Xba*I.

event (Figure 25). This result also demonstrates the monoclonal nature of this tumour. In the original study of v-*ras*/TPA tumours it was found that carcinomas were monoclonal although papillomas were frequently oligo or polyclonal (Brown *et al.*, 1986). The *Pst*I and *Eco*RI fragment sizes observed in X59 were identical to those detected in SN59 proving that this cell line was derived from SN59 (Figure 25).

Analysis of SN182, 186 and 197 with *Xba*I (Figure 24B) and *Pst*I or *Eco*RI (Figure 25) failed to positively identify HaMSV in all of these tumours. Several explanations are possible. First, HaMSV may have played no role in the development of these tumours. It may be significant that these tumours were obtained much later than SN59. The latter tumour was taken only 5 months after initiation whereas SN182, 186 and 197 were obtained 11, 12 and 14 months respectively after initiation. TPA treatment may therefore have selected out a few clones of cells which had suffered an initiation event unrelated to viral uptake. Such events have been detected (Pelling *et al.*, 1988) and may already be present prior to treatment with TPA or occur during TPA induced hyperproliferation. Spontaneous activation events frequently involve the cellular H-*ras* gene (Pelling *et al.*, 1988), but analysis of codons 12, 13 and 61 failed to detect c-H-*ras* mutations in the HaMSV negative tumours (Table 8). A second possibility is that SN182, 186 and 197 arose from uninfected cells which were influenced in some way by neighbouring infected cells. Thirdly, it is possible that HaMSV infection was the initiating event in these tumours but that the virus was lost during tumour progression. Finally, helper virus integration may have contributed to the development of these tumours.

Analysis of SN59 at polymorphic chromosome 7 loci showed that while there were no imbalances proximal to H-*ras*, under-representation of the NIH *Int*-2 allele had occurred (Figure 26, Table 11). However, no such imbalance was detected in the cell line X59 (Figure 26; Table 11). The calculated allele numbers, which varied depending on the IL3 band used to normalise for DNA amounts, did not clearly distinguish between mitotic recombination and deletion at the *Int*-2 locus.

Figure 25 Virus copy number in v-H-*ras* initiated tumours



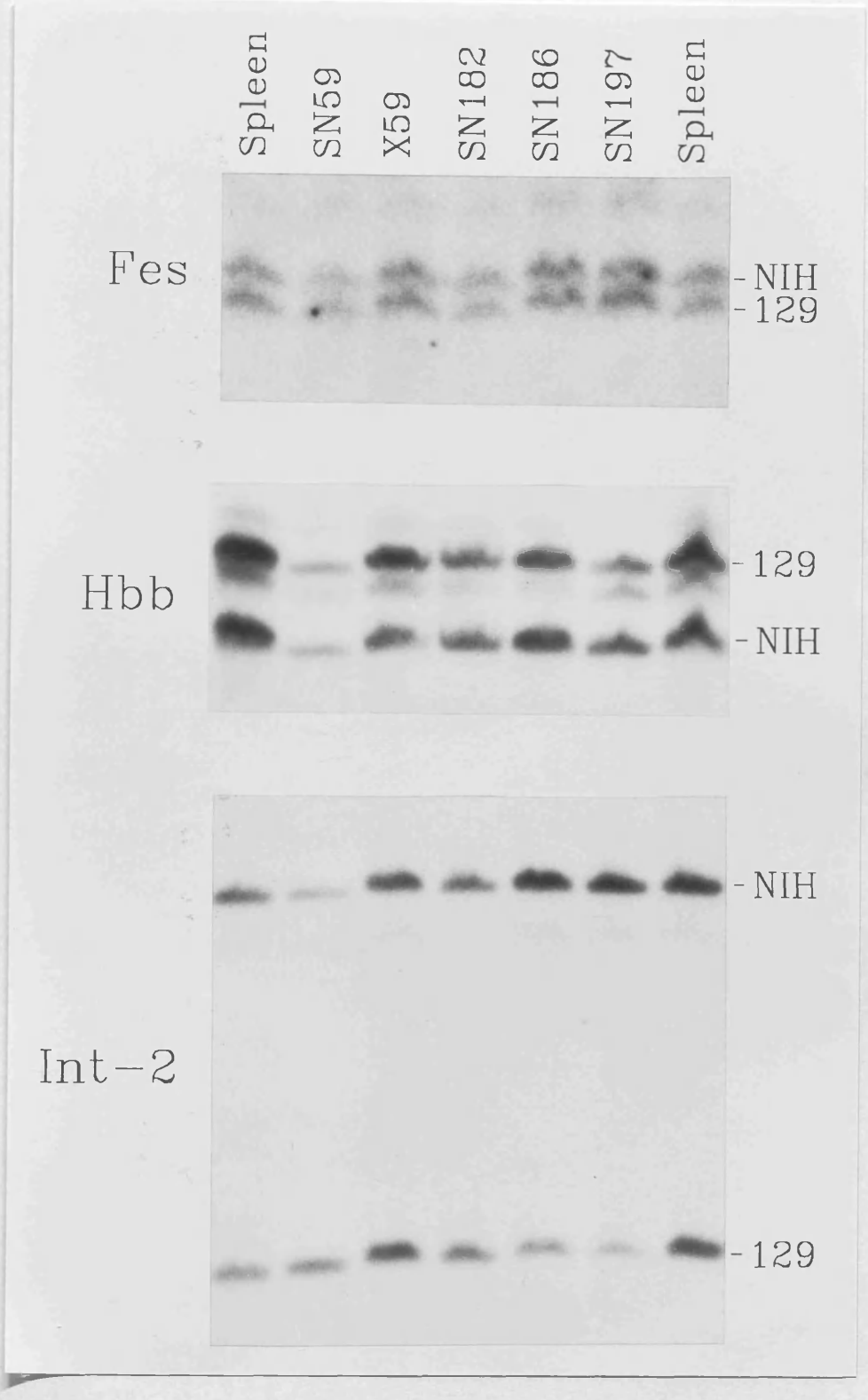
Southern analysis was carried out on v-*ras*-initiated, TPA-promoted tumours using *Pst*I and *Eco*RI. 3.5kb *Pst*1 and 4.5kb *Eco*RI viral fragments were detected in SN59 and X59. No viral fragments were detected in the other tumours. Cellular H-*ras* gene fragments are also indicated. Other faint bands are related sequences. The spleen was from an SN hybrid. The probe was BS9 (Table 4).

Nevertheless, SN59 is significant because it provides evidence that chromosome 7 changes are important for a reason other than elevation of mutant c-H-*ras*. The reason why the cell line derived from SN59 lacks chromosome 7 changes is unclear. One possibility is that it was derived from cells in which the *Int*-2 imbalance had not occurred.

No imbalances were detected in the mammary adenocarcinoma SN182 (Figure 26). However, a 2-3 fold excess of the NIH *Int*-2 allele was detected in the carcinoma SN186 (Figure 26; Table 11). Trisomy of chromosome 7 had not occurred since no imbalance was seen at *fes* or *Hbb* (Figure 26). The tumour had very little normal cell contamination (Table 11) so it appears that the 129 *Int*-2 allele was present within the tumour cells. Densitometric analysis indicated that there were 1.00-1.73 NIH *Int*-2 alleles in SN186 (depending on which IL3 band was used to normalise for DNA loading), and 0.42-0.73 129 *Int*-2 alleles. The total number of alleles at other (balanced) loci were 1.64-2.01 (*Hbb*) and 2.03-2.35 (*fes*). The values at the *Int*-2 locus are within the range expected for a tetraploid tumour in which mitotic recombination has occurred; the resultant 3:1 ratio at *Int*-2 would give calculated allele numbers of 1.5 (NIH allele) and 0.5 (129 allele). The tetraploid nature of many carcinomas (Pera and Gorman, 1984; Fusenig *et al.*, 1985), including v-H-*ras* initiated carcinomas (Aldaz *et al.*, 1988) makes this a feasible suggestion. However, given the variety of factors which can affect this type of assessment (section 1.13) other possibilities should not be excluded.

A similar pattern was detected in SN197, only in this case the 129 *Hbb* allele also appeared to be under-represented (Figure 26, Table 11). This carcinoma consisted almost entirely of tumour cells (Table 11) so, as in SN186, the weaker 129 *Hbb* and *Int*-2 alleles were probably still present in the tumour cells. Densitometric analysis suggested that there were 0.33-0.39 129 *Int*-2 alleles, 1.3-1.51 NIH *Int*-2 alleles, 0.63-0.67 129 *Hbb* alleles and 1.25-1.34 NIH *Hbb* alleles. The total number of *fes* alleles ranged from 2.01-2.46. Again, these are close to the expected figures for a mitotic recombination (distal to *fes*) in a tetraploid tumour, although the smaller

Figure 26 Chromosome 7 RFLP analysis of v-H-*ras*/TPA tumours



Details of tumours are given in Figure 24A and B. They were all from separate mice. The spleens were from SN hybrids. Other details as in Figures 7 and 9.

Table 11 Chromosome 7 allele ratios in v-H-ras/TPA tumours

Tumour Grade:		?	-	Mam.ad.	Spindle	Spindle
% contamination:		?	-	10	<5	<5
	Tumour:	**SN59**	**X59**	**SN182**	**SN186**	**SN197**
Allele ratios	**Fes**	1.28 (129)	1.01 (NIH)	1.2 (NIH)	1.06 (NIH)	1.00
	Hbb	1.17 (129)	1.35 (129)	1.00	1.28 (NIH)	1.98 (NIH)
	Int-2	2.46 (129)	1.34 (129)	1.13 (129)	2.37 (NIH)	3.93 (NIH)

The Table is in the same format as Table 6. Mam. ad. = mammary adenocarcinoma.

value for the 129 *Int*-2 allele (also evident from the allele ratios; Table 11) may mean that it has been deleted in some or all of the tumour cells.

It is difficult to deduce the precise mechanism behind the changes observed in SN186 and 197. Mitotic recombination in a tetraploid tumour would be indistinguishable from a diploid tumour in which only a proportion of the cells had suffered deletion or mitotic recombination involving the distal portion of chromosome 7. The large number of variables which can influence densitometric analysis (section 1.13) is another complication. However, the most intriguing aspect of these tumours is that they represent the only examples in this study of carcinomas in which an activated H-*ras* gene was not detected but chromosome 7 changes were.

3.8 Non-disjunction is common in papillomas, and also increases the number of mutant H-*ras* alleles

One of the main advantages of the mouse skin model is that molecular changes can be correlated with well-defined histological stages. The above sections describe analysis of the chromosome 7 status at the malignant stage of skin tumorigenesis. Studies were also carried out to determine if these events occurred before malignant conversion, ie during papilloma growth. Seven papillomas, induced in SN hybrid mice by DMBA-initiation and TPA-promotion, were analysed for H-*ras* mutations and chromosome 7 changes. 5/7 were positive for the *Xba*I 8+4kb mutant band (Figure 27) and one of the other papillomas (SN136) had an A:T->T:A transversion at the third nucleotide of codon 61 (Figure 28). The other papilloma (SN144) may also have had this mutation since a faint signal was observable after a long exposure of the "CAT" oligomer hybridised to amplified SN144 DNA (Table 8). RNase mismatch analysis of SN144 has confirmed that cells in this tumour express a mutant transcript, and the size of the digested RNA fragments suggests that the mutation is in codon 61 (S. Young, personal communication). As with the oligonucleotide

hybridisation signal, the mutant transcript detected by RNase mismatch analysis was faint.

Analysis of all the DMBA/TPA papillomas at polymorphic loci on chromosome 7 revealed an imbalance at every locus in 6/7 cases. For example, the ratio of NIH:129 alleles in SN146 was indicative of non-disjunction resulting in duplication of the NIH chromosome (Figure 27; Table 12). The initial impression gained from studying the allele ratios in this tumour (Figure 27; Table 12) is that non-disjunction has resulted in duplication of the chromosome carrying the normal H-*ras* allele. However, the ratio of normal : mutant H-*ras* alleles in this tumour was only 1.3:1 (Table 12). This excess of the normal allele is not great enough to support the proposition that the duplicated NIH chromosome carried the normal allele. Instead, this ratio, and the ratios at the other alleles, are close to the predicted ratios for a tumour which is 40-50% contaminated with normal cells and has two copies of the chromosome carrying the mutant allele and one carrying the normal allele (Table 16B; section 1.13.2). The same hypothesis fits the pattern seen in SN137, 145 and 146, only the amount of normal cell contamination appears to have varied from 15 to 50% (c.f. Table 12 and Table 16B).

The approximate 2:1 ratio of normal:mutant *ras* in SN134 suggests that this tumour was also highly contaminated with normal cells (Table 12). Unlike the papillomas discussed above, SN134 did not have an imbalance at *fes* or *Hbb* (Figure 27, Table 12). However, under-representation of the *Int*-2 129 allele was detected (Figure 27). The allele ratio was close to that predicted for a tumour 40% contaminated with normal cells, and heterozygous at H-*ras* with a deletion distal to this locus (Table 16F; section 1.13.2). However, calculation of the number of NIH *Int*-2 alleles suggested a figure of 1.73-2.65, (depending on the IL3 band used to normalise for DNA amounts), and as the total number of alleles at other (balanced) loci was also around two, this suggests that mitotic recombination had occurred. This discrepancy highlights the problems which can be associated with densitometric

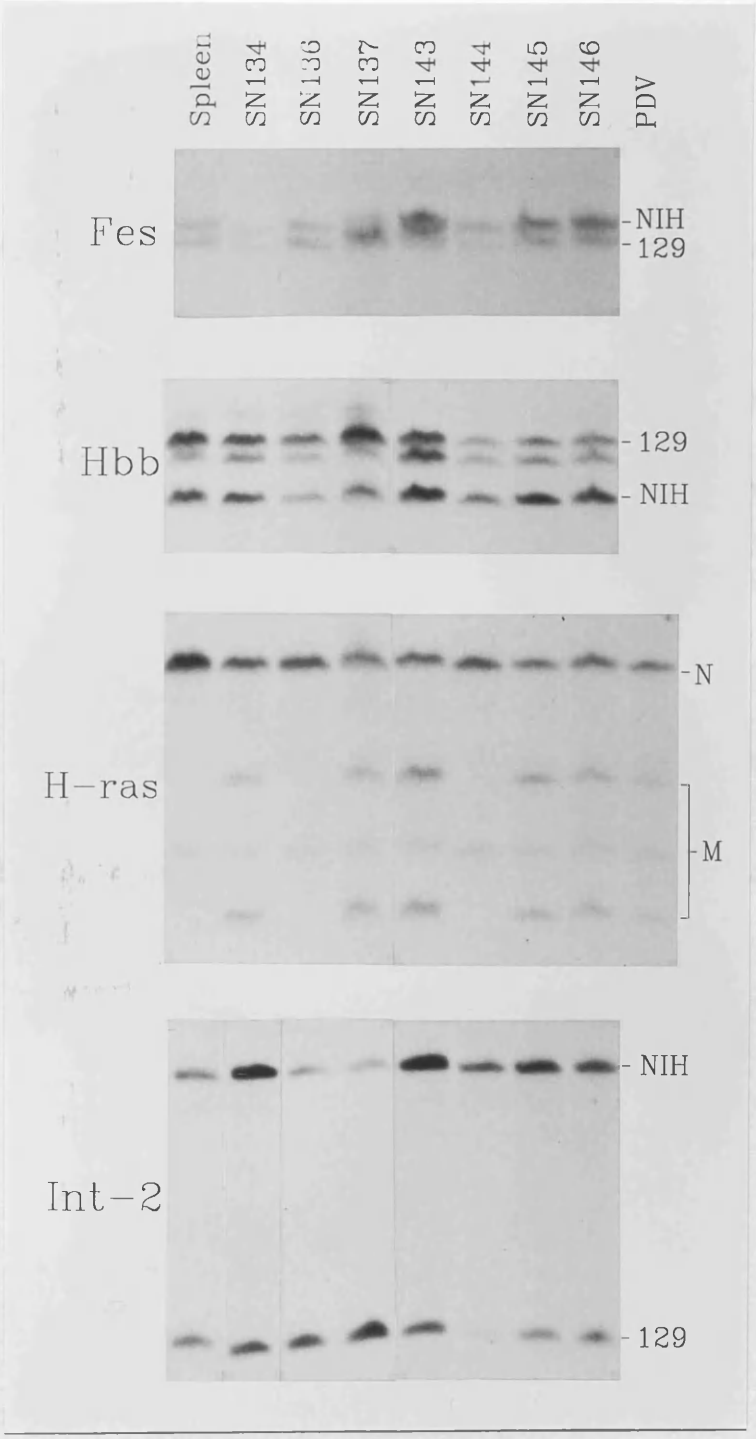
analysis of aneuploid tumours, highly contaminated with normal cells (section 1.13; Naylor *et al.*, 1987).

SN136, which had an A:T->T:A transversion at the third nucleotide of H-*ras* codon 61, showed an excess of 129 alleles, although the imbalance at *fes* was not as convincing compared with other alleles (Figure 27). Given the pattern seen in other papillomas it is likely, although not proven, that the mutant H-*ras* allele was also on the over-represented chromosome. SN144, which may have had the same mutation as SN136 (see above), was positive for a chromosome 7 imbalance; an excess of the NIH chromosome was detected (Figure 27). Oligonucleotide hybridisation and RNase mismatch analysis suggested that the mutant allele in this tumour was under-represented. It may therefore have been located on the 129 chromosome. This tumour may therefore be a rare example of non-disjunction resulting in over-representation of the normal allele. The selective advantage which this might confer is unclear. Duplication of chromosome 7 may give papillomas a selective growth advantage by increasing the copy number of some gene other than mutant H-*ras*. Another possibility is that the imbalance in this tumour may have been a consequence, rather than a cause, of tumour progression.

As discussed in section 3.4.4, the difficulties associated with interpreting the mutant : normal H-*ras* ratio in tumours contaminated with large numbers of normal cells are considerably simplified when analysing tumours from *spretus* x *musculus* crosses. This is because the normal alleles from each parent can be distinguished (Table 4 and Figure 14). Analysis of several papillomas from these hybrids has confirmed that the contamination level is high in benign tumours; in the majority of cases both normal parental alleles were detected, as well as a mutant allele (P.Burns, personal communication). By using the normal contaminating allele as a measure for the amount of stromal contamination it was possible to confirm that the mutant allele was over-represented in these papillomas (P.Burns, personal communication).

The detection of gross chromosome 7 changes in papillomas with activated H-*ras* shows that these events occur during the premalignant stage of tumorigenesis

Figure 27 Chromosome 7 changes in DMBA/TPA papillomas



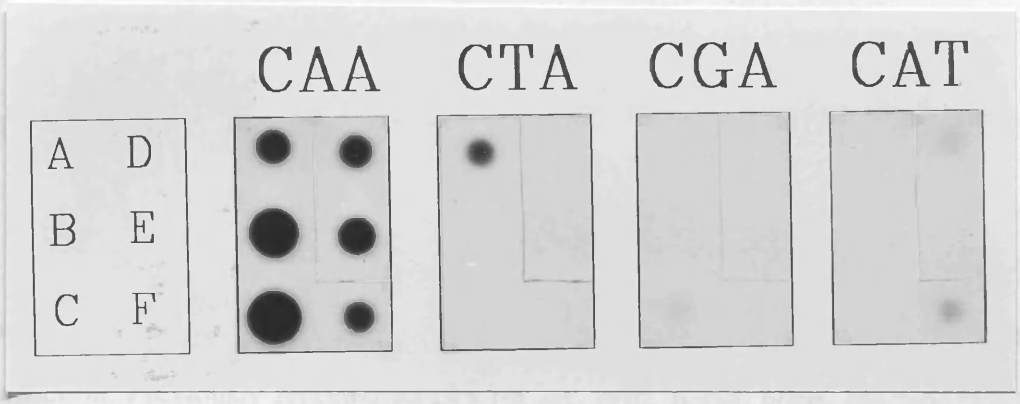
Chromosome 7 RFLP analysis of 7 SN DMBA/TPA papillomas. SN134, 136 and 137 were from a single mouse, as were SN143-146. The spleen was from an SN hybrid. Other details as in Figures 7 and 9.

Table 12 Chromosome 7 allele ratios in DMBA/TPA papillomas

	Tumour:	SN134	SN136	SN137	SN143	SN144	SN145	SN146
Allele ratios	Fes	1.10 (NIH)	1.37 (129)	1.71 (129)	2.00 (NIH)	1.77 (NIH)	1.84 (NIH)	1.57 (NIH)
	Hbb	1.08 (NIH)	1.64 (129)	1.55 (129)	1.61 (NIH)	1.81 (NIH)	2.21 (NIH)	1.89 (NIH)
	H-ras	2.01 (N)	NA	1.52 (N)	1.55 (M)	NA	1.46 (M)	1.26 (N)
	Int-2	2.27 (NIH)	1.78 (129)	1.99 (129)	2.18 (NIH)	2.28 (NIH)	1.81 (NIH)	1.64 (NIH)

The Table is in the same format as Table 6, except that no histological analysis was carried out; all tumour material was used to obtain nucleic acids.

Figure 28 An alternative codon 61 mutation in DMBA/TPA papillomas



DMBA-initiated, TPA-promoted papilloma samples were amplified with exon 2 amplimers and probed with oligomers specific for the normal codon 61 sequence (CAA) and mutant sequences (CTA, CGA and CAT). Grid letters correspond to the following amplified DNA samples: A) Control for CTA mutation (*Xba*I RFLP) B) SN spleen C) Control for CGA mutation D) SN136 E) SN144 F) control for CAT mutation. These tumours were negative for all other codon 12, 13 and 61 H-*ras* mutations (Table 8). The sequence of amplimers and hybridisation oligomers is given in Table 3, section 2.16.

and that other events are required to trigger further progression. It appears that one of the main contrasts between benign and malignant skin tumours is that the former frequently contain high amounts of normal cells, whereas most carcinomas contain mainly tumour cells (c.f section 3.4.2).

3.9 Chromosome 7 changes are non-random

To ensure that any chromosome 7 changes detected were non-random, efforts were made to find an RFLP on another chromosome for the hybrids used in tumour induction. Previously reported RFLPs for *src*, *myb*, K-*ras*, *erb*B, *fos*, and *neu* were non polymorphic in the hybrids we used (Table 13). In addition no polymorphisms were found using a *mos* probe (Table 13). However, using a probe for the catalase (*Cas*-1) gene, which maps to chromosome 2, two previously unreported RFLPs were discovered: a *BglI* RFLP was not appropriate for analysis of the hybrids used in tumour induction (Table 13), while a *BclI* RFLP was useful in analysing SB but not SN tumours, giving rise to a 7kb band in BALB/c mice and a 4.3kb band in SENCAR mice (Figure 29A and Table 13). Additionally, a previously reported RFLP for interleukin 3 (IL3) (Ihle *et al.*, 1987), which maps to chromosome 11, also proved useful in analysing SB but not SN hybrids. SENCAR mice had the 10.8kb fragment, BALB/c mice (as shown before) had the 8.5kb fragment (Figure 29B; Table 13).

The *Cas*-1 and IL3 RFLPs were used to analyse all the SB tumours. Only one tumour (SB137) showed an imbalance at *Cas*-1 (Figure 30A and Table 14). The ratio of BALB/c:SENCAR alleles, and the level of normal cell contamination (Table 14) suggested that this tumour had 2 or 3 BALB/c alleles to every SENCAR allele. This probably means that the SENCAR chromosome is under-represented and not lost, since a much higher ratio would be expected if complete loss had occurred, even in this tumour which is more highly contaminated with normal cells than most

Table 13 RFLPs on chromosomes other than 7

Format as in Table 4.

1. Locus: *Cas*-1 (chromosome 2)
Probe: 1.1Kb *Pst* I insert of pCAT4-1 (Boyd *et al.*, 1986)

		Mus musculus							*Mus*
	Enzyme	BALB	C57	NIH	NMRI	SENCAR	TFH	129	*spretus*
	*Bam*HI	ND	9.5, 7.0, 6.0	...	...	...	...	ND	ND
*	*Bcl* I	19, 7.0, 2.8	19, 4.3, 2.8	19, 4.3, 2.8	19, 4.3, 2.8	19, 4.3, 2.8	19, 4.3, 2.8	ND	ND
*	*Bgl* I	8.0, 4.2	7.5, 4.2	8.0, 4.2	8.0, 4.2	8.0, 6.9	8.0, 4.2	ND	8.3, 6.9
	Bgl II	1.0	...	...	...	...	...	ND	ND
	Eco RI	6.8, 4.6	...	...	...	...	...	ND	...
*s	*Hinc* II	ND	7.5, 6.7	ND	ND	ND	...	ND	6.7, 2.3
*s	*Hind* III	ND	ND.	8.0, 4.5	ND	ND	...	ND	9.7, 4.5
	Msp I	5.4, 1.3	...	...	...	...	...	ND	ND
	Pst I	ND	3.5, 2.5, 1.8, 0.9	ND	ND	ND	...	ND	ND
	Pvu II	1.8	...	...	...	...	...	...	...
*s	*Sac*I	8.0, 2.5	...	...	...	...	...	ND	8.0, 7.8
	Taq I	5.1, 1.4	...	...	...	...	...	ND	ND

2. Locus: *Src* (chromosome 2)
Probe: 800bp *Pvu*II insert of pvuIIE (Blatt *et al.*, 1984)

		Mus musculus					
	Enzyme	BALB	C57	NIH	SENCAR	TFH	129
*	*Hind* III	13	16.5, 14	13	13	13	13
	Kpn I	10.5	...	...	...	...	ND
	Msp I	3.4, 2.1	ND	...	...	...	...
	Pvu II	2.6	ND	...	...	...	...
	Taq I	2.5	ND	...	...	...	...
	Xba I	16	ND	...	...	...	ND

(NMRI and *Mus spretus* : ND)

3. Locus: *Mos* (chromosome 4)
Probe: 3Kb *Eco* RI fragment of pMS-1.

	Mus musculus					
Enzyme	BALB	C57	NIH	SENCAR	TFH	129
Eco RI	15	ND	...	15, (+19)	...	...
Pst I	2.0	ND	...	...	...	ND

(NMRI and *Mus spretus* : ND)

(SB hybrids lacked the 19Kb band detected in SENCAR)

4. Locus: IL3 (chromosome 11)
Probe: 1.1Kb *Eco* RI insert of pMu2a1, from A. Dunn & N. Gough.

	Mus musculus						
Enzyme	BALB	NIH	CBA	SENCAR	TFH	129	*Mus spretus*
* *Eco* RI	8.5 (16)	10.8 (16)	10.8 (16)	10.8 (16)	8.5 (16)	10.8 (16)	8.5 (13)
(*s) *Pst* I	1.0 (7.2, 1.4)	...	...	...	...	...	1.0 (5.3, 1.4)
(*s) *Xba* I	1.8 (9.8, 3.8)	...	...	...	...		1.8 (6.0, 3.8)

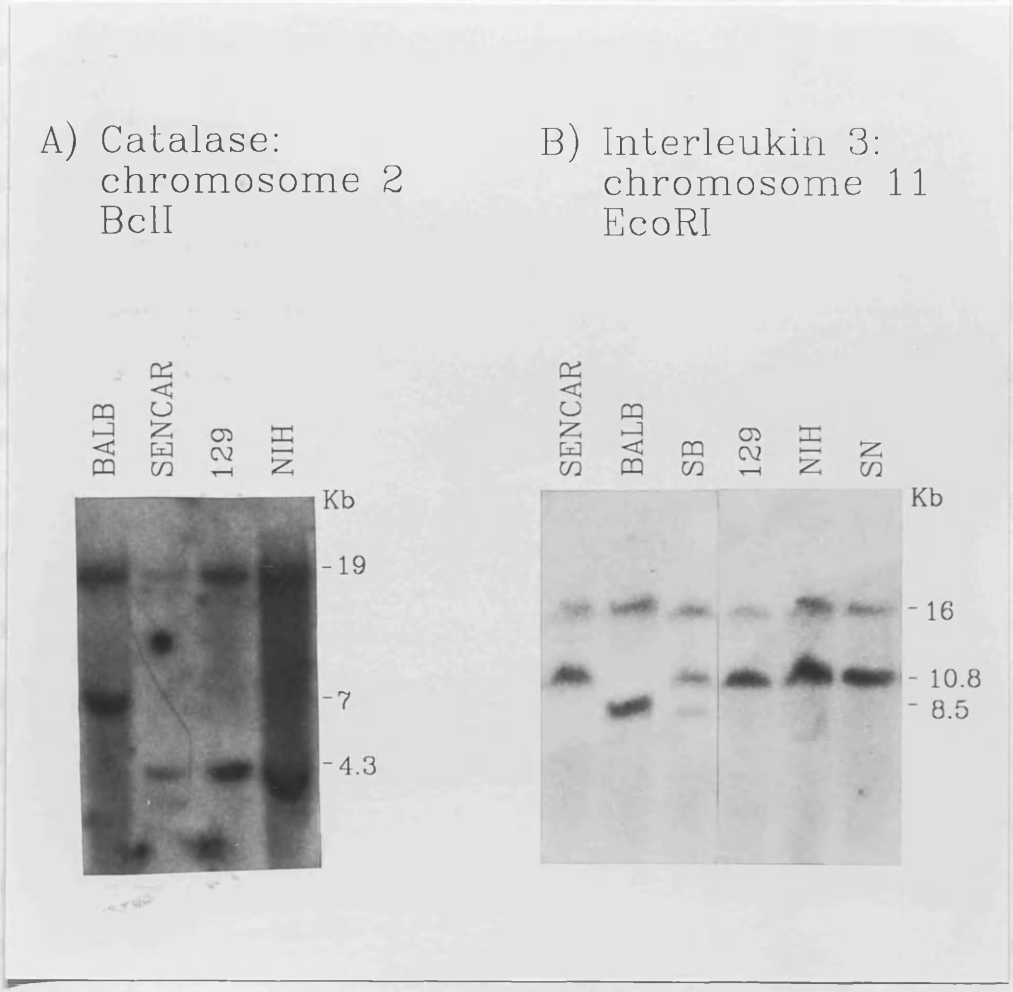
(C57 and NMRI: ND)

Figures in brackets represent fragments which hybridise to the unidentified fragment which was cloned with IL3 (see text).

Previously reported RFLPs at the following loci were also checked, but found to be uninformative for the crosses used in this study.

Locus	Chromosome	Original study	Enzyme
myb	10	Mock *et al.*, 1987	*Eco* RI
K-*ras*	6	Ryan *et al.*, 1986	*Eco* RI
fos	12	D'Eustachio, 1984	*Eco* RI
erb B	11	Ihle *et al.*, 1987	*Pst* I
neu	11	Xu *et al.*, 1986	*Hind* III

Figure 29 RFLPs on chromosome 2 and 11 in *Mus musculus* hybrids



A) SB hybrids are polymorphic for catalase using *Bcl*I, SN hybrids are not. The 19kb band is common to all strains.

B) SB hybrids are polymorphic for interleukin using *Eco*RI, SN hybrids are not. The band at 16kb corresponds to an unidentified DNA sequence cloned with the IL3 gene
(See Table 13 for details of clones).

carcinomas. The same tumour also showed an imbalance at IL3, with an approximate 2:1 imbalance in favour of the BALB/c allele Figure 30B; Table 14).

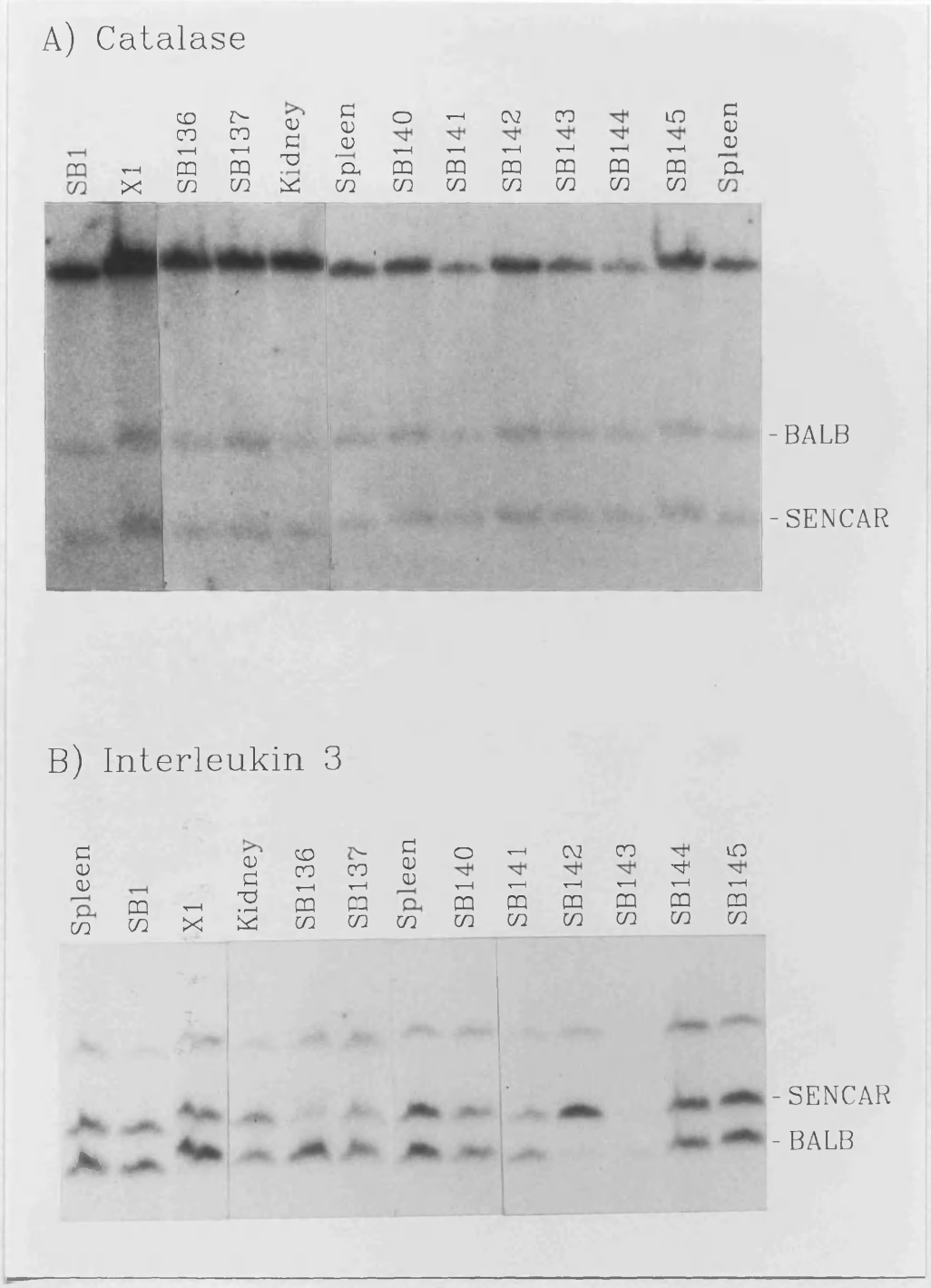
Two other tumours (SB136 and 142) showed complete loss of the one IL3 allele (Figure 30B). The approximate 5:1 (BALB/c:SENCAR) ratio in SB136 (Table 14) is consistent with loss of the SENCAR allele in a tumour 20-30% contaminated with normal cells; the possible mechanisms include hemizygosity at IL3 (c.f. Table 16C), and mitotic recombination or loss of the SENCAR chromosome with reduplication of the BALB/c chromosome (Table 16D). The latter two possibilities appear more likely, since the uppermost band in SB136 and SB137 (which corresponds to an unknown gene and acts as an internal control for DNA loading) are of similar intensity, whereas the BALB/c band is more intense in SB136 (Figure 30B). Loss of the BALB/c allele in SB142 (Figure 30B) is unambiguous since the ratio of SENCAR:BALB/c alleles is large (Table 14). Once again, comparison of the uppermost, internal control bands in SB141 and SB142 suggests that the SENCAR allele has been reduplicated in the latter (Figure 30B).

The detection of some changes on chromosomes 2 and 11 in some tumours was not unexpected given the highly aneuploid state of many carcinomas (Pera and Gorman, 1984; Conti *et al.*, 1986; Aldaz *et al.*, 1987). Although these changes may have some relevance to tumour progression, their relatively infrequent occurrence reinforces the significance of the chromosome 7 alterations commonly observed in carcinomas.

3.10 The Chromosome 7 alleles which are lost or under-represented can be derived from either parent

Different strains of inbred mice show varying degrees of sensitivity to tumour induction by the initiation-promotion protocol. For example, SENCAR is a sensitive strain while BALB/c is resistant (Slaga, 1984). If the molecular basis for this

Figure 30 Changes involving chromosomes other than 7 are rare



Chromosome 2 and 11 RFLP analysis of SB DMBA/TPA tumours.
(A) Catalase analysis
(B) Interleukin 3 analysis.
Spleen and kidney samples were from SB hybrids. Other details as in Figure 29.

Table 14 Catalase and interleukin 3 allele ratios in SB carcinomas

	Tumour Grade:	3	-	Lym.	2	1	1-2
	% contamination:	10	-	10	20	30	20
	Tumour:	**SB1**	**X1**	**SB122**	**SB136**	**SB137**	**SB140**
Allele ratios	**Catalase (chromosome 2)**	1.06 (S)	1.27 (B)	1.23 (S)	1.37 (B)	2.31 (B)	1.06 (S)
	Interleukin 3 (chromosome 11)	1.04 (S)	1.51 (B)	1.02 (S)	4.77 (B)	1.75 (B)	1.07 (B)

	Tumour Grade:	2	1-2	1	1-2	1
	% contamination:	<10	20	20	10	10
	Tumour:	**SB141**	**SB142**	**SB143**	**SB144**	**SB145**
Allele ratios	**Catalase (chromosome 2)**	1.01 (B)	1.2 (S)	1.13 (S)	1.16 (S)	1.18 (S)
	Interleukin 3 (chromosome 11)	1.05 (B)	18.3 (S)	1.24 (B)	1.3 (S)	1.06 (S)

The Table is in the same format as Table 6. S = SENCAR, B = BALB/c.

difference can be determined it could have important implications for the prevention and treatment of human cancers. One possibility is that susceptibility of certain key genes or chromosomes to mutation or loss is higher in some strains of mice than in others. This could be due to differences in chromosome and gene structure between strains. Alternatively, a more general explanation, such as the effiency of carcinogen metabolism, may apply, although there is no evidence to support this possibility (Naito and DiGiovanni, 1989). If the former is correct, and the intrinsic differences between chromosomes of various mouse strains are retained in F1 hybrid mice, then analysis of tumours from these mice should reveal preferential mutation/loss of alleles derived from the sensitive parent. SB hybrids were of particular interest in this respect because of the above mentioned difference in tumour susceptibility between these strains.

In 5/9 SB DMBA/TPA carcinomas, BALB/c chromosome 7 alleles were under-represented (and the SENCAR H-*ras* allele mutated), while the remainder showed the reverse pattern (Figures 9 and 11; section 3.4). Thus, there appears to be no evidence for preferential activation of the SENCAR H-*ras* allele or for preferential loss of chromosome 7 alleles from this tumour-sensitive strain. A similar pattern was observed in tumours from SN hybrids. In 4/7 DMBA/TPA carcinomas, 129 chromosome 7 alleles were under-represented or lost (and the NIH H-*ras* allele mutated), while the opposite was observed in in 3/7 of these tumours (Figures 9, 11 and 12; section 3.4).

The strain from which either chromosome 7 homologue is derived does not appear to influence the parental origin of the mutated H-*ras* allele, nor the direction of subsequent allelic imbalances involving this chromosome. However, in view of recent evidence that maternally-derived alleles on certain chromosomes are more frequently lost in some human tumours than paternally derived alleles (section 1.4.4), it remained possible that the sex of the parental strain did influence tumour-related genetic events. Intriguingly, the distal portion of chromosome 7 shows evidence of genomic imprinting (Searle *et al.*, 1989), a phenomenon which has been used to

explain the preferential loss of maternal alleles in human tumours (section 1.4.4). Despite this, no evidence was found for such a bias in DMBA/TPA carcinomas: of 5 such tumours obtained from a single SB hybrid (SB141-145) there was no indication of preferential chromosome loss (Figure 9).

Although negative in this case, these results demonstrate the potential of the F1 hybrid model for addressing the issue of genomic imprinting. Similar studies could be attempted in different tumour types and at different loci.

3.11 Analysis of DNA fingerprints in SB tumours

Human minisatellite regions (Jeffreys *et al.*, 1985a) are made up of tandemly repeated units of DNA. These units are rich in guanine residues, between 10 and 70 base pairs long, and are repeated 3-30 times in any one satellite (Jeffreys *et al.*, 1985a). A core region, recognisable in all the repeat units, is 10-15bp long and resembles the chi sequence, which functions as a recombination signal in *E. coli*. This core region may therefore orchestrate the generation of minisatellites, possibly through unequal sister chromatid exchange (Jeffreys *et al.*, 1985a). Hybridisation of minisatellite probes to DNA cleaved with an enzyme which does not cut within the satellite, gives rise to a pattern of bands (a "DNA fingerprint") which varies between different individuals (Jeffreys *et al.*, 1985b). As expected, more specific probes, which recognise a single minisatellite locus, detect a large variety of alleles. (Wong *et al.*, 1986).

The multi-allelic nature of minisatellite loci, and the resulting high heterozygosity, makes them potentially very useful for the analysis of allele loss in tumours. Loss of fragments from DNA fingerprints has been observed in a variety of human tumours (Thein *et al.*, 1987; de Jong *et al.*, 1988; Thein *et al.*, 1988). Minisatellite regions have also been identified in mouse (Jeffreys *et al.*, 1987) and so studies of this type are possible with skin tumours from this species. One advantage

over the human studies is that DNA fingerprints from mice of the same strain can be compared and an indication gained of the hypervariable loci which may be susceptible to random alteration during tumour development.

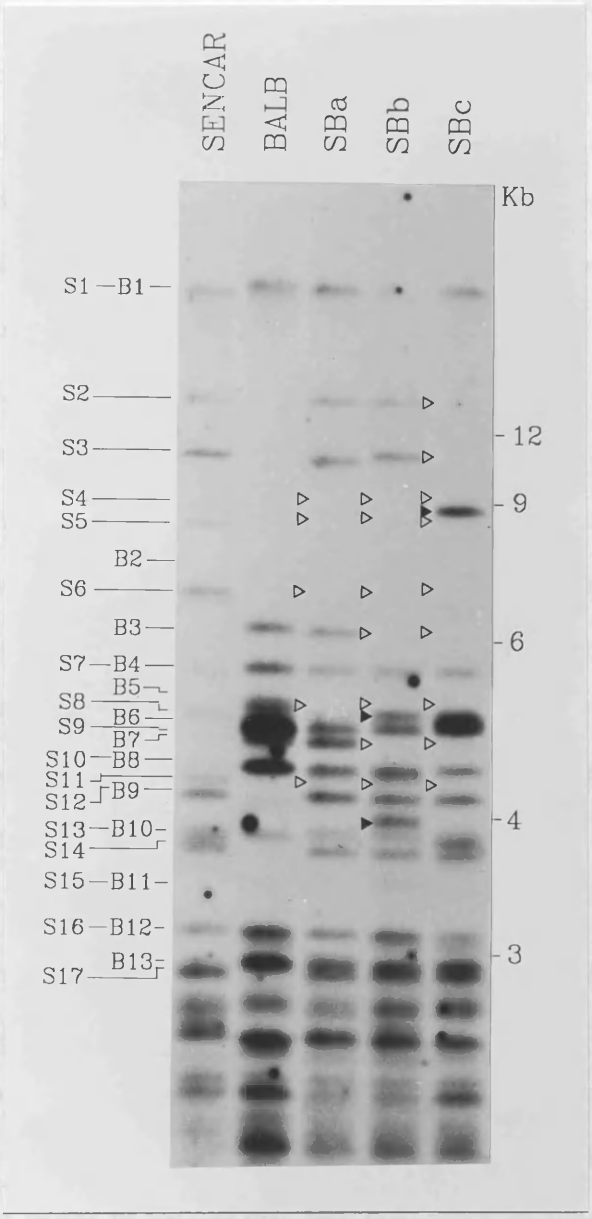
In this study, a minisatellite probe was used to analyse tumours derived from SB hybrids. All the tumours were induced by DMBA-initiation and TPA- promotion. The probe used (15.1.11.4) consisted of repeating units of the "core" sequence derived from the minisatellite in the λ33.15 clone (Jeffreys et al., 1985a). This clone hybridises to minisatellites on mouse chromosomes 4, 5, 14 and 17 (Jeffreys *et al.*, 1987). The ability to detect multiple loci with a single probe allowed the extent of genetic change in carcinomas to be assessed, thereby testing the significance of the high frequency of chromosome 7 changes observed in these tumours.

3.11.1 DNA fingerprints of parental strains

Typical DNA fingerprints for BALB/c, SENCAR and SB hybrid mice are shown in Figure 31. Fragments are labelled according to their parental origin to facilitate discussion. SENCAR and BALB/c mice have, as expected, different DNA fingerprints. The three SB hybrids shown have patterns which are clearly related to the parental strains. Heterozygosity in the parental strains may have been responsible for the absence of some bands in the hybrids. This is especially applicable in the case of SENCAR mice which, unlike BALB/c, are not inbred. This is reflected by the greater level of complexity of the SENCAR DNA fingerprint and the absence of more SENCAR bands than BALB/c bands in the hybrids (Figure 31). The absence of bands in hybrids may also have been caused by recombination. The new minisatellite fragments (Figure 31) may also have been generated in this way.

The differences between DNA fingerprints of separate SB hybrid mice demonstrates why tumour DNA must only be compared with constitutional DNA from the same mouse. Variations in DNA fingerprints of individual mice from inbred strains have also been detected, albeit minor ones (Jeffreys *et al.*, 1987).

Figure 31 DNA fingerprints of parental strains and F1 hybrids



Southern blot hybridisation of *Hinf*I digests was carried out using the minisatellite probe 15.1.11.4. SBa, b and c are SB141spleen, SB146kidney and SB140spleen respectively (also shown in Figures 32 and 33), all from different SENCAR x BALB/c hybrid mice. SENCAR specific bands are labelled S1-S17 and BALB/c specific bands are labelled B1-B13. The arrowheads indicate differences between parental and hybrid strains: Open arrowheads = bands absent in the hybrid that are present in either of the parental strains. Filled arrowheads = new bands in the hybrids that are not present in the parental strains; or bands that are present in both, but more intense in the hybrids. Sizes are indicated to the right in kb. DNA was run on 0.8% agarose gels

3.11.2 Tumour DNA fingerprints

Differences between tumour and constitutional DNA fingerprints were detected in 8/9 SB carcinomas and 1/1 SB lymphoma (Figures 32 and 33; Table 15). In most cases the alterations are clearly visible (e.g SB1, Figure 32). In others, changes are less obvious because of poor resolution (eg around B6, S9 and B7, Figure 32). In addition, changes in the level of intensity are more difficult to detect than complete loss of a fragment or appearance of an entirely new band (e.g B10 in SB142, Figure 32).

In SB1, band S6 was lost and two new bands generated (Figure 32). If the new bands were derived from S6 the mechanism probably involved more than one event because a) while one of the new bands is smaller the other is larger, and b) the intensity of the new bands suggests that extra copies of this minisatellite have been generated. An unequal cross over event could have generated the smaller and larger bands simultaneously. Normally, these should segregate to different cells. However, it is conceivable that in a tumour cell both sister chromatids could segregate to the same cell.

In SB140 a new band was detected at the same position as band B6 in BALB/c mice (Figure 32). This new fragment may have been derived by reduction in the size of a band at position S8, although the resolution was not good enough to permit unambigous interpretation (the possible loss of S8 is not indicated in Figure 32). The intensity of most bands in SB140 was less than those in control spleen, suggesting that less tumour DNA was loaded. However, bands at positions S4, B10/S13 and S14 were slightly more intense than expected (Figure 32). This may represent complete or partial duplication of one or more chromosomes, the latter being dependent on whether these fragments are linked or not. S4 and S14 are probably not linked since only one of these, S4, is present in SB1spleen; tight linkage would result in these bands being inherited together.

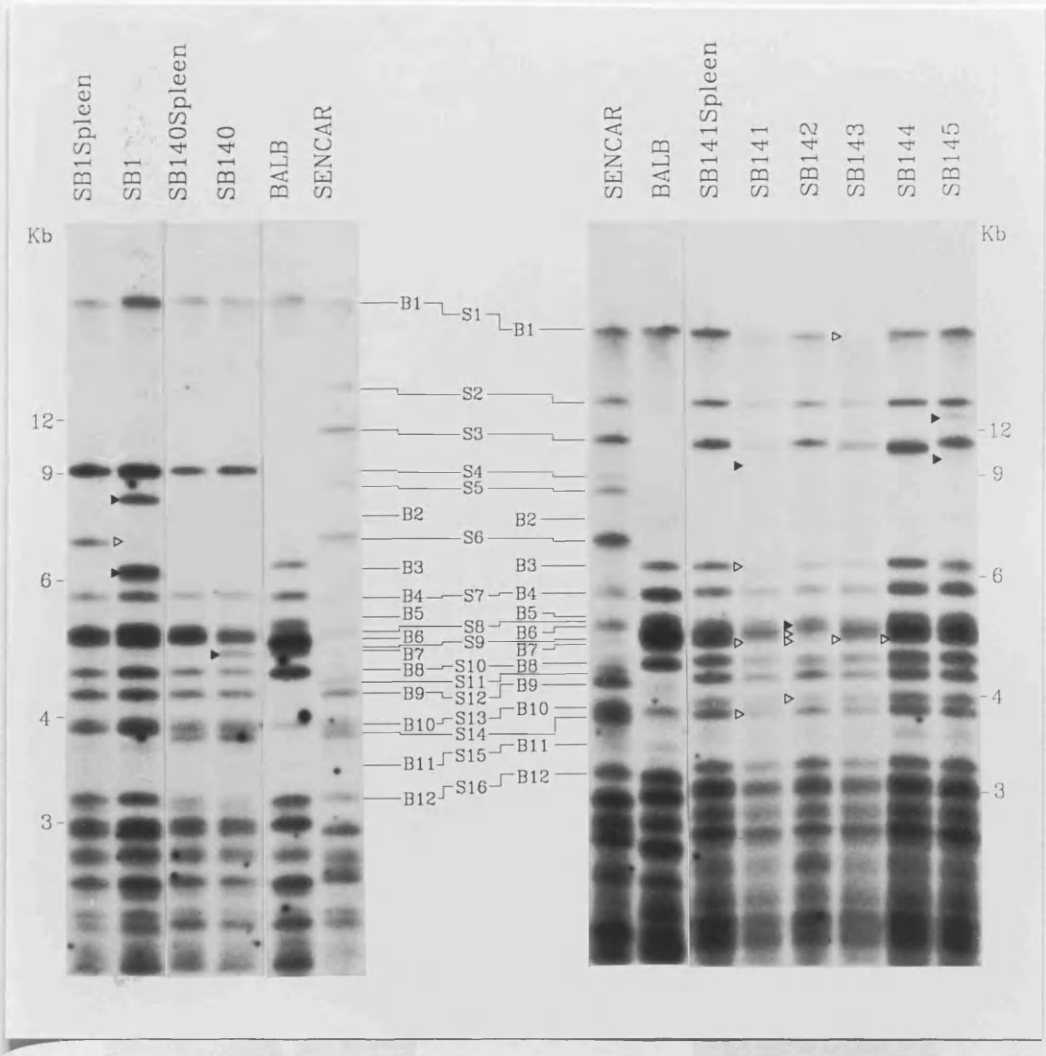
Tumours SB141-145 were taken from the same SB hybrid mouse. In SB141 and 142 one new band was detected, along with loss or reduction in intensity of three bands (Figure 32). The new fragment may have been derived from a recombination event involving one of the other marked bands. In SB143 and 144 the loss/reduction in intensity of two bands and one band respectively was not accompanied by the appearance of a new fragment (Figure 32). New unresolved bands may be present, or, alternatively, the losses may have occurred by deletion rather than recombination.

In SB145 two new bands, but no losses, were detected (Figure 32). Again, poor resolution could explain this result. These new bands were faint, and may have been derived from smaller fragments whose loss would be undetectable if they had comigrated with intensely hybridising fragments, of which there are many below about 6kb (Figure 32).

SB136 and 137 were shown from histological and RFLP analyses to be more contaminated with normal cells than the other SB tumours (section 3.4.2). This may explain the lower number of detectable alterations in these carcinomas (Figure 33). Band S12 appeared more intense in SB136, but no obvious changes were observed in SB137. An increase in the size of the band below S12 may also have occurred (Figure 33).

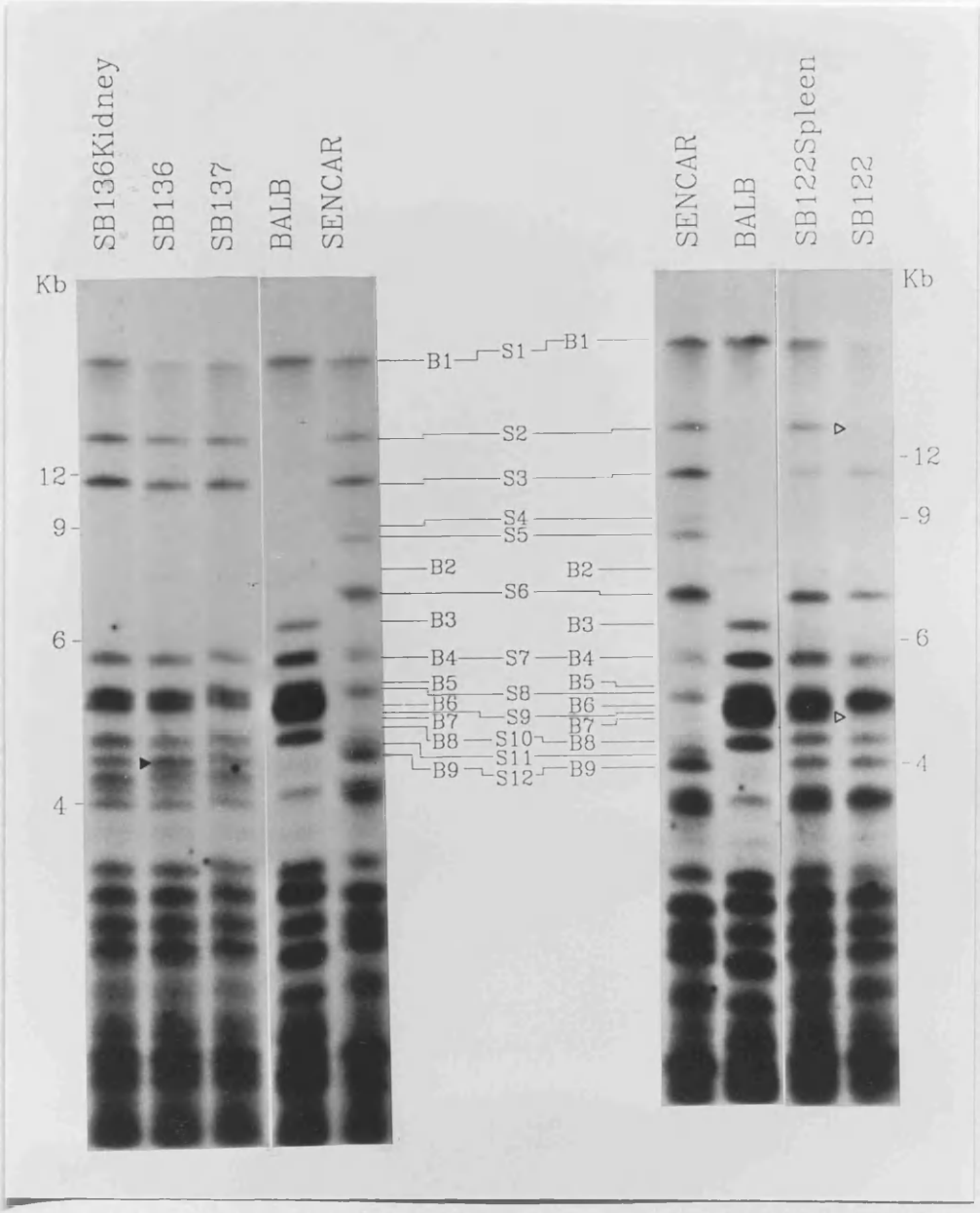
Histological analysis showed that SB122 was a lymphoma (Table 14). Two clear losses, involving S2 and B7, were detected in this tumour (Figure 14). The former may be linked to S3 since these two SENCAR-derived bands were either both present or both absent in the 5 normal tissue samples analysed in this study (Figures 31, 32 and 33). A larger number of animals would have to be analysed to test this possibility rigorously. If S2 and S3 are linked, the absence of new bands in SB122 could mean that S2 was lost by mitotic recombination or deletion of part of a chromosome.

Figure 32 DNA fingerprint analyses of SB carcinomas



Southern analyses of *Hinf*I digests using the 15.1.11.4. probe. Each tumour, or group of tumours, should be compared with a normal tissue sample obtained from the same mouse: SB1spleen was obtained from the same mouse as tumour SB1; SB140spleen was obtained from the same mouse as SB140; SB141spleen was obtained from the same mouse as SB141-145. Other details as in Figure 31.

Figure 33 DNA fingerprint analyses of an SB lymphoma and SB carcinomas which contained large amounts of normal cells



Southern analysis of *Hinf*I digests using the 15.1.11.4. probe. All tumours were derived by DMBA-initiation and TPA-promotion of the dorsal skin. SB136 and 137 are carcinomas, both obtained from the same mouse as SB136kidney. SB122 is a mammary adenocarcinoma obtained from the same mouse as SB122spleen. Other details as in Figure 31.

Table 15 Summary of minisatellite hybridisation data

Tumour	Bands lost or reduced in intensity	No. of bands at new positions	Bands of increased intensity
SB1	S6	2	-
SB140	-	1	-
SB141	B3, B7, S14	1	-
SB142	S9, B7, B10	-	B6
SB143	B1, B7	-	-
SB144	B7	-	-
SB145	-	2	-
SB136	-	-	S12
SB137	-	-	-
SB122	S2, B7	-	-

3.11.3 Stability of tumour DNA fingerprints during selection for tumour cells

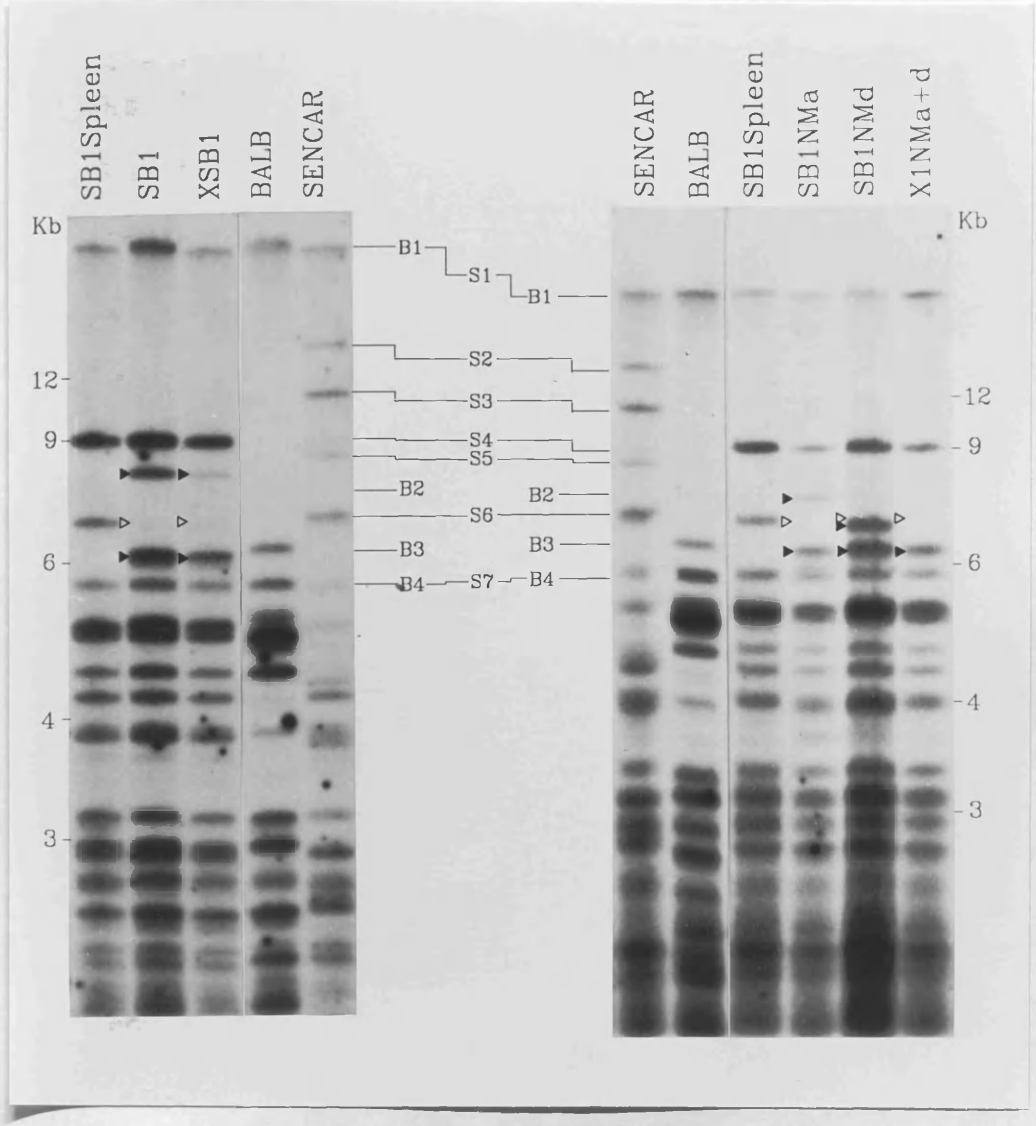
A cell line, XSB1 (also termed X1; section 3.4.1) was grown from an explant of SB1 as part of an experiment to remove the effect of contaminating normal cells on the interpretation of RFLP patterns in this tumour (section 3.4.1). Nude mouse tumours, SB1NMa and SB1NMd, were obtained from this cell line, and another cell line, X1NMa+d, grown from these (section 3.4.1). The stability of the DNA fingerprint originally observed in SB1 (Figure 32) was monitored at each of these selection stages.

The differences between SB1 tumour and constitutional DNA (loss of S6 and gain of two fragments) were preserved in the cell line XSB1. However, of the two extra bands detected in this tumour, the larger one appeared weaker in the cell line compared with the original tumour, even after taking into account the fact that less XSB1 DNA was loaded (Figure 34). This band was missing in the nude mouse tumours. It may have been reduced in size by recombination or deletion, since a smaller extra band was detected in SB1NMa and an even smaller band in SB1NMd. In the cell line derived from these tumours the larger of the two extra bands was absent (Figure 34). The other two changes originally detected in SB1, loss of S6 and gain of a band at about 6.2kb, were preserved in the nude mouse tumours and the cell line derived from these (Figure 34).

3.11.4 Somatic stability of DNA fingerprints

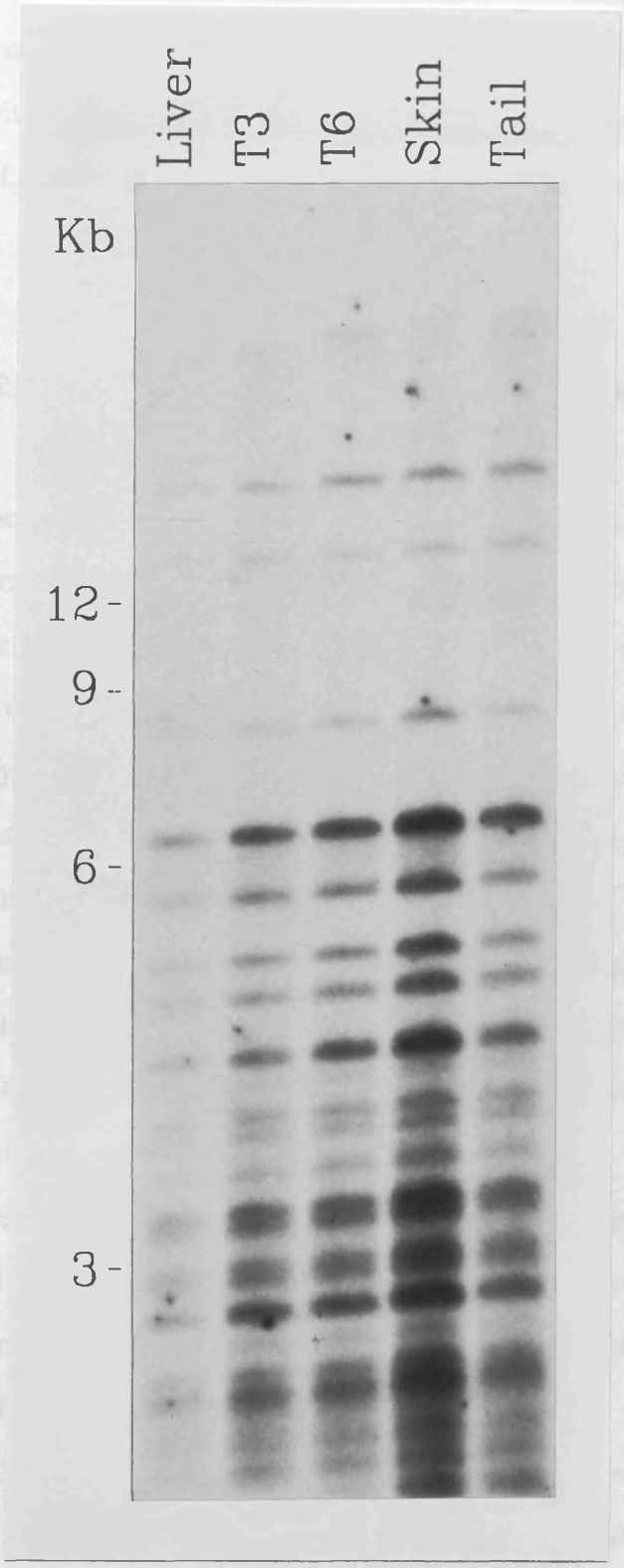
If somatic recombination involving minisatellite regions occurs, the significance of tumour-associated alterations in DNA fingerprints would be questionable. To investigate this possibility the DNA fingerprints of liver, skin, and tail from a single mouse were compared. No differences were detected (Figure 35), supporting the conclusion that DNA fingerprints are somatically stable.

Figure 34 DNA fingerprint analysis of SB1-derived cell lines and nude mouse tumours



Southern analysis of *Hinf*I digests using the 15.1.11.4. probe. The origin of the cell lines and nude mouse tumours is explained in Figure 10. SB1spleen was obtained from the same mouse as carcinoma SB1. Other details as in Figure 31.

Figure 35 DNA fingerprints of various tissues and tumours from a single transgenic mouse



Southern analysis of *Hinf*I digests using the 15.1.11.4 probe. DNA samples were from a transgenic mouse carrying the human T24 H-*ras* oncogene under the control of a suprabasal keratin promoter (Bailleul *et al.*, submitted). T3 and T6 were papillomas.

The tissues analysed in this experiment were obtained from a transgenic mouse carrying a mutant H-*ras* gene under the control of a suprabasal keratin promoter (Bailleul *et al.*, submitted). These mice were hyperkeratotic and developed papillomas without the application of TPA (Bailleul *et al.*, submitted). The DNA fingerprints of two of these papillomas were analysed. No alterations were detected (Figure 35).

3.12 Determination of DNA content in four cell lines

Carcinomas, and cell lines made from these, are frequently aneuploid or polyploid (Pera and Gorman, 1984, Fusenig *et al.*, 1985). Thus, allele ratios of, say 2:1, for a set of loci on one chromosome could represent trisomy of that chromosome, or, if the tumour was polyploid, a larger number of both homologues. Tumour ploidy is most accurately determined by cytogenetic analysis. Alternatively, an approximation of the DNA content of tumour cells can be obtained by flow cytometry. This gives an indication of the degree of genetic abnormality, but the effects of aneuploidy on individual chromosomes cannot be ascertained. However, it has the advantage of speed and simplicity, and, in addition, gives an estimate of the proportion of dividing cells in the tumour population. In this study, four tumour cell lines were analysed by flow cytometry.

Cells were fixed in methanol and the DNA stained using chromomycin A3. This chemical acts as a fluorochrome for G-C rich regions of DNA, is excited at 458nm and emits maximally at 555nm (see Shapiro, 1988). The fluorescent intensity is proportional to the cellular DNA content.

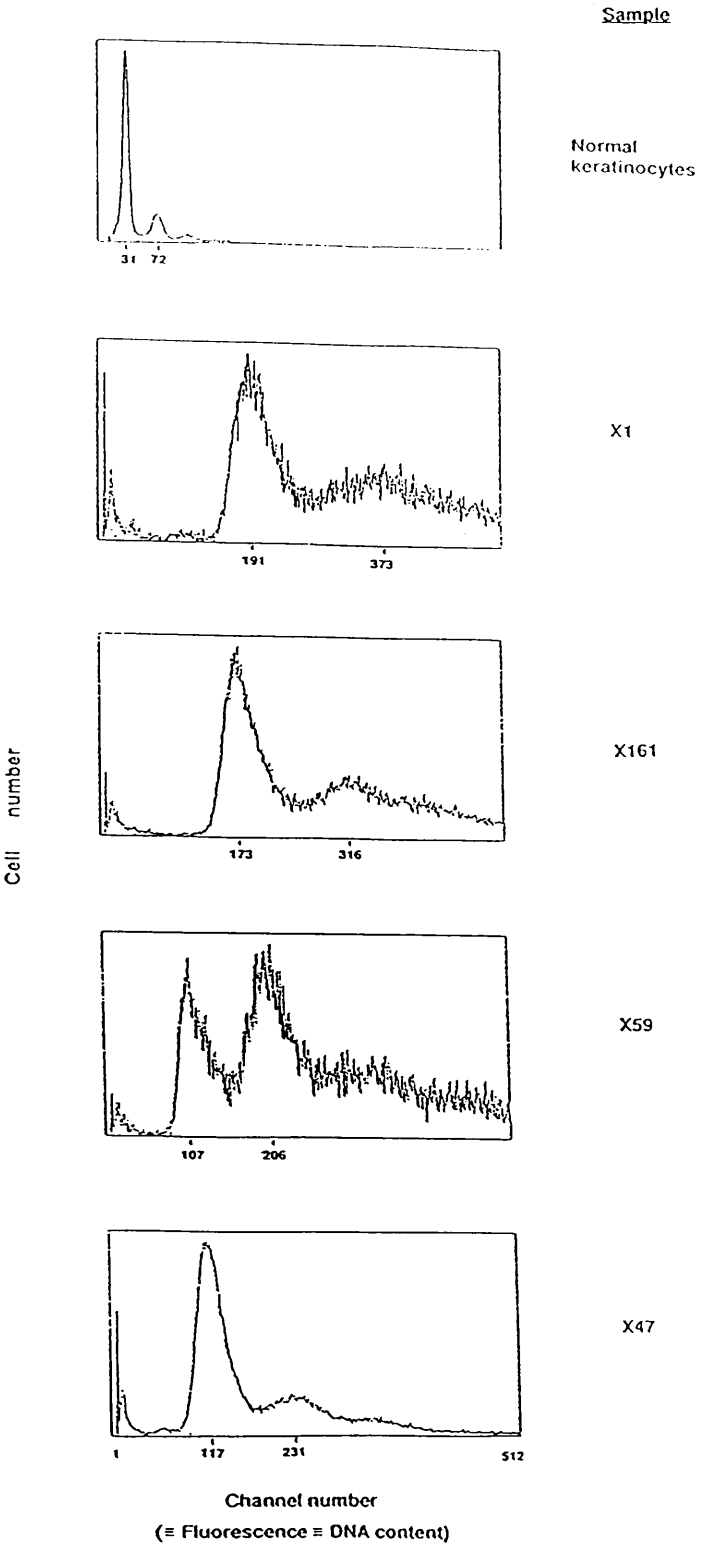
The cell lines analysed, X1, X161, X59 and X47, were derived from the DMBA/TPA carcinoma SB1, DMBA/TPA metastasis SN161, v-H-*ras*/TPA carcinoma SN59 and DMBA/DMBA carcinoma SN47 respectively. Mouse

keratinocyte nuclei were used to determine the position of G1 and G2+M peaks in normal cells.

In normal diploid cells, the fluorescence (DNA content) of the G2+M peak (4c) should be twice that of the G1 peak (2c). The abscissae in Figure 36 are divided into 512 channels. Maximum fluorescence of normal keratinocytes in G1 and G2+M was detected in channels 31 and 72 respectively. This suggests that the flow cytometer measurements were not precisely linear, as has been observed by others (Shapiro, 1988). However, non-linearity may be confined to low fluorescence peaks, since two extra peaks were detected in normal keratinocytes, to the right of the 2c and 4c peaks, which displayed maximum fluorescence in channels 111 and 150 (these numbers are not indicated in Figure 36). This suggests an approximate seperation of 40 channels for every complete set of chromosomes. These extra peaks were probably caused by doublets which persisted after cellular disaggregation. Additional shoulders were observed in the tumour cell line samples (Figure 36). These may also have been caused by doublets, but the possibility that they represent genuine polyploid populations cannot be excluded. The very low fluoresence peaks in these samples were probably caused by cell debris.

As expected, the tumour cell lines contained more DNA than normal keratinocytes. Assuming that integral multiples of the diploid complement were separated by 40 channels, with the first peak (2c) at channel 30, this means that the DNA content of X1 is close to 5c (channel 190), just under 5c in X161, and close to 3c (channel 110) in X59 and X47 (Figure 36). These cell lines are therefore hypo- or hyper-tetraploid, which is in close agreement with previous findings (Pera and Gorman, 1984). In this study, analysis of original tumour material by flow cytometry was not carried out. It is therefore possible that some of the alterations in ploidy occurred after establishment of cell lines. However, the fact that early passage cells were studied should minimise this. In addition, the results reported here resemble the DNA content of carcinoma tissue determined by flow cytometry (Goerttler *et al.*, 1976). Confirmation of the similarity between the DNA content of the cell lines

Figure 36 Flow cytometric analysis of four tumour cell lines



The positions of the G1 and G2+M peaks in normal keratinocytes and each cell line are indicated.

studied here and the tumours they were derived from could be obtained by using techniques developed for the analysis of paraffin embedded tumour tissue (Hedley *et al.*, 1983).

The tumour cell line peaks in Figure 36 are broad, suggesting that these lines may be heteroploid. Such mosaicism reflects a degree of genetic instability, with continual selection amongst new clones.

In the cell lines derived from chemically induced tumours (X1, X161 and X47) the proportion of resting (G1) to cycling (G2+M) cells was similar, with the bulk of cells in the former category (Figure 36). However, in the v-H-*ras*/TPA carcinoma cell line, X59, the proliferative fraction was higher (Figure 36). Although measurements were not made, the rapid growth of this cell line was noticeable during its establishment.

Finally, it is worth noting the absence of a 2c peak in all of the tumour cell lines (Figure 36). This suggests that very few normal cells survived explanting. The genetic stability of keratinocytes grown in supplemented medium has been demonstrated (Pera and Gorman, 1984).

3.13 Densitometric analysis: methods and problems

3.13.1 Calculation of T/N values and allele ratios

Calculation of the number of copies of an allele in a tumour (T) relative to the single copy present in normal tissue (N) permits accurate interpretation of results. Before T/N ratios can be calculated the density of tumour and normal alleles must be normalised for variation in DNA amounts loaded on the gel. In this study T and N values for each allele were normalised by division with the density of bands obtained after rehybridising blots with a chromosome 11 probe, IL3. After normalisation, T/N values were calculated. For *fes*, *Hbb* and *Int*-2 loci "N" was usually the equivalent

spleen allele. However, in the case of H-*ras*, N values for the 12 and 8+4kb allele were calculated using PDV and/or PDVC57. Before dividing the normalised T 8+4kb H-*ras* allele by the normalised PDV 8+4kb allele the latter was multiplied by 2. This was because PDV cells have 1 mutant H-*ras* allele in a tetraploid background, which is equivalent to only 0.5 copies in a diploid cell. Using the same logic, the value for the PDVC57 12kb allele was also multiplied by 2.

In addition to T/N ratios for individual alleles, the ratio of one allele to another at a single locus can also be determined. The allele ratio can be calculated by dividing the larger T/N value by the smaller value. These ratios are unaffected by normalisation for DNA amounts and this step can be left out if desired.

3.13.2 Factors influencing the reliability and interpretation of densitometry results

The reliability of T/N values and allele ratios depends on: (a) the quality of blots, (b) the existence of a linear relationship between the amount of radioactivity on a blot and the density of bands on film, and (c) the accuracy of measurement of band densities.

(a) The quality of blots on hybridisation with the probe of interest obviously affects both T/N and allele ratios, but the quality on rehybridisation with IL3 only affects the former.

(b) The linear relationship between amount of radioactivity and density of bands breaks down if bands are too faint or intense. The former problem is partially solved by preflashing film. However, much better results can be obtained using a phosphorimager which gives a linear response over a much larger scale than X-ray film.

(c) The measurement of band intensities can vary considerably if only part of a band is measured (e.g a line through a region of the band). The densitometer used in this study (Molecular Dynamics 300A) allowed the density of whole bands to be

measured thus minimising this problem. Whatever method is used, measurement of band density can be difficult if bands are close to each other (this was a problem in the quantitation of *fes* 12 and 13kb alleles and the *Hbb* 10kb allele).

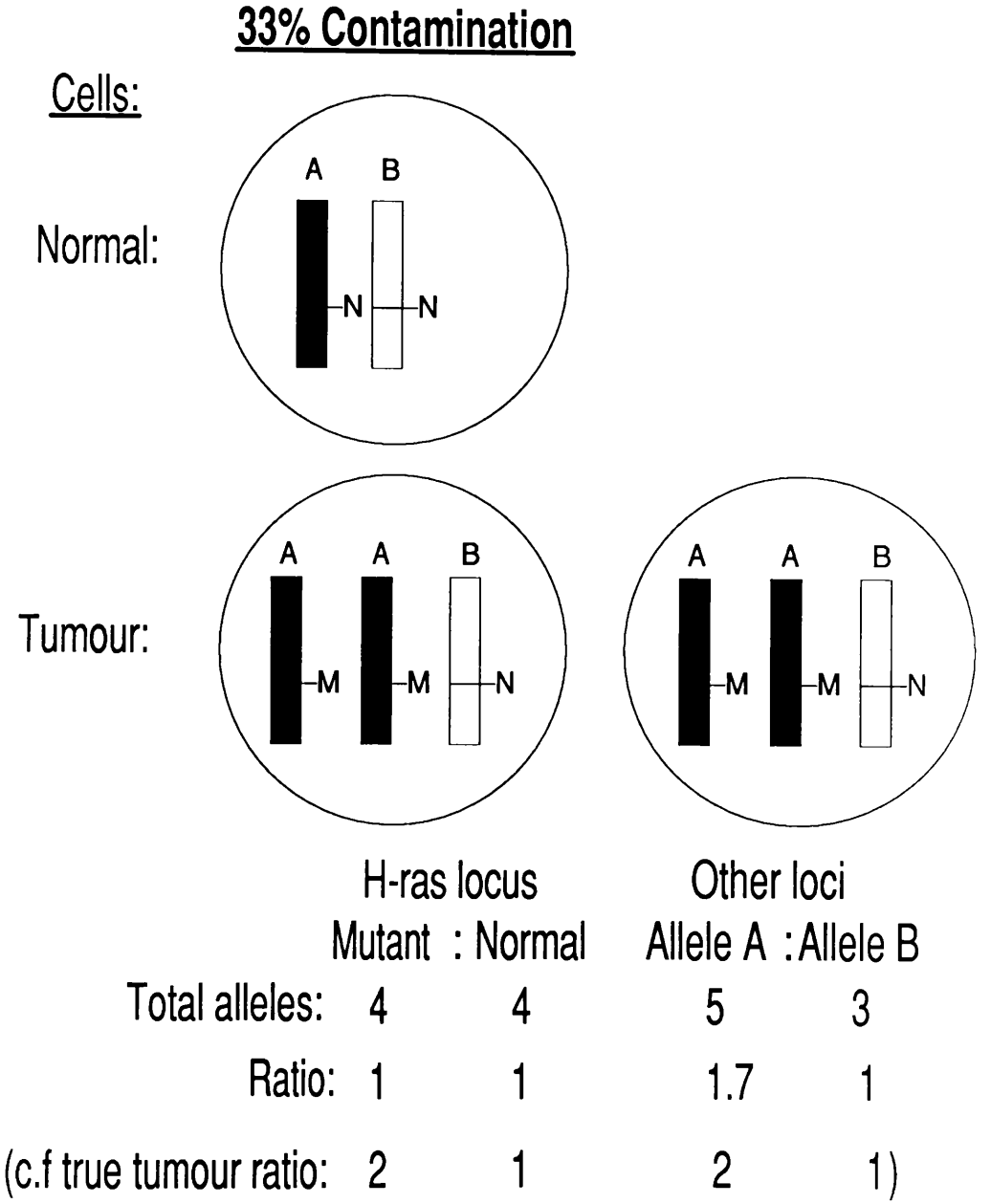
Even if these three factors do not distort T/N values and allele ratios, interpretation of results can still be confused by (a) the ploidy status of the tumour, and (b) the level of normal cell contamination of the tumour.

(a) The ploidy status will not alter allele ratios but it may affect T/N values. If, for example, chromosome 11 was trisomic in an otherwise near diploid tumour the T value would be reduced more than it should be by normalising with IL3. This would in turn reduce the T/N ratio. Conversely, underrepresentation of IL3 would increase this ratio. Another problem is that a T/N value of, say, 1 could either represent one allele in a diploid tumour or 2 in a tetraploid tumour.

(b) Normal cell contamination will reduce both T/N values and allele ratios in which there is an imbalance in favour of one allele. While the effect of contaminating cells on the analysis of tumour material is not considerable at polymorphic loci such as *fes* or *Hbb*, the distortion is twice as great when analysing the ratio of mutant to normal H-*ras*. This is because in SN and SB mice, normal H-*ras* is not polymorphic. Thus, each normal cell contributes two 12kb *Xba*I alleles compared with, for example, one 10kb and one 7.5kb *Hbb* allele. The effect of various amounts of contamination on the analysis of a range of tumour types is summarised in Table 16. One example is shown pictorially in Figure 37. These calculations illustrate the dramatic effect that contamination as low as 20% can have on the allele ratios. The numbers in Table 16 are calculated assuming that the tumours are near diploid. Several reports have shown that carcinomas are frequently aneuploid or near-tetraploid (Pera and Gorman, 1984; Conti *et al.*, 1986; Aldaz *et al.*, 1987). In such cases additional tumour DNA would dilute the effect of contaminating normal DNA.

Normal cell contamination is determined by morphological criteria. This has two main drawbacks. First, the tumour section taken for histology may not be representative of the whole tumour, and second, tumour cells may confer a neoplastic

Figure 37 **An example of the effect of contaminating normal cells on tumour allele ratios**

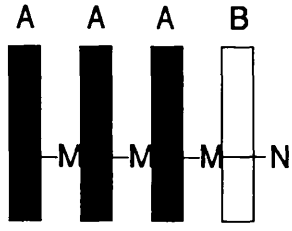


	H-ras locus		Other loci	
	Mutant	: Normal	Allele A	: Allele B
Total alleles:	4	4	5	3
Ratio:	1	1	1.7	1
(c.f true tumour ratio:	2	1	2	1)

Table 16 Effects of normal cell contamination on allele ratios in tumours with various chromosome 7 alterations

The H-*ras* locus is represented by M (mutant allele) or N (normal allele). All other alleles are termed A (black chromosome alleles) or B (white chromosome alleles).

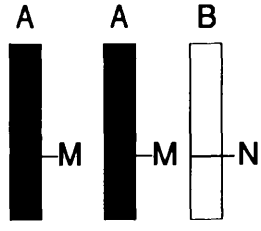
A) **Tumour status:** 3 chromosomes with mutant, 1 with normal H-ras



Allele ratios

	H-ras locus			Loci distal to H-ras		
Contamination	Mutant	:	Normal	Allele A	:	Allele B
0%	3	:	1	3	:	1
10%	2.5	:	1	2.8	:	1
20%	2	:	1	2.6	:	1
33%	1.5	:	1	2.3	:	1
40%	1.3	:	1	2.2	:	1
50%	1	:	1	2	:	1

B) **Tumour status:** trisomic



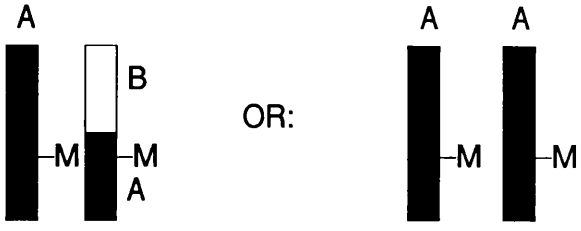
Allele ratios

	H-ras locus			Other loci		
Contamination	Mutant	:	Normal	Allele A	:	Allele B
0%	2	:	1	2	:	1
10%	1.7	:	1	1.9	:	1
20%	1.3	:	1	1.8	:	1
33%	1	:	1	1.7	:	1
40%	1	:	1.2	1.6	:	1
50%	1	:	1.5	1.5	:	1

C) **Tumour status:** Hemizygous

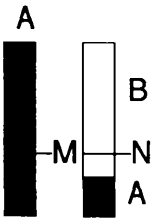
	Allele ratios					
	H-ras locus			Other loci		
Contamination	Mutant	:	Normal	Allele A	:	Allele B
0%	1	:	0	1	:	0
10%	4.5	:	1	10	:	1
20%	2	:	1	5	:	1
33%	1	:	1	3	:	1
40%	1	:	1.3	2.5	:	1
50%	1	:	2	2	:	1

D) **Tumour status:** Mitotic recombination proximal to ras, or chromosome loss and reduplication



	Allele ratios					
	H-ras locus			Other loci (Distal to H-ras in the case of mitotic recomb.)		
Contamination	Mutant	:	Normal	Allele A	:	Allele B
0%	2	:	0	2	:	0
10%	9	:	1	19	:	1
20%	4	:	1	9	:	1
33%	2	:	1	5	:	1
40%	1.5	:	1	4	:	1
50%	1	:	1	3	:	1

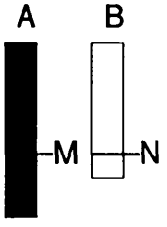
E) **Tumour status:** Mitotic recombination distal to H-ras



Allele ratios

	H-ras locus			Loci distal to H-ras		
Contamination	**Mutant**	:	**Normal**	**Allele A**	:	**Allele B**
0%	1	:	1	2	:	0
10%	1	:	1.2	19	:	1
20%	1	:	1.5	9	:	1
33%	1	:	2	5	:	1
40%	1	:	2.3	4	:	1
50%	1	:	3	3	:	1

F) **Tumour status:** Deletion distal to H-ras

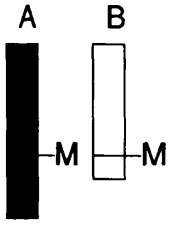


Allele ratios

	H-ras locus			Loci distal to H-ras		
Contamination	**Mutant**	:	**Normal**	**Allele A**	:	**Allele B**
0%	1	:	1	1	:	0
10%	1	:	1.2	10	:	1
20%	1	:	1.5	5	:	1
33%	1	:	2	3	:	1
40%	1	:	2.3	2.3	:	1
50%	1	:	3	2	:	1

G)

Tumour status: Deletion distal to H-ras, both H-ras alleles mutated



Allele ratios

	H-ras locus			Loci distal to H-ras		
Contamination	**Mutant**	:	**Normal**	**Allele A**	:	**Allele B**
0%	2	:	0	1	:	0
10%	9	:	1	10	:	1
20%	4	:	1	5	:	1
33%	2	:	1	3	:	1
40%	1.5	:	1	2.3	:	1
50%	1	:	1	2	:	1

phenotype on neighbouring normal cells. Results obtained from densitometric scanning should be viewed with these possibilities in mind.

3.13.3 Assessing the reliability of densitometric analysis

Given the range of problems that can upset densitometric analysis, it is important that the validity of data is tested. Reliable T/N values and allele ratios should meet the following criteria:

1. T/N ratios from a tumour allele should be the same irrespective of which IL3 band is used to normalise the T and N values for DNA amounts. For example, the *Int*-2 analysis uses *Pst*I. Rehybridisation with the IL3 probe gives bands of 7.2kb, 1.4kb and 1.0kb. T/N ratios should be the same irrespective of which of these three bands is used to normalise T and N values.

In the analysis of SN132, the T/N values for the *Hbb* alleles were fairly independent of the IL3 band (9.8, 3.8 or 1.8kb) used to normalise for DNA amounts (Table 17). Other examples exist however where the T/N values varied depending on the IL3 band used to normalise for DNA amounts. This variation was most commonly in the range of 1-2 fold (e.g, the *fes* NIH T/N values in SN97) . One possible explanation may lie in the nature of the IL3 probe. Only the smallest *Eco*RI, *Xba*I and *Pst*I fragments are IL3 specific, the others hybridise to the unidentified cDNA accidentally cloned with the IL3 cDNA. If this extra DNA represents one or more gene sequences on a chromosome or chromosomes other than 11, then changes at these loci not mirrored on chromosome 11 could produce the type of variation in T/N values described above. However, in general, deterioration of blot quality is a more likely explanation since the frequency of irregularities did appear to increase with the number of times a blot had been probed prior to hybridisation with IL3.

2. The T/N values and allele ratios should be the same at separate chromosome 7 loci affected by the same event. Thus, in a trisomy, T/N should be 2 at all loci on the duplicated chromosome and 1 at all loci on the single chromosome (or 1 and 0.5

respectively in a tetraploid tumour). The allele ratios should be 2:1 at every locus (this ratio is not different in diploid or tetraploid tumours unless there is contamination by normal cells).

Table 17 shows that the T/N values do vary at separate loci (this is compounded by the problem, outlined in point 1, of different T/N ratios depending on the IL3 band used to normalise for DNA amounts). The allele ratios, on the other hand, appear to be more consistent, but are not ideal. For example, in SN97 the allele ratios range from 2.3:1 at H-*ras* to 3.37:1 at *Int*-2. A lower *ras* allele ratio is expected because of normal cell contamination (section 3.4.2).

3. The T/N values and allele ratios at H-*ras* should be the same irrespective of which control (PDV or PDVC57) is used to adjust the ratio of mutant :normal alleles. Some variation, although not considerable, was observed (Table 17). An estimate of the true values can be obtained by combining the data for other alleles with information concerning the amount of normal cell contamination.

4. If the amount of normal cell contamination is known and a mechanism for loss/under-representation of chromosome 7 alleles has been suggested, then the allele ratios should correspond to the calculated values shown in Table 16 for that type of tumour. For example, in SN97 there may have been three copies of the NIH chromosome to one 129 chromosome. The tumour was 10% contaminated with normal cells. This would reduce the allele ratios to 2.5:1 at H-*ras* and 2.8:1 at other loci (Table 16A). The actual ratios shown in Table 17 only approximately fit the proposed chromosome 7 alteration in this tumour.

3.13.4 Summary

After full densitometric analysis of over 70% of the tumours discussed in this thesis (including several duplicate blots) it was clear that for several tumours deterioration of blot quality, aneuploidy, and the problem of contaminating normal cells meant that accurate quantitation of changes in allele ratios was not always

Table 17 T/N ratios and allele ratios in three DMBA/TPA carcinomas

		Tumour grade:	2	3-4	3
		% contamination:	10	10	50
			Tumour		
Locus	Allele	IL 3 normalising band(kb)	SN97	SN132	SN152
Fes	NIH	16	2.58	1.30	1.72
		10.5	2.13	0.50	0.84
	129	16	0.84	5.21	3.07
		10.5	0.69	1.98	1.50
	(Allele ratio)		(3.07)	(3.84)	(1.78)
Hbb	129	9.8	1.19	1.61	0.87
		3.8	0.71	1.61	0.60
		1.8	0.71	2.20	0.98
	NIH	9.8	3.73	0.50	0.40
		3.8	2.22	0.50	0.28
		1.8	2.21	0.60	0.46
	(Allele ratio)		(3.13)	(3.20)	(2.18)
H-ras	12 (PDV)	9.8	1.12	0.36	1.02
		3.8	0.64	0.24	0.56
		1.8	0.79	0.20	0.72
	8+4	9.8	2.78	0.93	0.64
		3.8	1.58	0.62	0.30
		1.8	1.90	0.52	0.44
	(Allele ratio)		(2.48)	(2.59)	(1.75)
	12 (PDVC57)	9.8	0.56	0.72	1.41
		3.8	0.22	0.68	0.93
		1.8	0.20	0.80	1.90
	8+4	9.8	1.22	1.74	2.26
		3.8	0.52	1.65	1.49
		1.8	0.46	1.94	3.05
	(Allele ratio)		(2.30)	(2.42)	(1.60)
Int-2	NIH	7.2	ND	ND	ND
		1.4	2.19	0.45	0.68
		1.0	3.03	0.64	0.80
	129	7.2	ND	ND	ND
		1.4	0.65	1.76	2.86
		1.0	0.90	2.50	3.36
	(Allele ratio)		(3.37)	(3.9)	(4.2)

The T/N ratios are shown opposite the IL3 band used to normalise for DNA loading. These were calculated as described in the text. Allele ratios were determined by dividing T/N values calculated using the same normalising IL3 band. H-*ras* T/N values were calculated using both PDV and PDVC57 as controls. ND = not determined.

possible. The most difficult problem is in determining the T/N ratio in tumours. Comparison of allele ratios at different loci is less prone to variation. In most cases approximate quantification of clearcut changes can be attempted assuming that all the variables have been taken into account. These results however can only be taken as rough estimates and it is debatable as to whether they represent an improvement on visual approximation.

The other tumours were only partially analysed; the allele ratios were calculated but the T/N ratios were not (these are the values which require normalisation for DNA values using the IL3 probe). The results are referred to in the text.

Chapter 4

Discussion

4.1 Gross chromosomal changes in skin tumour progression

H-*ras* activation is frequently the initiating event in chemically induced mouse skin tumours (Quintanilla *et al.*, 1986; Brown *et al.*, 1986). Previously, it has been reported that the wild-type H-*ras* allele is absent or under-represented in some carcinomas (Quintanilla *et al.*, 1986). Although *ras*-activation, together with loss of the remaining normal allele, has been detected in other other animal and human tumours (Capon *et al.*, 1983; Feinberg *et al.*,1983; Kraus *et al.*,1984; Santos *et al.*,1984; Guerrero *et al.*,1985; Bos *et al.*,1987; Diamond *et al.*,1988; Riou *et al.*,1988; Smit *et al.*,1988), the mechanisms responsible, except for one case, have remained undetermined. In the colon carcinoma cell line, SW480, a sequence polymorphism in the 5'region of the K-*ras* gene enabled Capon *et al.* (1983) to suggest that the normal allele had been lost by gene conversion. In the study described here, RFLP analysis of skin tumours from F1 hybrid mice has shown that subchromosomal events, such as gene conversion, are not commonly responsible for loss or under-representation of the normal H-*ras* allele. Instead, partial or complete loss of heterozygosity on mouse chromosome 7, suggestive of gross chromosomal changes, occurs at a very high frequency.

Gross chromosome 7 changes were detected in 26/28 carcinomas in which H-*ras* activation was also observed (Table 18, columns A and B). The mechanisms responsible for these changes are summarised below.

a) Non-disjunction resulting in over-representation of mutant H-*ras*. This alteration was detected in the majority of carcinomas.

b) Non-disjunction plus an an additional chromosome 7 change. In one DMBA/TPA carcinoma (SN184. section 3.4.3), mitotic recombination had occurred proximal to *Hbb* resulting in complete loss of the normal H-*ras* allele and one *Int*-2

allele. This had occurred in addition to trisomy of chromosome 7. A "double hit" was also detected in two DMBA/DMBA carcinomas. The simplest explanations for the observed allelic imbalances were trisomy of chromosome 7 followed by deletion of one *Int*-2 allele (SN47, section 3.6.2), and trisomy together with mitotic recombination distal to H-*ras* (SN31, section 3.6.2).

c) Duplication of mutant H-*ras*. This was detected in one carcinoma induced by repeated DMBA treatment (SN30, section 3.6.2). Amplification of mutant H-*ras* has also been detected in tumours induced by initiation/promotion (Quintanilla *et al.*, 1986; Brown *et al.*, 1990).

d) Changes distal to H-*ras*. Over-representation of the mutant H-*ras* allele was observed in all the tumours described above. However, two carcinomas were analysed in which alterations distal to H-*ras* were the only detectable gross chromosome 7 changes. One of these was induced by DMBA-initiation and TPA-promotion (SN158, section 3.4.3), while the other was obtained by treatment of DMBA/TPA papillomas with MNNG (SN80, section 3.6.3).

Structural and numerical changes to chromosome 7 clearly play an important part in the development of tumours initiated by activation of the H-*ras* gene. However, this does not appear to apply when H-*ras* is not involved in skin tumour growth. Nine carcinomas were analysed which lacked both H-*ras* mutations and chromosome 7 alterations (Table 18, column C). In fact, no chemically induced tumours were observed in which chromosome 7 changes had occurred in the absence of H-*ras* activation (Table 18, column D). This also applied to a lymphoma and a mammary adenocarcinoma which lacked activated H-*ras*. Further evidence for a correlation between the presence of mutant H-*ras* and chromosome 7 changes was provided by detection of an imbalance at *Int*-2 in a v-H-*ras*/TPA carcinoma (SN59, section 3.7). These results lead to the intriguing conclusion that gross chromosome 7 changes are *ras*-dependent, and that the initiating event, i.e. a *ras* mutation, can

Table 18 Correlation between H-*ras* mutations and chromosome 7 changes

			A	B	C	D
		H-*ras* mutation:	+	+	-	-
		Chromosome 7 changes:	+	-	-	+
Induction protocol		**No. carcinomas**				
Initiation	**Promotion**	**analysed**				
DMBA	TPA	18*	18	0	0	0
DMBA	DMBA/TPA	1	1	0	0	0
MNNG	TPA	4	2	0	2	0
MNNG	MNNG	5	0	2	3	0
DMBA	DMBA	8	4	0	4	0
DMBA	TPA -> MNNG [a]	1	1	0	0	0

*: Two metastases included.
a: MNNG application was started after papilloma formation.

influence the molecular nature of additional genetic changes occurring later in carcinogenesis.

Two other v-H-*ras* initiated carcinomas provided contrasting results to those described above. Despite the absence of v-H-*ras* and mutations in cellular H-*ras*, changes involving the distal portion of chromosome 7 were detected in these tumours. This suggests that a chromosome 7 gene other than H-ras may be involved in skin carcinogenesis. However, this conflicts with the evidence from chemically induced tumours in which chromosome 7 changes were only observed in those tumours which contained activated H-*ras*. Several explanations are possible, including a) the presence of mutations in the cellular H-*ras* gene at sites other than those analysed; b) "hit and run" involvement of the virus in tumorigenesis (c.f Smith and Campo, 1988), c) the presence of other initiating events (including insertional mutagenesis by the helper virus) able to cooperate with chromosome 7 changes in tumour induction. Irrespective of which explanation is correct, these tumours are obviously the exception rather than the rule, and as such may prove valuable in elucidating some of the unanswered questions concerning skin carcinogenesis.

4.2 Loss of heterozygosity: an alternative interpretation

Allele loss in human tumours is frequently equated with homozygous inactivation of a tumour suppressor locus, as described for retinoblastoma (section 1.4.2). However, the results presented here demonstrate that LOH may also indicate the presence of an activated oncogene. These alternatives can only be distinguished once the gene involved has been isolated. This is particularly relevant for human chromosome 11. Loss of loci at band 11p15, including one allele of H-*ras*, is frequently observed in a variety of tumour types (Mannens *et al.*, 1988; Koufos *et al.*, 1984; Riou *et al.*, 1988; Mackay *et al.*, 1988; Scrable *et al.*, 1987). Recently,

losses of this type have been observed in human basal cell and squamous cell carcinomas (Ananthaswamy *et al.*, 1989). It is important to note that lack of transforming activity in transfection assays does not necessarily mean that the remaining H-*ras* allele is completely normal. The gene could harbour weakly transforming mutations or alterations in regulatory regions (Cohen *et al.*, 1989) which may not be detected in such assays. A more detailed search for H-*ras* alterations in tumours showing loss of heterozygosity at this locus could therefore prove fruitful.

4.3 Role of mutant and normal *ras* in tumour progression

The results presented here clearly indicate that an increase in the copy number of mutant H-*ras* is involved in progression of mouse skin tumours. This complements *in vitro* studies which suggest that the level of mutant *ras* expression is proportional to the degree of morphological transformation, DNA synthesis, growth factor independence and metastatic capability (section 1.3.4.2). Comparison of four cell lines by FACS analysis in this report (section 3.12) revealed that a v-H-*ras* initiated carcinoma cell line contained the greatest proportion of dividing cells, consistent with the possibility that high levels of mutant *ras* lead to rapid proliferation.

What remains unresolved however, is whether over-expression of mutant H-*ras* is related in any way to overcoming a suppressive influence of the normal H-*ras* allele. Loss or under-representation of normal *ras* alleles has been seen in a wide variety of tumour types (Capon *et al.*, 1983; Feinberg *et al.*,1983; Kraus *et al.*,1984; Santos *et al.*,1984; Guerrero *et al.*,1985; Bos *et al.*,1987; Diamond *et al.*,1988; Riou *et al.*,1988; Smit *et al.*,1988; Burmer and Loeb, 1989). The

mechanism of suppression by normal *ras* could be through competition between mutant and normal *ras* alleles, either at the gene level, for a transcription activating factor, or at the protein level, for a cytoplasmic effector molecule. In support of this, a normal *ras*-related gene (K-rev) was recently identified which can induce reversion of the transformed phenotype of cells expressing mutant K-*ras* genes (Kitayama *et al.*,1989). Similarly, studies on yeast have shown that it is possible to interfere with the function of mutant *ras* alleles by introducing modified *ras* constructs carrying particular additional or alternative mutations (Michaeli *et al.*,1989).

In contrast, Paterson *et al* (1987) have introduced the normal N-*ras* protein into HT1080 cells, which express a mutant N-*ras* gene, but failed to detect any observable reversion of the transformed phenotype. Instead they found that the transformed phenotype was dependent solely on the level of mutant N-*ras*. However, the parental HT1080 cells already express high levels of normal N-*ras* P21 (Paterson *et al.*,1987) and it is therefore possible that these cells may have adopted another route to escape tumour suppression. Although it has been reported that the normal H-*ras* gene can act as an oncosuppressor (Spandidos and Wilkie, 1988) other groups have failed to suppress transformation by overexpression of normal *ras* (Ricketts and Levinson, 1988). Again, the possibility that some tumour cells have overcome the suppressive influence of normal *ras* should not be discounted. However, the fact that over-expression of normal *ras* can be transforming (Chang *et al.*, 1982; McKay *et al.*, 1986) also argues against a role for this gene in tumour suppression. Nevertheless, it is possible that very high levels of wild type *ras* might activate pathways unaffected by normal amounts of the protein.

Loss of the normal counterpart of an activated oncogene is not restricted to members of the *ras* family. Other studies have noted loss or under-representation of normal *myc* (Uno *et al.*, 1987), *pvt*-1 (Uno *et al.*, 1989), *neu* (Bargmann *et al.*, 1986), and p53 (Baker *et al.*, 1989; Takahashi *et al.*, 1989) alleles in tumours or cell hybrids

carrying activated forms of these genes. The detailed effects of these alterations are difficult to assess and require the development of sensitive assays that monitor key disturbances.

Although loss or under-representation of the normal H-*ras* allele may occur only as a consequence of gross chromosome events which increase the copy number of mutant *ras*, it is unlikely that the latter event is the only or main determinant of tumorigenicity. Some tumorigenic cell lines express low levels of mutant *ras* together with high levels of normal p21, whereas others express high levels of mutant p21 but remain non-tumorigenic (Paterson *et al.*,1987; Quintanilla *et al.*, in preparation). A similar situation is seen in hybrids between tumorigenic cells expressing mutant H-*ras* and normal cells. Some hybrids are phenotypically normal but continue to express the mutant gene at high levels (Geiser *et al.*,1986). These experiments show that other genetic loci exert a controlling effect on malignancy expressed through the *ras* pathway.

4.4 A tumour suppressor on chromosome 7?

A number of loci on mouse chromosome 7, including H-*ras*, are syntenic with a group of genes on the short arm of human chromosome 11 (Searle *et al.*,1989). This particular human chromosome contains at least two putative tumour suppressor genes, including the Wilm's tumour locus and a gene predisposing to Beckwith-Wiedemann syndrome (section 1.4.3; Searle *et al.*,1989). Recent comparative mapping data indicate that the mouse homologue of the Wilm's locus is located on chromosome 2 (Searle *et al.*,1989). The use of a catalase RFLP on chromosome 2 showed that SB carcinomas retain heterozygosity on this chromosome, suggesting that the Wilms' locus is an unlikely candidate suppressor gene in this system. This is perhaps not surprising given that WAGR patients do not contract cancers other than

Wilms' tumour at a higher frequency than the general population. Furthermore, recent characterisation of the Wilms' gene itself, has revealed that it is predominantly expressed in the kidney (Call *et al.*, 1990).

As described before, there is evidence that a tumour suppressor locus is located near to H-*ras* on human chromosome 11p (section 1.4.3). Loss of heterozygosity at chromosome 7 markers distal to *Hbb* in 2 DMBA/TPA carcinomas and 2 metastases is reminiscent of events involving human chromosome 11 in tumours such as rhabdomyosarcoma and hepatoblastoma, which frequently develop in sufferers of Beckwith-Wiedemann syndrome (Koufos *et al.*,1985). In addition, loss of alleles at around 11p15 is a common genetic feature of many other human tumour types (Mannens *et al.*,1988; Koufos *et al.*,1984; Riou *et al.*,1988; Mackay *et al.*,1988). In 2/4 of the mouse DMBA/TPA tumours in this category, the alteration included the H-*ras* locus, implying that the selective growth advantage conferred by these changes may have been related solely to the resultant increase in copy number of mutant H-*ras*. However, in one carcinoma, SN158, and possibly one of the metastases, loss of heterozygosity distal to H-*ras* was detected, supporting the proposal that a tumour suppressor gene could be located on chromosome 7. Further evidence for the involvement of such a gene in the growth of carcinomas was provided by the detection of alterations distal to H-*ras* in two DMBA/DMBA carcinomas (SN31 and SN47, section 3.6.2), and a DMBA/TPA/MNNG carcinoma (SN80, section 3.6.3). The identification of a chromosome 7 change in a v-H-*ras* initiated, TPA-promoted carcinoma (SN59, section 3.7) also supports the proposal that a gene other than H-*ras* on this chromosome is involved in skin tumour development. However, the detection of chromosome 7 changes in v-H-*ras* initiated tumours with no virus emphasises the need for a more comprehensive analysis of this tumour type.

If there is a tumour suppressor distal to H-*ras*, the apparent absence of chromosome 7 changes in skin tumours lacking H-*ras* mutations suggests that it

specifically suppresses the function of a mutant H-*ras* gene. This may not be unreasonable given that a revertant K-*ras* transformed cell line could be retransformed by fusion with cells transformed with some, but not other oncogenes (Noda *et al.*,1983).

If, as with classical tumour suppressor loci, complete inactivation of the putative chromosome 7 suppressor is needed for tumour progression, an explanation is required as to how this occurs in the many carcinomas in which trisomy of chromosome 7 is detected. One possibility is that these carcinomas are near-tetraploid and that chromosome 7 is actually under-represented. Interestingly, several *in vitro* transformed mouse keratinocyte cell lines have been shown to have only three copies of chromosome 7 in a near tetraploid background (Fusenig *et al.*,1985). The detection of hypo- or hypertetraploidy in four carcinoma cell lines (section 3.12) is consistent with this possibility. Alternatively, inactivation of the suppressor gene may occur by epigenetic as well as genetic mechanisms (Wilkins,1988). "Genomic imprinting" describes the epigenetic control of differential expression of maternally and paternally inherited alleles (section 1.4.4). Chromosome 7 imbalances in mouse carcinomas do not appear to result in preferential under-representation of either parental homologue (section 3.10), suggesting that inherited expression patterns do not influence this event. However, "random imprinting", i.e. with no preference for maternal or paternal alleles, would not be detected by RFLP analysis and so cannot be excluded as a potential mode of suppressor shut-down. Another possibility is that mutations at other loci might bypass the need to inactivate the chromosome 7 suppressor. Chromosome 11 may contain such a locus given that homozygosity for a marker on this chromosome was observed in 2/9 SB carcinomas. Several loci on this chromosome, including the tumour suppressor gene p53, map to human chromosome 17, which frequently suffers loss of heterozygosity during development of human carcinomas (Baker *et al.*,1989; Takahashi *et al.*,1989). The absence of minisatellite

bands in some carcinomas may also indicate loss of tumour suppressor genes on other chromosomes. However, it was concluded that these events were probably random (see below). Finally, it is of course possible that the chromosome changes observed in skin carcinomas signify the involvement of another oncogene in this system (c.f. section 4.2). Interestingly, several authors have reported amplification of a region on chromosome 11 which includes the *int*-2, *hst* and *bcl*-1 loci (Adelaide *et al.*, 1988; Tsuda *et al.*, 1989; Berenson *et al.*, 1989). However, no expression was detected using probes for these loci.

The paradoxical association of trisomy together with potential tumour suppressor inactivation is not peculiar to mouse skin tumours. In some cases of Beckwith-Wiedemann syndrome, partial trisomy of the terminal region of human chromosome 11p has been observed (reviewed by Reik, 1989); the same region which is also associated with LOH in tumours obtained from these patients

The possibility that there is a tumour suppressor gene close to H-*ras* may also apply to other members of the ras gene family, since mutation together with loss of heterozyg osity at K- and N-*ras* has been observed in several tumours (Santos *et al.*, 1984; Guerrero *et al.*, 1985; Bos *et al.*, 1987; Diamond *et al.*, 1988; Smit *et al.*, 1988; Burmer and Loeb, 1989). The F1 hybrid model could be applied to determine the mechanism of loss of these genes in chemically induced thymic lymphomas (Guerrero *et al.*, 1985; Diamond *et al.*, 1988).

4.5 Multiple carcinogen treatment: alternative routes to malignancy

One of the advantages which animal tumour models have over human studies is the ability to carefully control the carcinogens used to induce neoplasia. This was exploited in the work described above, in which different initiators were used to

establish the correlation between H-*ras* activation and subsequent gross chromosome 7 changes. In order to characterise further the types of molecular events which take place at the later stages of tumour progression, the effects of different post-initiation treatment protocols were analysed. In combination with the F1 hybrid model, which facilitates the analysis of molecular genetic changes during tumour progression, this approach has uncovered some intriguing differences between tumours induced by initiation/promotion and those obtained by repeated carcinogen treatment.

The most striking contrast was observed between MNNG/TPA and MNNG/MNNG tumours. 2/4 of the former were positive for activated H-*ras*, only one of which was likely to have been caused directly by MNNG. In both of these carcinomas non-disjunction of chromosome 7 was also detected (Table 18, column A). However, in 2/5 MNNG/MNNG carcinomas which were positive for mutant H-*ras*, no chromosome 7 changes were detected. In fact, of all the chemically induced carcinomas described in this study, these were the only examples in which activated H-*ras* was not accompanied by gross chromosome 7 changes (Table 18, column B). One explanation for this is that repeated treatment with MNNG mutates the gene which is affected by gross chromosome 7 changes in TPA-promoted tumours. This could involve mutation of the remaining wild type H-*ras* allele, or of a linked gene. Alternatively, MNNG treatment may, by altering a locus elsewhere in the genome, bypass the need for additional events involving chromosome 7. The changes this chemical is capable of inducing include deletions and sister chromatid exchange (Perry and Evans, 1975) as well as point mutations, so the affected gene(s) could be in the tumour suppressor or oncogene class.

There was no obvious difference in the H-*ras* mutation frequency when MNNG initiation was followed by TPA or MNNG treatment. However, the most striking difference between DMBA/TPA and DMBA/DMBA carcinomas was the relatively high proportion of the latter (50%) which *lacked* H-*ras* mutations. This

suggests that DMBA stimulates the growth of some initiated cells which TPA promotes either weakly or not at all. As with other H-ras mutation-negative tumours the molecular changes present in these cases remain to be determined. As expected, chromosome 7 alterations play no apparent role when H-*ras* is not mutated.

The DMBA/DMBA carcinomas in which H-*ras* mutations were detected resembled DMBA/TPA carcinomas, in that chromosome 7 changes were observed in every case (Table 18, column A). Furthermore, non-disjunction, resulting in over-representation of the mutant allele was detected in 3/4 of these; also the most common mechanism of change in DMBA/TPA carcinomas. However, in one of these three, a deletion distal to H-*ras* may have occurred in addition to trisomy. Finally, duplication of the mutant H-*ras* allele was detected in the other carcinoma. The variation in types of chromosome mechanisms is therefore higher in DMBA/DMBA tumours than DMBA/TPA tumours. In the former, three different types of chromosome 7 changes were detected in only four tumours; only two DMBA/TPA carcinomas had alternative or additional changes to non-disjunction, which was the sole event in 14/16 DMBA/TPA carcinomas.

The above findings are consistent with the proposal that repeated DMBA treatment induces the changes that are also found in TPA-promoted tumours, but at a much greater frequency. This, taken together with its ability to stimulate the growth of tumours initiated with DMBA, but lacking H-*ras* mutations, may explain the higher proportion of malignant tumours obtained by repeated DMBA treatment. However, it is also likely that this phenomenon is due in part to increased mutation of loci other than those commonly affected by TPA treatment.

4.6 Gross chromosome 7 changes occur at a premalignant stage of tumorigenesis

The detection of gross chromosome 7 changes in DMBA/TPA papillomas as well as carcinomas, suggests that over-representation of mutant H-*ras* is an early event in tumour progression, and probably confers a selective growth advantage on papilloma cells. The expansion of these cells presumably increases the frequency of other progression-related events. In the majority of papillomas duplication of the mutant H-*ras* gene was mediated by non-disjunction of chromosome 7. This complements a recent cytogenetic study in which it was found that sequential trisomy of chromosomes 6 and 7 occurs early in the development of papillomas (Aldaz *et al.*, 1989). One of the advantages of RFLP analysis is that it detects events which karyotyping would miss. For example, in this study, an allelic imbalance distal to H-*ras* was detected in one DMBA/TPA papilloma. If this was due to mitotic recombination or a small deletion, karyotyping would not have detected it. The question of whether these chromosomal changes are induced by TPA, or are an indirect consequence of rapid cell proliferation has already been discussed (section 1.5.3.1b).

The detection of similar genetic events in benign and malignant skin tumours represents molecular evidence that carcinomas are derived from papillomas. This is supported by histological, biochemical and cytogenetic findings (Aldaz and Conti, 1989). This point has been contested by Reddy *et al.* (1987) who claim that as many as 40% of carcinomas may not be derived from papillomas previously observed at the same site (section 1.5.1). Further insight into the problem may come from analysis of tumours from *spretus/musculus* hybrids. The H-*ras* polymorphism in this cross permits unambiguous identification of the parental identity of the mutant H-*ras* allele in skin tumours. Thus, a comparison of the mutated allele in same-site

papillomas and carcinomas should provide evidence with which to resolve this long-standing debate.

Although gross chromosome 7 changes can occur during benign skin tumour growth, alterations involving the H-*ras* locus are not limited to a single stage of tumour progression. In one tumour, generation of homozygosity for mutant *ras* apparently occurred during metastatic progression. In addition, the ratio of mutant:normal *ras* was above 2:1 in some carcinomas studied here, and in some others the mutant allele was amplified (Quintanilla *et al.*,1986; Brown *et al.*,1990), implying that selection for additional mutant H-*ras* genes can occur after the initial conversion to trisomy.

4.7 Tumour clonality

Much of the evidence gathered to date concerning the clonality of tumours suggests that the majority are unicellular in origin (Heim *et al.*, 1988; Nowell, 1989). The techniques used to address this question rely on the detection of markers which differentiate cells within the same tissue. One approach exploits the mosaic pattern of X-chromosome inactivation. The markers in this case include electrophoretic enzyme variants (Reddy and Fialkow, 1983) and RFLPs caused by methylation differences (Fearon *et al.*, 1987). Another technique involves the analysis of markers in embryo aggregation chimaeras (Winton *et al.*, 1989). These approaches have an important drawback: the "patch" size of cells in a tissue (the number of neighbouring cells which share the same polymorphism) may result in the growth of tumours which, appear monoclonal, but are in fact derived from more than one cell. An alternative approach is described by Griffiths *et al.* (1988). They analysed 141 dimethylhydrazine (DMH) –induced colon tumours and noted two in which a marker phenotype had been altered in every cell; consistent with mutation of the marker

gene in a single neoplastic progenitor cell. Five tumours were also described which displayed a mixed phenotype. This was attributed to mutation of the marker gene in a cell some time after the start of tumour growth, but could also be explained by polyclonality. In any case, the low frequency with which mutation of the marker gene is concurrent with neoplastic transformation limits the usefulness of this approach.

All of the above approaches share an additional caveat; in the cases where different markers are present in the same tumour, suggesting polyclonality, the possibility of normal cell contamination cannot be ruled out. Histological assessment of the degree and sites of normal cell infiltration does not permit an unambiguous conclusion, since paracrine effects of the tumour population may give normal cells a neoplastic appearance.

The best markers with which to assess tumour clonality are the initiating genetic aberrations which give rise to neoplastic growth. This permits unequivocal identification of normal and tumour cells. Analysis of the events which occur at later stages of tumorigenesis suffers from the problem of genetic convergence (Heim *et al.*, 1988), i.e. selection of a single clone from a polyclonal tumour.

The main drawback with using the initiating event to study tumour clonality is that in the vast majority of cases it is completely unknown. One exception is the mouse skin carcinogenesis model, in which H-*ras* mutation has been identified as a frequent initiating event (Quintanilla *et al.*, 1986; Brown *et al.*, 1986). The second advantage of this system is that a polymorphism in F1 *spretus/musculus* hybrid mice can be used to distinguish the parental H-*ras* alleles. If tumours induced in these hybrids are polyclonal, a proportion are bound to contain both *spretus* and *musculus* mutant H-*ras* alleles. In contrast, tumours of unicellular descent will consist of cells which contain a mutant H-*ras* allele of identical parental origin. Analysis of several tumours obtained from these hybrids has demonstrated the monoclonal origin of both

papillomas and carcinomas (P. Burns, R. Bremner and A. Balmain, in preparation). This contrasts with a recent study in which it was found that immunohistochemical markers derived from both parents in embryo aggregation chimaeras were present in papillomas (Winton *et al.*, 1989). However, these results can be explained entirely on the basis of normal cell contamination. A similar explanation may therefore apply in the case of human tumours which are apparently polyclonal (Heim *et al.*, 1988)

Previously, it has been suggested that epithelial tumours may be subject to genetic convergence (Heim *et al.*, 1988). In this proposal, reduction to monoclonality is seen as a product of selection pressures experienced late in tumorigenesis, e.g. upon invasion into the dermis. This appears to be the case in v-H-*ras* initiated mouse skin tumours. A single viral integration event was detected in carcinomas, indicating monoclonality (section 3.7; Brown *et al.*, 1986). However, 2 or more integration events were detected in v-H-*ras*/TPA papillomas, suggesting a multicellular origin (Brown *et al.*, 1986). In the case of chemically induced tumours from F1 hybrid mice, a different pattern is observed. Carcinomas, and papillomas analysed soon after their appearance, consistently display mutation of only one of the parental H-*ras* alleles, but never both. Thus it appears that monoclonality applies from the very beginnings of tumour growth.

4.8 Minisatellite analysis: random rearrangement of hypervariable loci

The disadvantage of using single copy DNA probes is that a maximum of 50% heterozygosity is possible if, as in most cases, there are only two alleles in the population. Several RFLPs were unusable in SN and/or SB hybrids because of this. Minisatellite loci, on the other hand, are highly polymorphic because of variation in the number of repeat units which make up a single allele. Although probes which

hybridise to single minisatellite loci are available (Thein et al., 1988), a probe which hybridises to several loci was used in this study. This meant that the status of several chromosomes could be monitored simultaneously. The probe detects loci on chromosomes 4, 5, 14 and 17 in C57BL/6J and DBA/2J mice (Jeffreys et al., 1987). It was therefore considered a useful tool to a) screen tumours for consistent band losses, and b) compare the level of random chromosomal alterations with the level of changes involving chromosome 7. Loss of chromosomes 4 alleles would be of particular interest, given the tumour suppressing properties assigned to it by cell fusion studies (Table 1, section 1.4). Chromosome 14 is also of potential interest since its under-representation was noted in several transformed keratinocyte cell lines (Fusenig *et al.*, 1985). Furthermore, the mouse retinoblastoma locus has been mapped to this chromosome. It should be noted however, that differences between minisatellite alleles in the strains used in this study and those in which mapping studies were carried out (Jeffreys *et al.*, 1987), prevent assignment of band losses in SB hybrids to one of the four chromosome mentioned above. In the mapping study, ten fragments totalling 90kb were assigned to the chromosome 4 minisatellite locus, while each other locus was represented by one band only. Thus, random losses in SB tumours would probably map to chromosome 4.

Of 9 SB carcinomas and 1 SB lymphoma, induced by DMBA- initiation and TPA-promotion, all but one carcinoma had detectable alterations in the DNA fingerprint detected by the probe described above. Gross chromosome 7 changes were also detected in all of these SB carcinomas. This resulted in over-representation of the chromosome carrying activated H-*ras*, mutated at initiation by DMBA. The alterations detected by the minisatellite probe could therefore represent events, such as loss of suppressor genes, involved in the post-initiation stages of carcinogenesis. The loss of minisatellite bands in the SB lymphoma represents the only genetic

alteration detected in this tumour. It lacked a mutant H-*ras* gene, and no imbalances or losses were detected using polymorphic markers on chromosomes 2, 7 and 11.

It is unlikely that the differences between tumour and normal tissue fingerprints were the result of somatic mutations prior to tumour induction, since the DNA fingerprint of separate tissues of a single mouse were identical. This complements results from analysis of different human tissues (Thein et al., 1987). However, the possibility that micropopulations of cells within the skin already contained the mutations observed in the tumours cannot formally be excluded.

No preference for loss of SENCAR or BALB/c bands was detected, even in the five carcinomas (SB141-145) which were derived from a single mouse. This complements the chromosome 7 analyses in which no bias was detected concerning the loss or under-representation of parental alleles.

Loss of minisatellite bands may have been caused by events associated with the loss of tumour suppressor loci, such as non-disjunction, mitotic recombination and deletion. In theory, mitotic recombination could even be mediated by interaction between minisatellite alleles. However, two other mechanisms could explain the changes observed. First, *Hinf*I is methylation sensitive and will not cleave if the cytosine of the CpG dinucleotide in the GANTCG recognition site is methylated (McClelland and Nelson, 1985). Thus, the *Hinf*I DNA fingerprints of SB tumours may have been altered by this mechanism, especially since methylation patterns are known to be altered by transformation (Jones, 1986). However, in a study of several different human tumour types, cleavage of DNA with methylation insensitive enzymes such as *Alu*I and *Hae*III, which give similar DNA fingerprints to *Hinf*I, detected comparable shifts in minisatellite fragment sizes (Thein et al., 1987). This suggests that changes in minisatellite bands were probably the result of genetic rather than epigenetic events. Even if the latter did apply, this would not detract from the

potential interest of such changes given the role of methylation in the control of gene expression (Jones, 1986; Holliday, 1987).

The tumour-related changes in DNA fingerprints could also be the result of sister chromatid exchange. Depending on the fidelity of this process, it might only affect the size of minisatellite alleles and not other loci. Such alterations would probably be a consequence, rather than a cause, of tumour progression. Four factors suggest that this may be the case. First, the majority of bands which were altered in tumours were large, and therefore potentially hypervariable (Jeffreys et al., 1985b). The variation in these bands between SB hybrids supports this possibility (although some of this variation may have been due to parental heterozygosity rather than germ line recombination). Second, in only one case was the loss of a particular band observed in more than one tumour. Similar results were obtained in a study of carcinomas from NIH mice (P. Burns, personal communication). Finally, secondary alterations involving a new carcinoma-specific band were observed in cell lines and nude mouse tumours obtained from this tumour, suggesting hypervariability.

These findings are consistent with the hypothesis that large hypervariable minisatellite fragments, which are particularly susceptible to recombination during germ line transmission, undergo frequent rearrangement in the genetically destabilised environment of a carcinoma. Such increased recombination could be mediated through deregulated expression of one or more minisatellite recombinases, which, on the basis of the similarity between minisatellite core sequences and the *E. coli* Chi sequence (Jeffreys et al., 1985a) may resemble the recombinase which interacts with the latter.

It cannot be excluded that the apparently random loss of hypervariable minisatellite bands could be related to the loss of different tumour suppressor genes in separate tumours. Alternatively, different minisatellite bands which were lost in separate tumours may be from a single locus. Loss of different minisatellite bands

may therefore accompany loss of a single tumour suppressor locus. It is of interest that the chromosome 4 locus detected by the probe used in this study was represented by 10 fragments totalling 90kb in C57BL/6J mice, although the other loci which were mapped were represented by one band only (Jeffreys et al., 1987). However, it is not possible to conceive a simple mechanism for the loss of different *Hinf*I fragments in separate tumours, all from the same locus, while simultaneously deleting a linked suppressor gene (or activating an oncogene). A simpler explanation is that these changes were generated by sister chromatid exchanges at different regions of a large minisatellite locus. Unless these loci are important regulatory elements, it is unlikely that their rearrangement would have a significant effect on tumour progression.

The difficulties in interpreting tumour associated DNA fingerprint alterations can only be properly resolved by cloning deleted or new fragments, assigning their chromosomal location and using other single copy polymorphic markers to test for chromosomal alterations. This procedure could be simplified by using hypervariable minisatellite probes which detect single loci on a known chromosome. The inability to detect multiple loci simultaneously with this approach can be overcome by rehybridising the same blot with different hypervariable probes. LOH has been detected in 10/118 cases of myelodysplastic syndrome (MDS) using one probe of this type which maps to human chromosome 7 (Thein et al., 1988). In at least five of these cases the loss was known, from cytogenetic analyses, to have been caused by complete loss of one chromosome. However, that LOH in the other tumours was related to loss of a linked gene requires confirmation by use of single-copy polymorphic markers.

No changes were detected in the DNA fingerprints of two papillomas containing an activated H-*ras* transgene. The latter was under the control of a suprabasal keratin promoter (Bailleul et al., submitted). A similar result was obtained

from the analysis of several papillomas from NIH mice induced by DMBA-initiation and TPA promotion (P. Burns, personal communication). This could be related to the relative genetic stability of papillomas compared to carcinomas (Conti *et al.*, 1986; Aldaz *et al.*, 1987) and/or the restriction of suppressor gene loss to a small subset of papillomas which progress to carcinomas.

Finally, one purpose of the DNA fingerprint analyses was to assess the frequency of random genetic alterations in carcinomas. Most bands in the tumour DNA fingerprint were unaltered and the rearrangements that were detected involved putative hypervariable loci. These results consolidate the findings from analysis of chromosome 2 and 11 markers suggesting that gross chromosome 7 alterations are non-random.

4.9 Mouse skin tumorigenesis: an emerging pattern of genetic events

Mutation and RFLP analyses have given rise to a multistep model of carcinogenesis in which activation of one or more oncogenes, together with loss of several tumour suppressor genes, occurs in a stepwise manner, eventually resulting in full expression of the malignant phenotype (Bouck and Benton, 1989; Vogelstein, 1989). The results described in this thesis suggest that tumour progression in mouse skin follows a similar pattern. Following initiation by H-*ras* activation, there is selection for additional copies of this gene. Subsequently, there may be inactivation of tumour suppressor loci on chromosome 7 and/or other chromosomes, of which chromosome 11 is one candidate. The F1 hybrid model should continue to provide valuable insight into the molecular nature of these additional changes.

4.10 Future prospects

The F1 hybrid model described in this study was originally developed to assess the role of allele loss on chromosome 7 in mouse skin tumorigenesis. Its wider application, to the study of other loci, tumour types, tumour induction protocols and animal models, may result in the eventual identification of new tumour suppressor and oncogenes. In addition, the model should also provide valuable insight into issues such as genomic imprinting, tumour clonality and the relationship between benign and malignant tumours of similar lineage. The limiting factor in all of these is the level of heterozygosity at relevant loci. This can be maximised by using *spretus/musculus* crosses, which are sufficiently divergent to ensure heterozygosity at virtually every locus.

There are several matters raised by the study described here which require further investigation. First, there is the problem of whether wild-type H-*ras* suppresses tumour progression. Gene targeting may help to to resolve this issue. If a single H-*ras* allele can be disabled in mice and/or epithelial cells lines, the resultant effect on tumorigenicity may indicate the role, if any, of the normal gene in tumour growth. Second, over-representation of mutant H-*ras* needs to be confirmed at the expression level. RNase mismatch analysis is one approach which can be used to address this issue. This technique may also uncover some novel mutations in apparently "normal" *ras* genes. This may apply to the v-H-*ras* tumours in which gross chromosome 7 changes were detected despite the absence of codon 12 , 13 and 61 mutations.

Finally, the evidence presented for the role of a locus distal to H-*ras* in mouse skin tumorigenesis requires further study. If more tumours which demonstrate such alterations can be obtained from *spretus/musculus* hybrids, it should be possible to map this locus more closely.

Chapter 5

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