The role of p53 in human epidermal keratinocyte terminal maturation.

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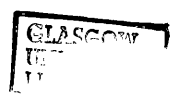

To my parents
for all their love and encouragement
- and the family tank !

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Abbreviations

APS	Ammonium persulphate
BSA	Bovine serum albumin
CMV	Cytomegalovirus promoter enhancer
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra-acetic acid
EBV	Epstein-Barr virus
HEK	Human epidermal keratinocyte
HPV	Human papilloma virus
LOH	Loss of heterozygosity
Neo	Neomycin
PAb	antibody
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SCC	Squamous cell carcinoma
SCC-HN	Squamous cell carcinoma of the head and neck.
SDS	Sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylethylenediamine

bp	base pair
kb	kilobase pairs
OD	optical density (absorbance)
nm	nanometres
SD	Standard deviation
w/v	weight per unit volume

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Abstract

Two cell lines, SCC12F and SCC12B were derived from the same squamous cell carcinoma of the facial epidermis and shown to display different phenotypes. SCC12B is more resistant to the induction of terminal differentiation and also more tumorigenic than SCC12F. The aim of this thesis was to investigate any genetic differences between SCC12B and SCC12F that could be responsible for their different phenotypes and therefore possibly be an important genetic event in the progression of squamous cell carcinomas of the head and neck (SCC-HN).

Microsatellite analysis of SCC12B and SCC12F revealed no differences in loss of heterozygosity (LOH) at the loci investigated on chromosome 3p and 9 which have previously been reported to show frequent LOH in SCC-HN. No overexpression of cyclin D1 was observed in the two cell lines and others have reported the absence of any H-*ras* mutations or abnormalities in Rb-1. Analysis of the tumour suppressor gene p53 however revealed different levels of the protein between the two cell lines. SCC12B was shown to express much higher levels of p53 protein than SCC12F. Sequencing of p53 revealed a novel heterozygous mutation at codon 216, a T→G transversion substituting a valine for a glycine. Interestingly, whilst the mutant allele was visible in both cell lines SCC12B appeared to express much more mutant p53 than SCC12F which mostly retained wild-type p53 expression. Dot blot analysis suggested that mutant p53 expression in SCC12B was double that of SCC12F.

Investigations were then undertaken to investigate whether the variations in this mutant to wild-type gene dosage could explain the different abilities of SCC12F and SCC12B to undergo suspension induced terminal differentiation. A clone of SCC12F, clone 19, expressing low levels of p53 and therefore presumably expressing a more normal phenotype was used as a target for alterations in mutant p53 expression. The use of this clone had the advantage that it retained a related genetic background to SCC12F and SCC12B and therefore is a more relevant target cell for investigating the effects of increased mutant $p53^{216}$ on terminal differentiation.

The results in this thesis show that increasing the mutant $p53^{216}$ dosage in clone 19 decreased the cells ability to express involucrin and form cornified envelopes and increased cell survival in response to suspension induced terminal maturation. An increase in tumorigenicity was however not observed.

Taken together these results therefore suggest that the acquisition of a p53 mutation is an early event in this SCC and its accumulation leads to a dramatic progression of this cancer. Inactivation of p53 appears to inhibit the cells ability to terminally differentiate and the possible roles of p53 in tumour progression are discussed.

Chapter 1

Introduction

1.1. Squamous cell carcinoma of the head and neck (SCC-HN).

Squamous cell carcinomas of the head and neck (SCC-HN) are still today a major cause of death in developing nations. It appears to be more frequent in India and SE Asia, comprising 40-50% of total malignancies (Pinborg, 1984), than in the West where it comprises only 5% of all malignancies (Million *et al.*, 1989). Premalignant lesions which can progress to SCC-HN are papillomas, leukoplakias and erythroplakias although 50% of SCC tend to develop without the prior premalignant lesion. Tumours develop at a low frequency from leukoplakias (1-5%) but there is a higher probability of progression from erythroplakias (30-55%) and for this reason the erythroplakias are the only premalignant lesion that is surgically removed. Squamous cell carcinomas can develop into the more aggressive spindle cell carcinoma although these tend to be recurrent or metastatic tumours.

The aetiological risk factors of development of SCC-HN in India appears to be chewing tobacco (Jussawalla and Deshpande, 1971) whilst in the West cigarette smoking, the use of snuff and alcohol have proved to be the main causes (Wynder and Stellman, 1977). Although the epidemiology of SCC-HN has been studied, the genetic mechanisms that are involved in its progression are poorly understood.

1.1.1. Cultivation of SCC-HN cell lines.

Rheinwald and Green, 1975b, developed an efficient method for the cultivation of normal HEK's as well as squamous cell carcinomas by growing them on a lethally irradiated 3T3 feeder layer. Several oral SCC cell lines have been established (Rheinwald and Beckett, 1981; Easty *et al.*, 1981a&b; Rupniak *et al.*, 1985; Prime *et al.*, 1990; Tatake *et al.*, 1990). However most of these lines (except Rheinwald and Beckett, 1981) were developed under suboptimal conditions in the absence of a feeder layer or cholera toxin rendering the selection of fitter variants or more aggressive phenotypes likely (Rheinwald and Beckett, 1981). Most were also derived from

recurrent or irradiated tumours; normal tissue from the same patient is not available for comparison and tumour stage has not been recorded. These cell lines therefore do not provide a good system in which to study the genetic changes that give rise to SCC-HN. Recently a more detailed collection of cell lines have been cultivated from both premalignant erythroplakias and SCC-HN at different stages of tumour progression (Edington *et al.*, 1994). These cell lines are currently being examined to highlight the phenotypic and genetic changes which occur during development and progression of SCC-HN.

Cell lines derived from premalignant erythroplakias retain many of the properties associated with cultured normal keratinocytes such as a requirement for high serum levels, hydrocortisone, cholera toxin, anchorage and an irradiated 3T3 feeder layer for optimal *in vitro* growth. (Edington *et al.*, 1994). They also have a normal diploid karyotype, a limited lifespan ending in senescence and are non-tumorigenic. They differ from normal HEK's in that they form poorly stratified cultures and also appear to be resistant to suspension-induced terminal differentiation and cell death (Edington *et al.*, 1994). These latter phenotypes are also characteristic of later stage SCC cultures (Parkinson *et al.*, 1983; Edington *et al.*, 1994) and therefore resistance to terminal maturation appears to be a phenotype acquired early in SCC-HN development.

Cells from more advanced tumours possessed an altered morphology and all gave rise to immortal cell lines (Edington *et al.*, 1994). This late development of *in vitro* immortality is also observed in colon cancer (Paraskeva *et al.*, 1984) and melanoma (Mancianti and Herlyn, 1989). SCC cell lines also displayed an aneuploid karyotype and a reduced requirement for serum growth factors. The majority of cell lines from advanced SCC-HN formed tumours in nude mice and there appears to be a correlation between stage of tumour from which the cell line is derived and degree of tumorigenicity (Edington *et al.*, 1994).

1.1.2. SCC12B and SCC12F: A target system in which to study the affect of tumour suppressor genes in SCC progression.

SCC12B and SCC12F were originally derived from the same SCC tumour of the facial epidermis (SCC12) (Rheinwald and Beckett, 1981). This tumour was taken from a 60 year old male kidney transplant recipient who had been treated with immunosuppressive drugs for the previous seven years. SCC12 was shown to be partially defective in its ability to undergo terminal differentiation in response to suspension culture. Compared to normal HEK, SCC12 cells exhibited a reduced rate and extent of cornified envelope formation. Only 40% of cells developed cornified envelopes after five days in suspension compared to 80% of normal HEK's (Rheinwald and Beckett, 1980). SCC12 also had a much longer survival half-life in suspension than normal keratinocytes. The colony forming ability of normal HEK after 24 hours in suspension is completely lost. SCC12 cells showed a rapid decay during the first day followed by a stable retention of colony forming ability of the remaining cells (Rheinwald and Beckett, 1980). This sharp drop in survival was due to the presence of a non-tumorigenic fraction of cells in SCC12 that were also immortal in culture. SCC12 was therefore divided into two cell lines, one of which appeared to have a more malignant phenotype (SCC12B) than the other (SCC12F). These two cell lines therefore represent phenotypically distinct cell populations within the same tumour that are at different stages of tumour progression.

SCC12B and SCC12F have been shown to share common traits which is important evidence in support of their monoclonal origin. Both cell lines share identical rearrangements of the β-polymerase and EGF receptor (Weichselbaum *et al.*, 1988) and do not express mutant *ras* (Clark *et al.*, 1993). Cytogenetic analysis showed that both cell lines were triploid and had lost one copy of chromosomes 8, 13, and 15 (Jaffe *et al.*, 1992).

Despite these similarities SCC12F and SCC12B differ in their tumorigenic potential and their ability to respond to terminal differentiation signals. SCC12B is highly tumorigenic (Parkinson *et al.*, 1984) inducing tumours in all mice injected within

20 days (Jaffe *et al.*, 1992). In comparison SCC12F is non-tumorigenic. SCC12B was much more resistant to the induction of terminal differentiation than SCC12F. SCC12B had a much longer survival half-life in suspension of 24 hours compared to that of SCC12F which showed loss of colony forming ability T1/2 of 8.6 hours (Parkinson *et al.*, 1983). Similar results were obtained when the cell lines were induced to terminally differentiate by exposure to PMA (Parkinson *et al.*, 1983). Studies into the loss of colony forming ability in reponse to treatment with PMA showed that 70% of SCC12B cells were resistant to the effects of PMA compared to only 21% resistance of SCC12F cells. Consistent with this reduced ability to terminally differentiate, SCC12B had a lower degree of cornified envelope inducibility (Rubin and Rice, 1986) and involucrin synthesis as compared to SCC12F (Jaffe *et al.*,1992). After exposure to 100mM PMA for six days SCC12F showed a 10 fold increase in cornified envelope formation over their controls whilst SCC12B only showed a three fold increase (Parkinson *et al.*, 1983).

SCC12F can be converted towards a phenotype displayed by SCC12B by transfection with the Epstein Barr virus (EBV) latent membrane protein (LMP1) or Ha-ras (Dawson *et al.*, 1990). Both proteins were shown to impair the terminal differentiation of SCC12F but only Ha-ras gave rise to malignant transfectants.

Somatic cell hybrids generated by fusing SCC12F with SCC12B resulted in hybrids with a much lower tumorigenic potential than SCC12B suggesting that SCC12F has donated a tumour suppressor function to the hybrid (Jaffe *et al.*, 1992).

Taken together these observations suggest that SCC12B was derived from a population of cells in the original tumour that had progressed further towards malignancy than the population from which SCC12F was derived. Comparisons of these two cell lines therefore provide an ideal system in which to investigate the genetic changes that occur in SCC-HN during tumour progression.

1.1.3. Terminal differentiation in keratinocytes : a good *in vitro* model

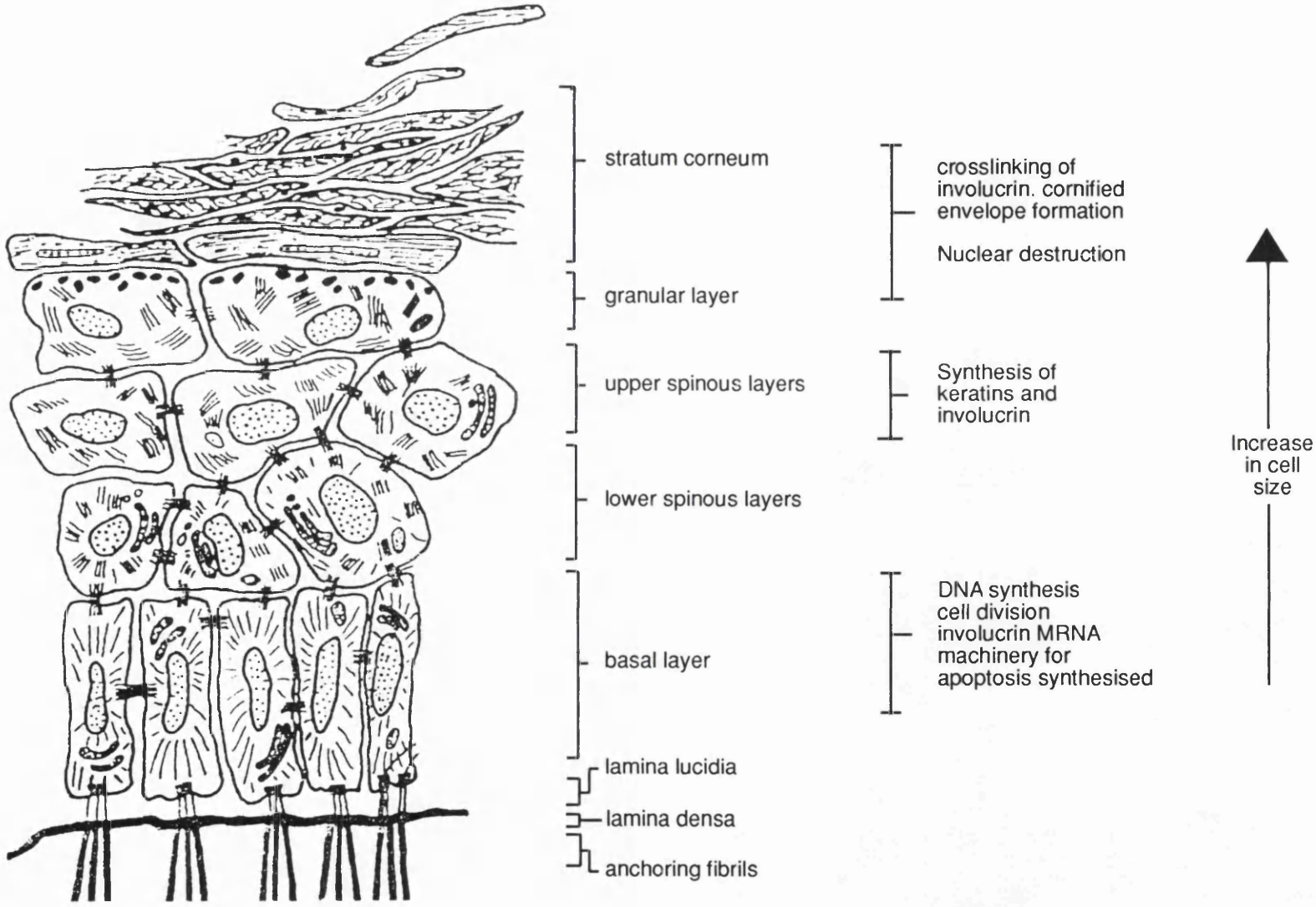
Normal HEK's have been successfully cultured in the presence of a 3T3 feeder layer with an *in vitro* lifespan of more than 150 population doublings (Rheinwald and

Green, 1975b). More efficient cell growth has been obtained by using a medium (DMEM) supplemented with 20% fetal bovine serum, hydrocortisone (Rheinwald and Green, 1975b), cholera toxin (Green, 1978) and EGF (Rheinwald and Green, 1977). By such a method each colony consists of keratinocytes forming a stratified squamous epithelium in which the dividing cells are confined to the lowest layer as in the epidermis *in vivo*.

A simplified diagram of the epidermis is shown in figure 1.1. The epidermis consists of several layers whose cells possess distinct properties. Cells in the lower basal layer are the only proliferating cells and as they mature they migrate up towards the surface of the skin losing their ability to multiply and begin to terminally differentiate. As the cells enter the spinous layer they increase in size (Rowden, 1975) and begin to synthesise involucrin (Watt and Green, 1981). As the cells enter the granular layer, they begin to destuct. The cytoplasmic organelles are eliminated (Lavker and Matoltsy, 1970) and their chromatin becomes marginated and clumped. The plasma membrane bcomes permeable to calcium and this influx activates an endogenous endonuclease which fragments the nuclear DNA into 200bp fragments- a hallmark of apoptosis (McCall and Cohen, 1991). Keratins of various types are present at all stages of differentiating keratinocytes (Sun and Green, 1978). The cells in the outer layers contain larger keratins than those of the inner layers (Fuchs and Green, 1980). The calcium influx activates the protein proflaggin to bundle keratin fibrils together (Fuchs and Byrne, 1994) and triggers the cross-linking of involucrin by a transglutaminase, ultimately forming an insoluble cornified envelope (Sun and Green, 1976) surrounding a cytoplasm composed of mostly keratins. This cornified layer acts as a mechanical waterproof barrier preventing damage to the underlying cells.

Studies have shown that loss of cellular contact with the basal lamina (Sun and Green, 1976) (perhaps by alterations in adhesion molecule expression) and alteration of cell shape are sufficient to trigger terminal differentiation (Watt *et al.*, 1988). This response can be mimicked *in vitro* by suspending cells in methylcellulose which prevents cell to cell contact and the cells become unresponsive to mitogen stimulation. Basal cells mediate attachment to the basal lamina mostly via members of the integrin family

Figure 1.1 **The epidermis showing sequence of terminal differentiation events**



receptors. Integrins are heterodimeric transmembrane proteins consisting of α and β subunits (Hynes, 1992). Different α and β associations determine the ligand binding specificities of the integrin heterodimers for various extracellular matrix proteins including fibronectin, laminin and collagen. Alterations in ligand binding to the $\alpha_5\beta_1$ fibronectin receptor has been implicated in epithelial cell proliferation and differentiation signalling. Terminal differentiation induced by suspension culture can be inhibited by immediate addition of fibronection (Adams and Watt, 1989) which binds to the integrin receptor and prevents negative growth signalling. However, any delay in this addition has no effect as the cells have already been committed to terminal differentiation and the ability of the receptor to bind fibronectin is decreased (Adams and Watt, 1990). Detachment of epithelial cells from extracellular matrix contacts or growth in suspension has also been shown to elicit an apoptotic response termed anoikis (Meredith *et al.*, 1993; Frisch and Francis, 1994). As well as being part of the terminal differentiation pathway this controlled cell death would also be important in preventing cells from becoming dislodged and inappropriately reattaching and thereby helps to maintain the polarity and organisation of the epithelium. The signalling pathway mediated by the integrin receptor is yet to be identified in detail but may be mediated via changes in cellular pH, calcium fluxes and tyrosine phosphorylation events (Meredith *et al.*, 1993; reviewed in Juliano and Haskill, 1993).

In order to study terminal differentiation of keratinocytes in an *in vitro* situation it is important to establish to what extent these cultures resemble normal epidermis. They retain the fundamental characteristics in that they are stratified, with proliferation restricted to the lowest layer and cells undergoing terminal differentiation as they move upwards through the layers. However in culture morphology is poor compared to normal epidermis in that the basal cells tend to be flattened, no proper stratum corneum is formed and culture shed nucleated squames (Green, 1977). Proper cornified and granular layers similar to those in vivo can be restored to cultures by depleting the culture medium of vitamin A which has been shown to suppress keratinocyte differentiation (Fuchs and Green, 1981). In vitro the spatial distribution of involucrin (Watt, 1983) and keratins (Kopan *et al.*, 1987) are changed. In culture cells express only

small keratins, no large keratins are expressed in the outer layers (Fuchs and Green, 1980) and certain keratins are not seen at all (Sun and Green, 1978).

The complete *in vivo* differentiation program can best be seen *in vitro* by culturing the keratinocytes on collagen rafts (Kopan *et al.*, 1987). Once confluent, the epithelium becomes raised above the medium and is only fed through the collagen and the ventral surface of the epithelium. It is thought that in this situation a vitamin A gradient is set up across the layers similar to an *in vivo* situation and therefore normal morphology and protein expressions are regained.

Keratinocytes grown *in vitro* are therefore a very good system in which to study the terminal differentiation programme. Cells grown in a monolayer show the majority of the characteristics of terminal differentiation. But this can be improved by growing cells on a raft culture or be induced by placing cells from the monolayer into suspension culture. Many markers of terminal differentiation are available for study using this system namely, involucrin synthesis; cornified envelope formation; loss of colony forming ability, which is usually the first indicator of terminal differentiation as keratinocytes kept in suspension culture lose their ability to form colonies with a half-life of 3 hours (Rheinwald, 1980) and apoptosis.

1.2. Carcinogenesis is a multistage process

It is clear from analysis of the phenotypes of tumours in experimental models that there are three distinct stages involved in tumour progression i.e. initiation, promotion and progression. It would therefore be surprising if only one genetic event was responsible for all stages and it is more likely that a number of genetic changes act in concert with each other to develop a malignant phenotype. Such a model for a multistage process was first proposed by Foulds in 1954 and only recently has technology made it possible to identify the molecular events involved in carcinogenesis.

Early evidence to support this multistep model came from the transformation of primary rat embryo fibroblasts (REF) with cooperating oncogenes. Transfection of both *ras* and *myc* into REF cells lead to their transformation (Land *et al.*, 1983), but neither oncogene could act on its own. It was proposed that each step in the tumorigenic process reflects a mutation leading to the activation of one or more cellular oncogenes. The resulting oncogenes then work together to induce the full neoplastic phenotype.

Weinberg (1985) proposed that for full transformation to occur one oncogene should be nuclear and one cytoplasmic i.e. *myc* being the nuclear protein and *ras* the cytoplasmic one in the above experiment. It is now thought that the nuclear oncogene can be substituted by an inactivated tumour suppressor gene (reviewed by Weinberg, 1989) and this has been confirmed by the ability of the tumour suppressor gene p53 to cooperate with *ras* in the transformation of REF cells in its mutant form (Eliyahu *et al.*, 1984; Parada *et al.*, 1984), but suppress transformation in its wild-type form (Finlay *et al.*, 1989; Eliyahu *et al.*, 1989).

Until recently the only tumorigenic mutations studied in detail were those that activated oncogenes. Oncogenes, which are mutant alleles of the wild-type genes proto-oncogenes, have acquired novel or aberrant activities that promote malignancy. Their ability to function in a genetically dominant manner and the selective growth advantage that they confer have facilitated the identification of a long list of oncogenes e.g. *src*, *fos*, *myc*.

More recent efforts have been made to identify tumour suppressor genes e.g.p53, Rb-1, CDKN2/p16^{INK4}. These tumour suppressor genes or anti-oncogenes normally act to inhibit growth and therefore are more difficult to identify. The inactivations of tumour suppressor genes by mutations was first observed by Knudson in 1971 from studies on familial retinoblastoma, who proposed that inactivation of both copies of the gene was required and this occurred by a 'two hit' mechanism. In inherited Rb, one mutant allele is inherited from the parent. A second mutation occurs in the other allele as an independent event in the offspring. In sporadic Rb, both mutations occur as two events in the same cell population. If a person has an initial recessive mutation in one copy of Rb, reduction to homozygosity can be obtained by several chromosomal mechanisms (Cavenee *et al.*, 1983). Firstly, mitotic nondisjunction with loss of the wild-type allele would result in hemizygosity at all loci on the chromosome. Mitotic nondisjunction with duplication of the mutant chromosome would result in homozygosity at all loci on the chromosome. Alternatively mitotic recombination may occur between chromosomal homologues, with a breakpoint between the tumour locus and the centromere which would result in heterozygosity at loci in the proximal region and homozygosity throughout the rest of the chromosome including the tumour locus. Additionally other genetic events such as gene conversions, deletions and mutations can also occur.

In line with the proposal that carcinogenesis is a multistep process, inactivation of one gene by the mechanisms previously described is not enough and additional events are required to move this homozygous defective cell towards full neoplasia. It has been proposed (Nowell, 1976) that neoplasms develop from a sequential selection of mutant subpopulations from a single cell origin. In such a model an initial genetic event will give a cell a growth advantage over its neighbours. This pre-neoplastic cell proliferates further and from time to time as a result of genetic instability in the expanding population, genetic variants acquiring other genetic events are produced. Nearly all these variants are eliminated because of metabolic disadvantage or immunologic destruction, but occasionally one has an additional advantage and becomes the precursor of the new predominant subpopulation. This sequential selection proceeds over time until a clone has arisen that has a full aneuploid malignant phenotype. Some genetic events

are common among different tumours e.g. p53 mutations, but others are specific to that cell type.

It is well known that primary mouse fibroblasts grown in culture frequently produce immortalised clones of cells (Hermann and Rice, 1983). However the spontaneous immortalisation of human cells in culture is very rarely seen (Baden *et al.*, 1987). The interpretation of this observation maybe that fewer independent mutations are required in mice than in humans and therefore they become immortalised much more frequently.

Studies of cells from premalignant and malignant SCC-HN indicate that acquisition of an immortal phenotype in this cancer is normally a late event (Edington *et al*, 1994) and cell hybrid experiments have also shown that immortality was genetically recessive to senescence (Berry *et al.*, 1994) thereby supporting the existence of tumour suppressor genes. These results therefore support the hypothesis that multiple genetic changes are required for HEK immortality. This is in line with a recent model of tumorigenicity proposed for colorectal cancer (Fearon and Vogelstein, 1990) in which at least four genetic alterations involving both tumour suppressor genes and oncogenes must occur befor the onset of tumour formation. Studies are currently being undertaken to identify these genetic events in SCC-HN and the results are discussed further in section 1.3.

Other evidence for the requirement of multiple mutations in carcinogenesis come from the study of transgenic mice. Transgenic mice have been bred that express *c-myc* at high levels in breast epithelial cells (Leder *et al.*, 1986). Although these mice express a large amount of *myc* protein, they do not develop breast tumours until late in life. Therefore *c-myc* predisposes breast epithelial cells to become tumorigenic but this is delayed until other genetic mutations have accumulated. In much the same way, p53 knockout mice are viable but do not develop tumours until they are six months old (Donehower *et al.*, 1992). Again suggesting that a p53 mutation alone is not sufficient for malignancy. The latent period is increased in mice heterozygous for p53 (Donehower *et al.*, 1992) presumably because they have to also acquire loss of the p53 wild-type allele.

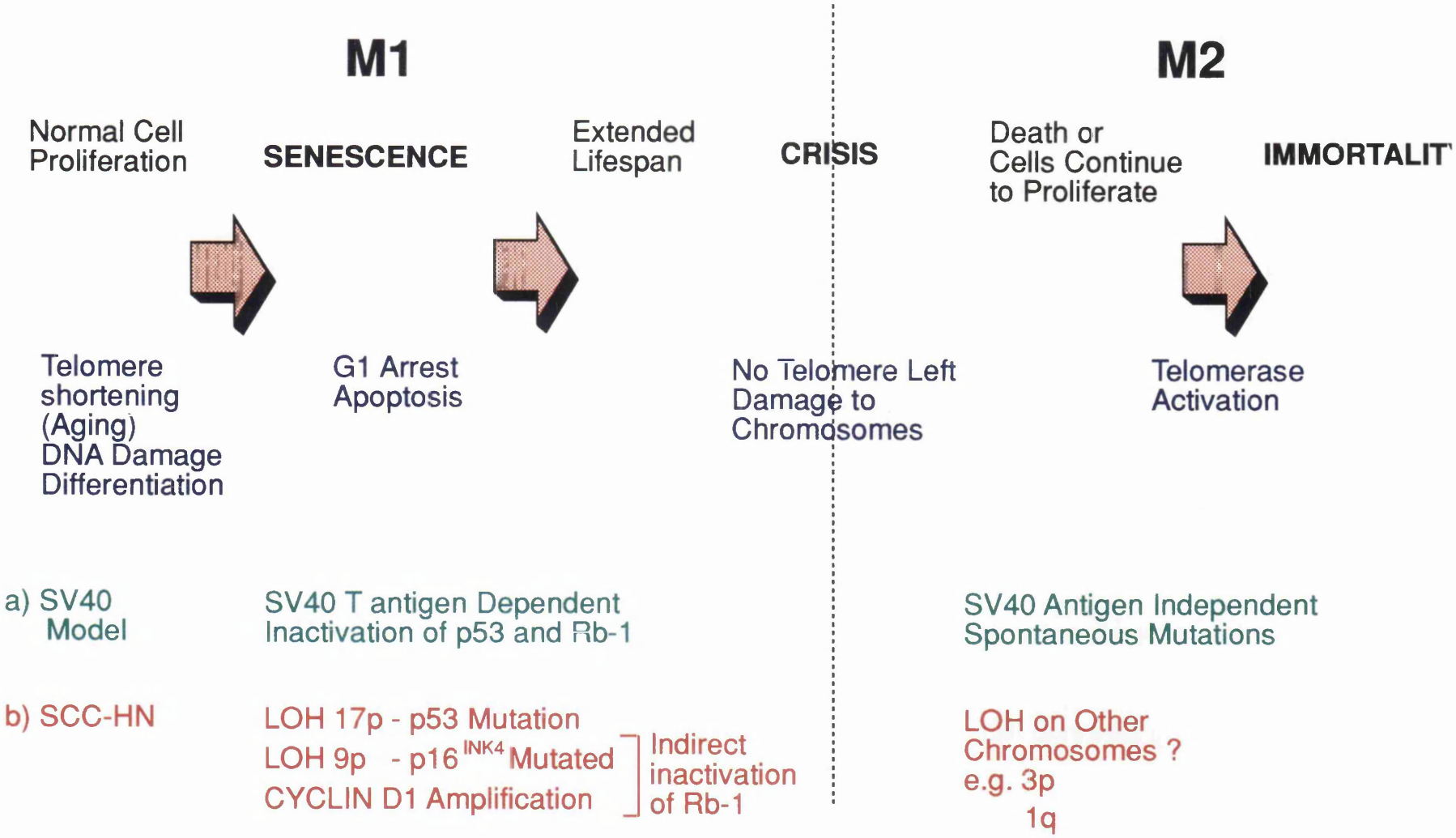
1.2.1. SV40 model of immortalisation (M1 and M2)

Normal human fibroblasts infected with SV40 virus show an extended lifespan in culture and after a period of crisis, immortal variants occasionally appear. It was shown by two independent groups that this effect was due to the expression of SV40 T antigen (Wright *et al.*, 1989; Radna *et al.*, 1989) and its continued expression was required for cells during the extended lifespan prior to crisis and following immortalisation. These results led to a proposal of a two stage model to explain this escape from senescence (Wright *et al.*, 1989) figure 1.2. The first stage of this so called mortality mechanism, M1, leads to senescence and it can be inactivated or bypassed by SV40 T antigen. This allows cells to continue proliferating until crisis occurs. Crisis is caused by the onset of stage two (M2) of this mortality mechanism. Rare inactivations of unknown genes in this stage give rise to immortal variants.

SV40 large T antigen has been shown to bind to both Rb-1 (De Caprio *et al.*, 1988) and p53 (Lane and Crawford, 1979; Linzer and Levine, 1979). By use of T antigen mutants it has been shown that the binding of p53 and Rb-1 is involved in the bypass of the M1 mechanism (Figure 1.2) and that both activities of the T antigen are required for proliferation (Shay *et al.*, 1991). Furthermore adenovirus E1B and E1A, or HPV E6 and E7 (each pair of which is known to bind to p53 and Rb-1 respectively) are able to replace T antigen functions and permit cell proliferation (Shay *et al.*, 1991). This remarkable similarity between these viruses cannot be by chance and highlights the necessity of p53 and Rb-1 inactivation for continued cell proliferation and the bypass of M1.

The genetic events that occur in order to overcome M2 are less well characterised. Recent experiments have implicated the necessity for reactivation of the enzyme telomerase in immortal cells. Telomerase activity is absent in somatic cells and is only normally found in germline cells (Allsopp *et al.*, 1992) where its function is to maintain chromosome length (Greider *et al.*, 1990). In human cells transfected with SV40, the telomeres continue to shorten as in a normal cell even though the cell now has an extended lifespan (Counter *et al.*, 1992). When these cells pass through crisis telomere

Figure 1.2 ***Model of Human Cellular Immortalisation in a) SV40 Model b) SCC-HN***



shortening is arrested and the cells have the potential to become immortal (figure 1.2). Telomerase has indeed been shown to be re-activated in ovarian carcinoma cells in vivo (Counter *et al.*, 1994).

Genes other than Rb-1 and p53 whose inactivation is essential for immortalisation are continually being discovered. The first step in their discovery is by identifying areas of frequent loss on chromosomes suggesting the presence of a frequently inactivated tumour suppressor gene (section 1.4.1.).

Somatic cell genetics have also shown that when fibroblasts immortalised by SV40 virus are fused with normal fibroblasts, hybrids which senesce result (Pereira-Smith and Smith, 1981). These hybrids were shown to continue expression of SV40 T antigen in their nucleus. This is evidence for the presence of recessive tumour suppressor genes or senescence genes which are donated to the hybrid by the normal cells and which must also become inactivated for immortalisation to ensue. Micro-cell mediated chromosome transfer, where a chromosome induces a senescent phenotype when transferred into a cell line, is also currently being used to identify such genes.

Genes and chromosomal regions discovered to be important in immortalisation in SCC-HN are discussed further below and are fitted into the M1/M2 mechanism in figure 1.2.

1.3 The genetic analysis of immortalisation of human squamous cell carcinoma.

1.3.1. *ras*

Mutations in the *ras* gene at amino-acids 12 or 61 produce a protein that binds GTP and is constantly signalling for cell growth. Mutations in all three family members N-*ras*, K-*ras* and more rarely H-*ras* have been detected at different frequencies in different cancer types. It also appears that cancers have a preference for activation of only one of the *ras* proteins, for example 50% of colorectal carcinomas and large adenomas harbour a K-*ras* mutation (Vogelstein *et al.*, 1988). Experimental models have also shown that chemically induced mouse tumours exhibit H-*ras* mutations at an

early stage and are thought to be the initiating event in carcinogenesis (Balmain *et al.*, 1984).

In SCC-HN conflicting results have been obtained as to whether *ras* mutations are an important step in carcinogenesis of this tumour type. In India 35% of SCC-HN were found to have H-*ras* mutations (Saranath *et al.*, 1991). However the incidence of H-*ras* mutations in patients from the West were not detected (Clark *et al.*, 1993) or detected at an extremely low rate (Sheng *et al.*, 1990). This discrepancy may be explained by the exposure of the patients in India to different carcinogens (Chang *et al.*, 1991) or tumour promoters (Clark *et al.*, 1993). Although this has yet to be tested, the chewing of betel quid in India is a common habit that does not exist in the West and this may contain a carcinogen that causes *ras* mutations and gives these cells a selective advantage analagous to the mouse epidermal model (Clark *et al.*, 1993). Therefore it appears that *ras* mutations are not a common genetic event in the initiation or progression of SCC-HN although it is clear that when they occur such mutations can give a selective advantage to keratinocytes in SCC.

1.3.2. Epidermal growth factor receptor (EGFr).

The EGFr binds the ligands epidermal growth factor (EGF) (Savage *et al.*, 1972) and transforming growth factor-α (TGF-α) (DeLarco and Todaro, 1978) which activate the intracellular domain tyrosine kinase and promotes cellular growth. Studies have shown that the EGFr is frequently overexpressed in SCC-HN (Stanton *et al.*, 1994; Weichselbaum *et al.*, 1989; Cowley *et al.*, 1984 and 1986; and Ozanne *et al.*,1986). Inhibition of ligand binding to the EGFr by specific antibodies prevents growth of SCC in culture (Masui *et al.*, 1984) and therefore it has been suggested that overexpression of the EGFr may enable cells to respond to low levels of EGF and TGF-α and give them a selective growth advantage over their neighbours.

1.3.3 Cyclin D1

The human CCND1/cyclin D1 gene, also known as PRAD1, is found on chromosome 11q13 (D11S287) and has been identified as a G1 cyclin due to its induction in G1 by growth factor stimulation (Matsushime *et al.*, 1991; Won *et al.*, 1992). There are three members of the D-type cyclins, D1, D2 and D3 all of which have been shown to interact with Rb-1 (see section 1.4.2) (Dowdy *et al.*, 1993; Kato *et al.*, 1993). The D type cyclins bind Rb-1 together with their catalytic partners cdk-4 or cdk-6 (Matsushime et al., 1994; Meyerson and Harlow, 1994) causing Rb phosphorylation and allow cells to enter S phase (Kato *et al.*, 1993). These G1 cyclins are therefore putative proto-oncogenes as their overexpression would lead to deregulation of the cell cycle.

Deregulated expression of CCND1 was first identified in parathyroid adenomas (Arnold *et al.*, 1989) by a translocation event that had fused the DNA from D11S287 to the 5' regulatory region of the parathyroid hormone resulting in a dramatic increase in cyclin D1 expression. Chromosomal translocations of 11q13 have also been identified in B-cell lymphomas and leukemias (Tsujimoto *et al.*, 1985) and called BCL-1. CCND1 has since been identified as the gene in this BCL-1 region (Rosenberg *et al.*, 1991; Withers *et al.*, 1991) and CCND1 mRNA has been shown to be overexpressed in B-cell lines carrying this translocation. The chromosomal region of 11q13 has also been shown to be amplified in a variety of human tumours (Lammie and Peters, 1991) such as breast cancer (Fantl *et al.*, 1990), bladder cancer (Proctor *et al.*, 1991), SCC of the lung (Berenson *et al.*, 1990) and esophageal cancer (Jiang *et al.*, 1992). The cyclin D1 gene is also amplified in SCC-HN (Jiang *et al.*, 1992). A more recent study of the above SCC-HN cell lines (section 1.1.1.) has also shown a consistent increase in the level of cyclin D1expression (Nickolic *et al.*, submitted). This overexpression of cyclin D1 does not correlate with elevated levels of EGFr.

Cyclin D1 overexpression therefore appears to be an important event in progression of SCC-HN.

1.4 Involvement of tumour suppressor genes in SCC-HN.

1.4.1. Identification of potential tumour suppressor genes by amplification of microsatellites.

The use of microsatellite markers has become favoured over other methods for detecting loss of heterozygosity (LOH) on chromosomes e.g. restriction fragment length polymorphisms (RFLPs), because it is a faster method and as it is polymerase chain reaction (PCR) based it uses relatively small quantities of DNA. Microsatellite sequences are CA-GT repeats of unknown function. They are highly polymorphic in length and occur approximately every 30-60kb throughout the genome (Weber and May, 1989). Their frequency allows most regions of every chromosome to be examined in detail. They are inherited in a Mendelian fashion and therefore due to their differences in length, the paternal and maternal microsatellite sequences can be distinguished.

The sequences are amplified using PCR and are separated on a polyacrylamide gel. If the individual is heterozygous for a particular marker then both alleles will be seen (Weber and May, 1989). Any LOH can be identified by comparing amplified sequences from the tumour DNA with that from the same patients normal DNA. If however a patient is not heterozygous at a particular marker then only one allele will be seen and it is then impossible to identify any LOH. In this case the microsatellite is said to be uninformative and another has to be used. PCR allelotyping is not suitable for the detection of gene amplifications, small deletions or point mutation and therefore this method used in combination with one that does e.g comparative genomic hybridisation (Kallioniemi *et al.*, 1992) would be more sensitive.

Although identification of LOH does not confirm the existence of a gene involved in malignancy, repeated LOH at any locus in several different tumours is strong evidence supporting the involvement of loss of this gene in carcinogenesis. However a gene deletion could also be due to genomic instabilty and not an important genetic event in cancer. The comparison of various tumour stages and the identification of sequential events is more successful in identifying relevant genes. Identifying regions of LOH is not

sufficient to identify novel suppressor genes. These genes have to be cloned and their function and inactivation studied in more detail.

1.4.1.1. Identification of regions of LOH in SCC-HN

Loss of heterozygosity studies have been carried out on the above SCC-HN cell lines (Edington *et al.*, 1994; Loughran *et al.*, 1994 and by others) Frequent loss has been observed at chromosome 17p13 (Edington *et al.*, unpublished data) corresponding to the p53 gene and on further investigation nearly all immortal SCC-HN were found to have p53 missense mutations (Burns *et al.*, 1993). The relevance of inactivation of p53 in the progression of SCC-HN is discussed further in section 1.4.3. and by the results in this thesis.

Frequent LOH have been shown on chromosome 9. LOH between markers D9S171 and D9S157 on 9p21 was identified in 100% of immortal SCC-HN cell lines (Loughran *et al.*, 1994) but in none of the senescent cultures. Other reports have shown similar LOH at 9p21 in SCC-HN (Zhang *et al.*, 1994; Van der Riet *et al.*, 1994b; Nawroz *et al.*, 1994), melanoma (Holland *et al.*, 1994), bladder cancer (Cairns *et al.*, 1993) and malignant mesotheliomas (Cheng *et al.*, 1993). Further support for the importance of deletions in this region comes from experiments that introduce a normal chromosome 9 into mouse A9 cells by monochromosome transfer which showed that it was not tolerated without deletions between the markers IFN and D9S171 (Cuthbert et al., 1995).

Recently the Multiple Tumour Suppressor 1 (MTS-1) gene has been mapped to this region and found to encode the $p16^{INK4}$ gene (Kamb *et al.*, 1994; Nobori *et al.*, 1994; Serrano *et al.*, 1993). $p16^{INK4}$ is a specific inhibitor of cdk4 which is known to interact with cyclin D. Loss of $p16^{INK4}$ would therefore enhance the cyclin D-cdk4 kinase activity. This complex is known to phosphorylate and inactivate Rb-1 (see section 1.3.3) and therefore allow progression of the cell into S phase.

Frequent LOH has also been shown on chromosome 9q in SCC-HN. Several markers have shown high frequency of loss between 9q22.3-9q33 (between markers

D9S127and GSN) (Loughran *et al.*,1994). Other studies have also shown similar loss on 9q in SCC-HN (Ah-see *et al.*, 1994; Quinn *et al.*, 1994) and in basal cell carcinomas (Van der Riet *et al.*, 1994a) but as yet no gene has been identified.

Consistent losses have also been identified on chromosome 3 in immortal SCC-HN cultures (Loughran *et al.*, unpublished data) at 3p25-pter, 3p21 and 3p13-14. Similar losses have been reported in SCC-HN by others. Latif *et al.*, 1992 showed loss at 3p14-3p26; Maestro *et al.*, 1993 showed loss mapping to 3p24-ter, 3p21,3 and 3p14-cen and El-Naggar *et al.*, 1993 showed losses at 3p21 and 3p24 in SCC-HN. A putative tumour suppressor gene has been mapped to 3p21, namely the receptor protein-tyrosine phosphatase γ (LaForgia *et al.*, 1991) which functions by controlling the levels of phosphorylation in the cell cycle. Loss of one allele has been shown in renal and lung carcinomas but further investigations are required to study whether its loss plays any part in SCC-HN. Interestingly, introduction of normal chromosome 3 by microcell-mediated chromosome transfer into an ovarian carcinoma cell line induced senescence and growth arrest as well as suppression of tumorigenicity (Rimessi *et al.*, 1994). Tumours induced by chromosome 3 monochromosomic hybrids consistently showed loss in two regions 3p23-24.2 and 3p21.1-21.2. Taken together these results strongly suggest the presence of an as yet unidentified tumour suppressor gene on 3p that is inactivated in a variety of cancers including SCC-HN.

Further studies have recently shown that there is also LOH on chromosomes 1q42, 6q13-15, 7q and 4q in SCC-HN, but not on the X chromosome (Loughran *et al.*, In preparation). Interestingly, putative senescence genes have been proposed to reside on these chromosomes (chromosome 1, Sugawara *et al.*, 1990; chromosome 4, Ning *et al.*, 1991; chromosome 6, Volz *et al.*, 1994; chromosome 7, Ogata *et al.*, 1993 and chromosome X, Wang *et al.*, 1992) but it is not yet clear if these genes are involved in SCC-HN.

1.4.2. Rb-1

The functional inactivation of the retinoblastoma gene (RB-1) in many cancers suggests that it operates as a tumour suppressor gene and a negative regulator of cell growth (Weinberg *et al.*, 1991). Rb-1 protein is highly regulated during the cell cycle by phosphorylation (Buchkovich *et al.*, 1989; Chen *et al.*, 1989). The underphosphorylated form of Rb-1 is present in early G1 and prevents progression into S phase by interaction with growth promoting proteins via its pocket domain e.g. E2F (Chellappan *et al.*, 1991). Rb-1 becomes hyperphosphorylated by cyclin-cdk complexes such as cyclin E-cdk-2, or cyclin D-cdk4 (Kato *et al.*, 1993) in mid to late G1 and remains phosphorylated until the cells exit mitosis. This hyperphosphorylation has been shown to release proteins bound in the pocket domain and thereby allow entry into S phase. DNA tumour virus oncoproteins i.e. SV40 large T antigen (Decaprio *et al.*, 1988), adenovirus E1A (Whyte *et al.*, 1988) and human papilloma virus E7 (Dyson *et al.*, 1989) all prevent the growth suppressive effect of Rb-1 by binding to the underphosphorylated form in early G1 and displacing the bound growth promoting proteins.

Loss of heterozygosity on 13q has been identified in 50% of SCC from the esophagous (Boynton *et al.*, 1991; Huang *et al.*,1993). A high incidence of loss on 13q has also been identified in SCC-HN (Yoo *et al.*, 1994). This loss did not correlate with absence of Rb-1 protein suggesting that the loss seen in these studies is associated with another as yet unknown tumour suppressor gene which lies distal to the RB-1 gene. The Rb-1 protein also appeared normal in the series of SCC-HN cell lines described in section 1.1.1. (Edington *et al.*, 1994) displaying normal phosphorylation patterns and nuclear expression (Nikolic *et al.*, submitted). These cell lines were also shown not to be infected with HPV 16 or 18 and therefore Rb-1 has not been inactivated by the E7 oncoprotein (Edington *et al.*, 1994).

D-type cyclins have been shown to interact with Rb-1 protein (Dowdy *et al.*, 1993; Kato *et al.*, 1993) and as described in section 1.3.3. cyclin D1 is overexpressed in SCC-HN. There has also been shown to be LOH as the $p16^{INK4}$ locus in SCC-HN (section 1.4.1.1.) and this inactivation of $p16^{INK4}$ protein indirectly promotes

phosphorylation of RB-1 through continuous cyclin D activity. Therefore the functional inactivation of Rb-1 has not occurred through direct mutation of Rb-1 in SCC-HN but instead via overexpression of cyclin D1 and inactivation of $p16^{INK4}$.

1.4.3. p53

More mutations have been reported in the tumour suppressor gene p53 than in any other gene to date. Such mutations have arisen in a wide variety of cancers (reviewed by Caron de Fromentel and Soussi, 1992) and have outlined the importance of p53 inactivation in tumour progression.

1.4.3.1. p53 mutations in human cancer.

The most frequent method of p53 inactivation is by a missense mutation or deletion with concurrent loss of the remaining p53 allele. It has been shown that the wild-type p53 is phenotypically dominant to mutant p53 (Chen *et al.*, 1990) highlighting the necessity for inactivation of both alleles before malignancy can proceed. This is however not always the case and rare heterozygous mutations, as reported in this thesis, have been reported. It is believed that in these situations the mutation has not lead to a functionally inactive p53 protein but to one that can override the effect of the wild-type allele and has also possibly gained a function giving the cell a selective advantage.

Conflicting evidence has implied that mutation of p53 is both an early and late event in tumorigenesis depending on the type of cancer studied. In colon cancer 75% of carcinomas show loss of chromosome 17p in the region of the p53 gene (Vogelstein *et al.*, 1988) but such loss is relatively infrequent in adenomas. Similarly p53 mutations were found to be more common in carcinoma than adenoma (Baker *et al.*, 1989). Several groups have documented the occurrence of p53 mutations in astrocytomas. Ohgaki *et al.*, 1993, reported that 3 of 11 low grade astrocytomas harboured p53 mutations and a similar rate was also documented in grade II and III tumours (Von Deimling *et al.*, 1992). In another study one group of tumours showed that pairs of low

grade and recurrent tumours contained the same p53 mutation (Sidransky *et al.*, 1992). However another group of tumours in the same study showed that while at first appearance, progression to high grade was associated with a *de novo* p53 mutation, closer analysis revealed that a subpopulation of cells were present in the low grade tumour containing this same mutation, therefore supporting the idea that progression is associated with a clonal expansion of cells that have acquired a p53 mutation giving them a selective growth advantage. A third low grade tumour was shown to have a small population of cells with a heterozygous mutation and progression to a higher grade occurred by acquiring a different p53 mutation in the second allele. P53 mutations therefore seem to occur as both early and late events in astrocytomas.

Chronic myelogenous leukemia (CML) is a myeloproliferative disorder which progresses from an initial long chronic phase to an accelerated phase, eventually leading to a blast crisis when acute leukemia supervenes. In CML the p53 gene is not inactivated by a single point mutation as described in the previous cancers; instead a high occurrence of gross structural changes, rearrangements or deletions have been reported to occur in the late blastic phase (Feinstein *et al.*, 1991; Ahuja *et al.*, 1991 and Kelman *et al.*, 1989).

Some types of cancer arise because of obvious aetiological contributions from exogenous as well as endogenous mutagens. In hepatocellular carcinomas (HCC) for example, hepatitis B and aflatoxin B1, a potent liver carcinogen, are well known risk factors. Murakami *et al.*, 1991, found that p53 mutations were only present in advanced HCC. Studies also identified a mutational hotspot at codon 249, a G→T transversion shown to be caused by exposure to aflatoxin B1 (Hollstein *et al.*, 1991).

P53 mutations have been shown to occur as relatively early steps in non-small cell lung cancer (NSCLC) with G→T transversion also being a common event (Suzuki *et al.*, 1992). This mutation has been shown to correlate with exposure to carcinogens in tobacco such as benzopyrene (Hollstein *et al.*, 1991).

This thesis is concerned with the role that p53 mutations play in the progression of squamous cell carcinomas of the head and neck (SCCHN) and it has become clear from a variety of studies that this is a very common event. Work in our group has shown

that 33% of our human squamous cell carcinomas (SCC) and 50% of our cell lines derived from SCC overexpressed p53 protein. 80% of these cell lines were found to harbour p53 mutations (Burns *et al.*, 1993) and the remainder fail to express a normal level of p53 protein (Edington *et al.*, 1995) In this study we could not detect a correlation between tumour stage, p53 mutation or treatment history. Our group has also shown that p53 mutations detected in primary SCC of the tongue are also detected in the corresponding lymph node metastases indicating that keratinocytes harbouring these p53 mutations possess a selective advantage throughout SCC progression. Other studies showed similar results to ours (Gusterson *et al.*, 1991) and slightly higher cases of elevated p53 expression were reported by Field *et al.*,(1991) and Maestro *et al.*,(1992) of 67% and 60% respectively. In the case of SCC of the upper aerodigestive tract mutations were commonly G→A transitions or G→T transversions consistent with the known mutational spectra produced by cigarrette smoke (Burns *et al.*,1993; Maestro *et al.*, 1992 and Sakai *et al.*, 1992). SCC of the epidermis have also been reported to have a high incidence of p53 mutations (Brash *et al.*, 1991; Pierceall *et al.*, 1991 and Moles *et al.*, 1993). Mutations in these SCC were commonly CC→TT double base changes suggesting UV light to be the aetiological risk factor.

Inactivation of p53 not only occurs by genetic mutations but also by the interaction of viral oncoproteins. SV40 large T antigen, Adenovirus E1B and human papilloma virus (HPV) E6 oncoproteins have all been shown to sequester p53. The DNA of certain HPV types including HPV 16, 18, 31, 33 and 39 is found in 85% of cervical carcinomas (Riou *et al.*, 1990). These viruses are considered to be high risk because of their association with cancer and high grade intra-epithelial lesions. Low risk HPV types e.g. HPV 6 and 11 have a similar tissue specificity, yet a lower affinity for p53 and are associated primarily with benign lesions that are low risk for malignant progression. In primary HPV positive cervical carcinomas, the p53 gene is wild-type (Wrede *et al.*, 1991). E6 forms a protein complex with p53 and targets it for ubiquitin-mediated degradation (Scheffner *et al*, 1990). In HPV negative tumours p53 mutations are frequent (Srivistava *et al.*, 1992a; Crook *et al.*, 1992, and Kaebling *et al.*, 1992) and these tumours are associated with poor prognosis.

Families have been discovered with inherited autosomal dominant p53 mutations. This has been termed the Li-Fraumeni syndrome (LFS) after its discoverers (Li *et al.*, 1988) and families are defined as containing an individual (the proband) with a sarcoma diagnosed before 45 years, a first degree relative with cancer before 45 and with an additional first or second degree relative with cancer before 45 or a sarcoma at any age. LFS patients display diverse tumour types such as soft tissue sarcomas, breast carcinoma, brain tumours, osteosarcoma, leukemia and adrenocortical carcinoma. Such tumours develop at an unusually early age and multiple primary tumours are common.

Germline p53 mutations were first reported by Malkin *et al.*, 1990, all of which occurred in exon 7 and were located between codons 245-258. More recently this region has been extended (Law *et al.*, 1991; Malkin *et al.*, 1992; Birch *et al.*, 1994.) and a hotspot for mutation has emerged at codon 248. P53 is found in a heterozygous state in the normal cells of an LFS individual (Malkin *et al.*, 1990; Srivistava *et al.*, 1990 and 1992b) and the same mutant allele is retained in tumours with loss of the wild-type allele. This finding supports the two hit hypothesis for tumour progression (Knudson *et al.*, 1971) in that the loss of p53 heterozygosity constitutes the "second hit" in tumours arising in LFS individuals. Mutant p53 protein and RNA is expressed at a low level in normal fibroblasts from family members (Malkin *et al.*, 1990; Srivistava *et al.*, 1992b) and does not exert a trans-dominant effect on the wild-type p53 present (Milner and Medcalf, 1991) stressing the need for loss of wild-type p53 during tumour progression. Indeed the murine $p53^{245}$ mutant (corresponding to human $p53^{248}$ hotspot mutant) has been shown to retain most of the properties of wild-type p53 but was found to be transcriptionally inactive (Hao *et al.*, 1993) suggesting LFS mutations may be loss of function mutants.

It is also important to note that when normal fibroblasts from LFS individuals are placed in culture they develop changes in morphology, anchorage independant growth, chromosome abnormalities and escape senescence (Bischoff *et al.*, 1990a) suggesting that the cells are prone to spontaneous mutations that predispose them to immortalisation. As with LFS fibroblasts, fibroblasts from p53 null mice when placed in

culture also overcome senesence and become immortal (Harvey *et al.*, 1993; Tsukada *et al.*, 1993).

Kemp *et al.*, 1993 used p53 null and heterozygous mice to study the initiation, promotion and progression of carcinogenesis *in vivo* and to attempt to answer the question of whether p53 inactivation is an early, or initiating event in tumour development or a later event inducing progression. In this study mice treated with the chemical carcinogen DMBA followed by repeated treatments of the tumour promoter TPA developed well-differentiated benign skin papillomas. A proportion of such papillomas developed into SCC and further progression lead to a highly invasive spindle cell carcinoma. It was observed that papilloma yield was similar in wild-type and heterozygous mice but reduced in p53 null mice. In contrast, the rate of progression to carcinoma was increased in the null and heterozygous mice compared with the wild-type mice. The appearance of carcinomas in the heterozygous mice was associated with the loss of the wild-type allele. Therefore the loss of p53 does not increase the incidence or shorten the latency of appearance of papillomas but it does appear to greatly enhance the malignant progression. In this system then, p53 inactivation does not appear to act as an initiation event in carcinogenesis but allows the cells to progress towards a more malignant phenotype.

Asking the same question in a human *in vivo* study however suggested that p53 mutations were an early and maybe an initiating event (Nees *et al.*, 1993). This study identified p53 overexpression at epithelial sites distant from the primary SCC-HN tumour in cancer patients. Such overexpression was not due to a normal response to DNA damage and correlated with a p53 mutation, an increased proliferation rate and a dedifferentiated phenotype. Furthermore different p53 mutations were detected at different tumor-distant sites suggesting not a monoclonal but a multifocal polyclonal process occurs in SCC-HN development. These results strongly suggest that p53 inactivation maybe an early event in carcinogenesis and these cellular changes remain inconspicuous until other genetic events have accumulated either from genetic instability or by exposure to a carcinogen such as alcohol in the oral cavity. These conclusions are in contrast to the findings using the mouse model described above. In this system H-*ras*

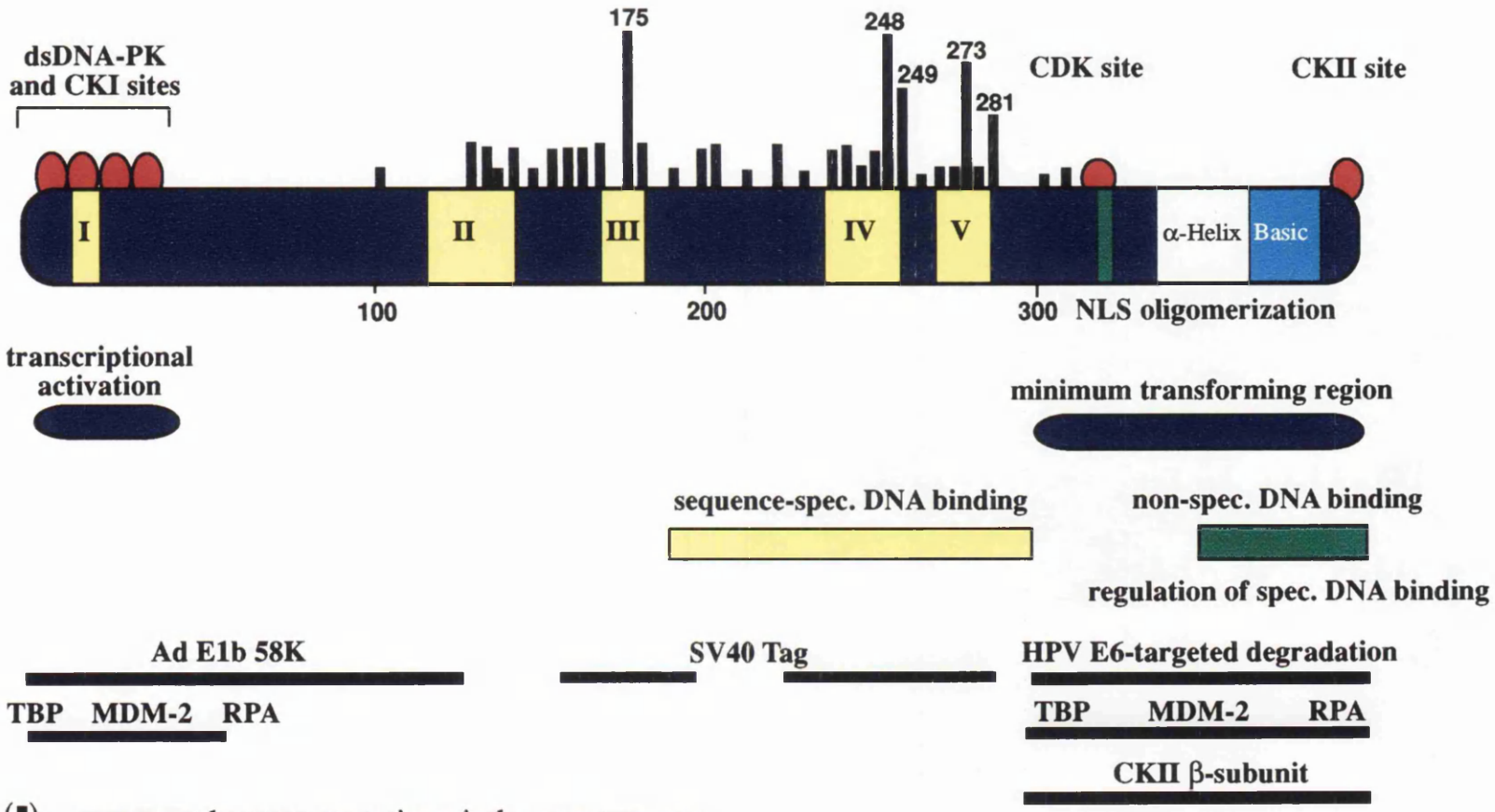
mutations have been identified as an initiating event (Balmain *et al.*, 1984) and as discussed in section 1.3.1. this has little relevance to SCC-HN except in the presence of a tumour promoter (Clark *et al.*, 1993). It would be interesting to ascertain whether the timing of a p53 mutation is dependent on the presence or absence of a tumour promoter and therefore whether in the above mouse model a p53 mutation has occurred earlier but lies dormant until other genetic events induced by exposure to the tumour promoter have occurred

As the progression to malignancy is a multistep pathway involving the activation of oncogenes and inactivation of tumour suppressor genes, it appears to not be important when p53 is inactivated, as this appears to be different depending on the system studied. It does however appear that its inactivation by whatever means is essential if carcinogenesis is to proceed.

1.4.3.2. Structure of p53.

The human p53 gene is found on chromosome 17p13.1 (McBride *et al.*, 1986) and this region is commonly deleted in tumours (Baker *et al.*, 1989, Nigro *et al.*, 1989) illustrating another mechanism for p53 inactivation. The gene is large (20kb in length) and is comprised of eleven exons , the first of which is non-coding and separated from the rest by a large intron (10kb). The p53 gene has been conserved through evolution and comparison of sequences from man, rat, mouse, frog, chicken and bony fish has identified five highly conserved domains (Soussi *et al.*, 1990) among amino acid residues 13-19, 117-142, 171-181, 234-258 and 270-286 (I-V illustrated in figure 1.3). A comprehensive study of mutations found in various tumour types, as discussed in the previous section, has shown that the majority of p53 mutations occur in four of these conserved regions (II-V) indicating that these areas must be important functional areas of the p53 protein (Caron de Fromentel and Soussi, 1992). Several amino acid hotspots for mutations have also been identified as shown in figure 1.3.

Figure 1.3 **Structural Domains of Human p53**



(▌) represent hotspot mutations in human tumours

phosphorylation sites

I-V conserved domains

NLS nuclear localization signals

The amino-terminal region of p53 (residues 1-73) when fused to Gal 4 DNA binding domains has been shown to activate the transcription of a reporter construct *in vitro* (Farmer *et al.*, 1992) and in mammalian cells (Fields and Jang, 1990; Raycroft *et al.*, 1990; Kern *et al.*,1992). Various p53 mutants however were shown to be transcriptionally inactive or temperature sensitive in this assay (Raycroft 1991., Unger *et al.*, 1992) suggesting that they are loss of function mutants or conformationally altered.

The central region of the p53 protein is very hydrophobic and proteolytic digestion of p53 has revealed a protease resistant fragment (residues 102-292) which is capable of binding DNA (Bargonetti *et al.*, 1993; Pavletich *et al.*, 1993). This DNA binding has been shown to be sequence specific and p53 consensus binding sites have been identified by a variety of groups. Bargonetti *et al.*, 1991, first showed that p53 recognises a GC-rich domain (**gggCGG**) near the origin of SV40 DNA replication. Another binding site was identified (Kern *et al.*, 1991) in the ribosomal gene cluster (RGC) sequence (**TGCCTTGCCTggactTGCCTggcctTGCCT**) and binding was shown to be disrupted by guanine methylation (capitals represent essential nucleotides for p53 binding). A palindromic p53 binding sequence (**ggaCaTGcccgggCATGtc**) was isolated (Funk *et al.*, 1992) by amplification of DNA bound to p53. By a similar method a different consensus binding site for p53 has been established (El-Deiry *et al.*, 1992) consisting of two copies of a 10 bp motif **PuPuPuC(A/T)(T/A)GPyPyPy** separated by 0-13 base pairs. The heterogeneity of these binding sequences outlines the complex nature by which p53 binds DNA in a sequence specific manner and hinders the finding of genes activated specifically by p53 (discussed further later).

The p53 protein has been shown to form homotetramers (Stenger *et al.*,1992; Friedman *et al.*,1993). The region involved in this oligomerization (see figure 1.3) has been identified as an amphipathic alpha helix followed by a stretch of basic amino-acids at the carboxy-terminus of the protein (Sturzbecher *et al.*, 1992). Use of the yeast two hybrid system showed that the smallest region in this fragment sufficient for oligomerisation is amino-acids 331-393 (Iwabuchi *et al.*, 1993) and this has recently been shortened to residues 319-360 (Sakamoto *et al.*, 1994). A 3D analysis of this oligomerisation domain (residues 319-360) by NMR spectroscopy (Clore *et al.*, 1994)

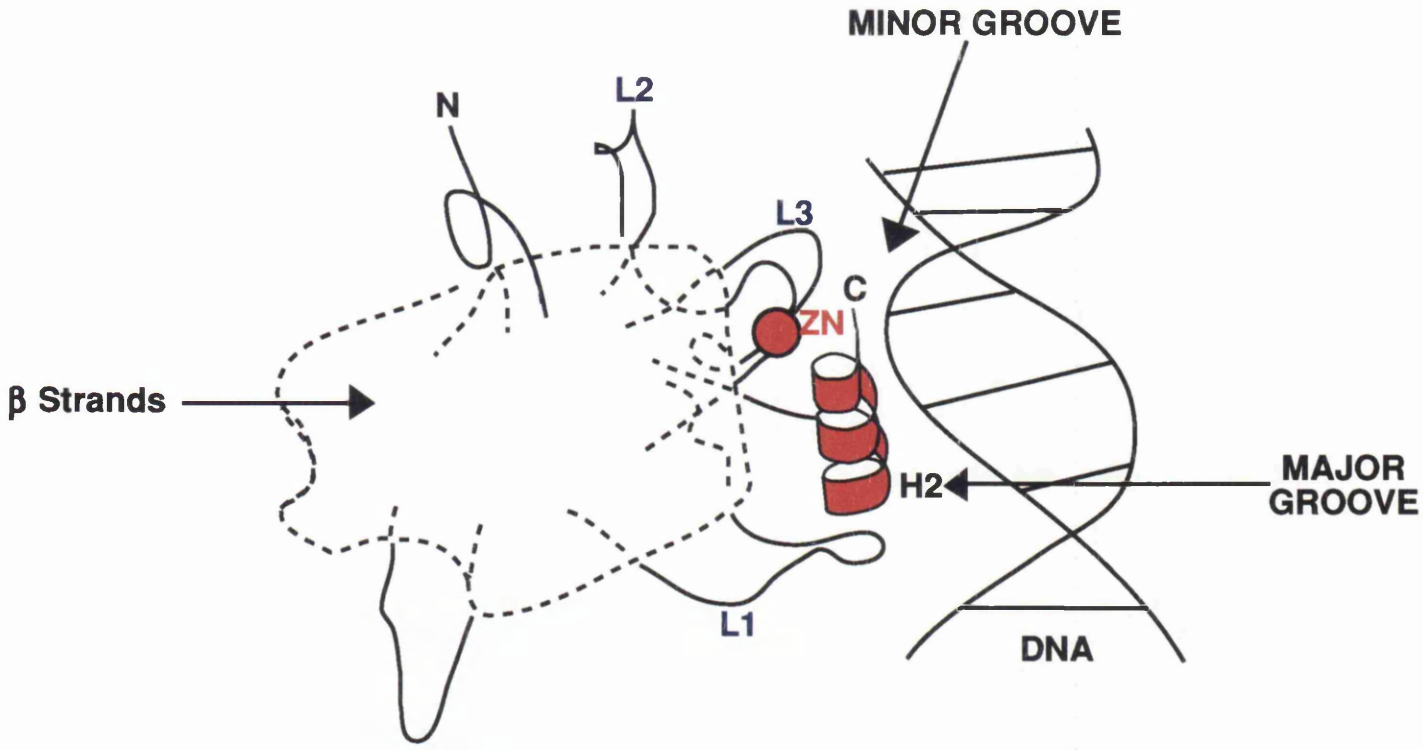
showed that it formed a symmetric tetramer made up of a dimer of dimers. Each monomeric unit contains a β strand and an α helix. Each subunit interacts with another subunit such that the helices and β strands are antiparallel.

The carboxy terminal region of p53 (residues 330-393) which is highly basic appears to be involved in non-specific DNA binding (Pavletich *et al.*, 1993; Wang *et al.*, 1993) and together with the oligomerization domain this region may facilitate the correct binding to DNA.

The conformation of p53 appears to be disrupted by metal chelating agents (Pavletich *et al.*, 1993), an organic mercurial reagent which targets cysteinyl residues and low concentrations of soft metals such as mercury or cadmium (Hainaut and Milner, 1993) suggesting that metal ions, most probably zinc, bind to cysteinyl residues stabilizing the tertiary structure.

Recent determination of the 3D structure of p53 has helped understand how the above functional domains of p53 spatially fits together and has interestingly identified the physical relevance of the hotspot mutations discussed previously (see figure 1.4). The crystal structure of a complex containing the core domain of human p53 and a DNA binding site (El-Deiry *et al.*, 1992) has been determined by Cho *et al.*, 1994. The core domain structure (see figure 1.4) consists of a β sandwich that serves as a scaffold for two large loops and a loop sheet helix motif (LSH) The two loops (L2 and L3) are indeed held together in part by a tetrahedrally coordinated zinc atom, the ligands for which are Cys^{176}, His^{179} on L2 and Cys^{238}, Cys^{242} on L3. It has been revealed that the structures involved directly in DNA binding correspond to the conserved regions of the core domain and contain the mutational hotspots. The L1 loop and β hairpin that follows it correspond to conserved region II (residues 117-142) and the H2 helix motif corresponds to region V (residues 270-286). These two structures make contacts with the major groove of the DNA (figure 1.4). Minor groove contacts in the AT rich region of the binding site are made by L3 which corresponds to conserved region IV (residues 234-258). L2 at the end of the β sandwich does not directly interact with DNA but is involved in extensive interactions with L3 and it coincides with region III (residues 163-195). Mutations are most frequent in L3 (30% of mutations in tumours), the LSH motif

Figure 1.4 **Structure of the p53 Monomer Core Domain**



A model of the p53 core as it binds to DNA.
The α helix (H2), three loops (L1, L2, L3) and Zinc atom (ZN) are labelled and described in the text. The β strands are represented as a block for simplicity.

L1 corresponds to conserved region II; L2 to region III, L3 to region IV and H2 to region V.

(25%) and L2 (17%). Six hotspot mutations are known Arg^{248}, Arg^{273}, Arg^{175}, Gly^{245}, Arg^{249}, Arg^{282} and two of these Arg^{248} and Arg^{273} which have the highest mutational rate directly contact the DNA The other four residues appear to play a critical role stabilizing the structure of the DNA binding surface of p53. The β sandwich is not a frequent target for mutation but some are found in the hydrophobic core closest to the DNA binding surface.

The conformation of the p53 protein can be identified by a variety of antibodies. PAb 1620 recognises the wild-type p53 protein whilst PAb240 recognises a so called "mutant" form of p53 and these are frequently used in immunocytochemical assays to detect mutant p53. The epitope for PAb240 (residues 212-217) (Stephen and Lane, 1992) has been shown in this crystal structure to lie in the hydrophobic core and is therefore inaccessible to an antibody. In order for the antibody to bind, the protein has to unfold and therefore this antibody recognises more a denatured form of p53 than a "mutant" form. Two classes of mutant have been identified by this study. Class I (e.g. Arg^{273}) mutants which do not bind hsc70 are $PAb240^{-}/1620^{+}$, yield a stable core upon proteolytic digestion and fail to bind DNA but still transactivate reporter constructs (Zhang *et al.*,1993; Chen *et al.*, 1993). Class II mutants (e.g.Arg^{175}) which are unfolded are $PAb240^{+}/1620^{-}$, bind hsc70, are highly sensitive to proteolysis and do not transactivate.

The p53 protein is a nuclear protein and three nuclear localisation signals are clustered at the carboxy terminus (Shaulsky *et al.*, 1990) as shown in figure 1.3. NLSI is the major nuclear localisation signal and is conserved between species. NLSII and III are less effective singularly and efficient nuclear localisation requires all three signals.

The p53 protein is extensively post-transcriptionally modified, mostly by phosphorylation. This enables the cell to control p53's many possible roles. Most known phosphorylation sites are at the N-terminus (see figure 1.3) where DNA-PK (double-stranded DNA activated protein kinase) phosphorylates human p53 at serines 15 and 37 and mouse p53 at serines 4 and 15 (Lees-Miller *et al.*, 1992) and 7, 9, 18 and 37 (Wang *et al.*, 1992). Mutation of Ser-15 to Ala has been shown to partially block the ability of p53 to arrest cell cycle progression at G1/S and also leads to stabilisation of the

protein. Therefore phosphorylation at this site may contribute to the activation of p53 as a growth suppressor (Fiscella *et al.*, 1993). Casein kinase I (CKI) has also been shown to phosphorylate the N-terminal region at serines 4, 6 and 9 in mouse p53 (Milne *et al.*, 1992a). A DNA repair-defective mutation in *Saccharomyces cervisiae* is linked to a protein kinase similar to CKI offering support for a link between DNA damage, p53 and phosphorylation (discussed in section 1.4.3.6.). A third kinase, MAP (mitogen-activated protein) kinase has been shown to phosphorylate mouse p53 at threonine 73 and 86 (Milne *et al.*, 1994). It has been shown that MAP kinase can be activated by exposure of cells to UV radiation, possibly suggesting that regulation of gene transcription following UV damage may be mediated through the MAP kinase pathway.

The C-terminal DNA binding domain is phosphorylated *in vitro* at Ser-315 in humans and at the corresponding Ser-309 in mice by both the cyclin A and cyclin B associated forms of $p34^{cdc2}$ (Bischoff *et al.*, 1990b; Sturzbecher *et al.*, 1990) suggesting that p53 is regulated by cell cycle progression. Indeed p53 is known to become highly phosphorylated upon entry into S phase suggesting that in fact a G1/S cyclin dependant kinase (which has as yet not been identified) may actually be the kinase that phosphorylates p53 *in vivo*. P53 is known to be a substrate for two isoforms of Protein kinase C (PKC II and III) but as yet no sites have been defined although they are thought to lie within the C-terminal regulatory domain. Casein kinase II (CKII) targets many nuclear proteins involved in growth regulation e.g. jun, myc, max and also the transforming proteins of the DNA tumour viruses Adenovirus E1A, HPV E7 and SV40 large T antigen. It is constitutively active but also stimulated by growth factors and is therefore an important player in regulating growth. CKII has been shown to phosphorylate mouse p53 at Ser-389 and human p53 at Ser-392 (Meek *et al*, 1990). Binding by CKII and coincident phosphorylation activates the specific DNA binding function of p53 (see figure1.3). Hupp *et al.*, 1992 has shown that this activation can be mimicked by other agents including deletion of the C-terminal 30 amino-acids, and by binding of dnaK (the bacterial homologue of hsp70) or the monoclonal antibody PAb421. It is thought that these interactions may cause a conformational shift in the C-terminus of p53 thereby exposing and activating a cryptic DNA binding activity.

Mutation of this CKII phosphorylation site in mouse (Milne *et al.*, 1992b) abolishes the growth suppressor activity of p53 in SV3T3 cells. However, apparently contradictory results showed that mutation of Ser-392 on human p53 produced a mutant that was indistinguishable from wild-type p53 (Fiscella *et al.*, 1994) concluding that phosphorylation of Ser-392 was not necessary for wild-type functions *in vivo*. A possible explanation for this difference maybe that mouse and human p53 differ in their phosphorylation requirements and proper regulation may only occur on the appropriate cellular background. One other possible function of p53 not yet investigated maybe to target the CKII protein to specific intracellular locations. Phosphorylation may then release the enzyme after p53 has bound to specific DNA sites.

1.4.3.3. Wild-type p53 is anti-proliferative.

Early experiments showed that in conjunction with activated c-Ha-*ras*, wild-type p53 could transform early passage rodent cells (Eliyahu *et al.*, 1984, Jenkins *et al.*, 1984 and Parada *et al.*, 1984). However as more cDNA clones were sequenced it became apparent that these experiments were carried out using mutant p53's. Wild-type p53 was unable to cooperate with *ras* and furthermore wild-type p53 actually inhibited the transformation induced by other oncogenes and *ras* (Eliyahu *et al.*, 1989 and Hinds *et al.*, 1989). Introduction of wild-type p53 into colon (Baker *et al.*, 1990) and bone tumour cell lines (Chen *et al.*, 1990 and Diller *et al.*, 1990) confirmed that overexpression of wild-type p53 was antiproliferative. When a wild-type p53 under the control of an inducible promoter was expressed in a human glioblastoma cell line expressing an endogenous mutant p53, the cells were induced to growth arrest in G1 (Mercer *et al.*, 1990). These experiments were elegantly confirmed by using a mouse temperature sensitive p53 ($p53^{val135}$) which behaves as a mutant at 37.5-39.5°C and as a wild-type at 32°C. Rat embryo fibroblasts were found to be transformed by this mutant and an activated *ras* gene at 37°C, but were growth arrested in G1 when shifted to 32°C (Michalovitz *et al.*, 1990; Ginsberg *et al.*, 1991; Martinez *et al.*, 1991). P53

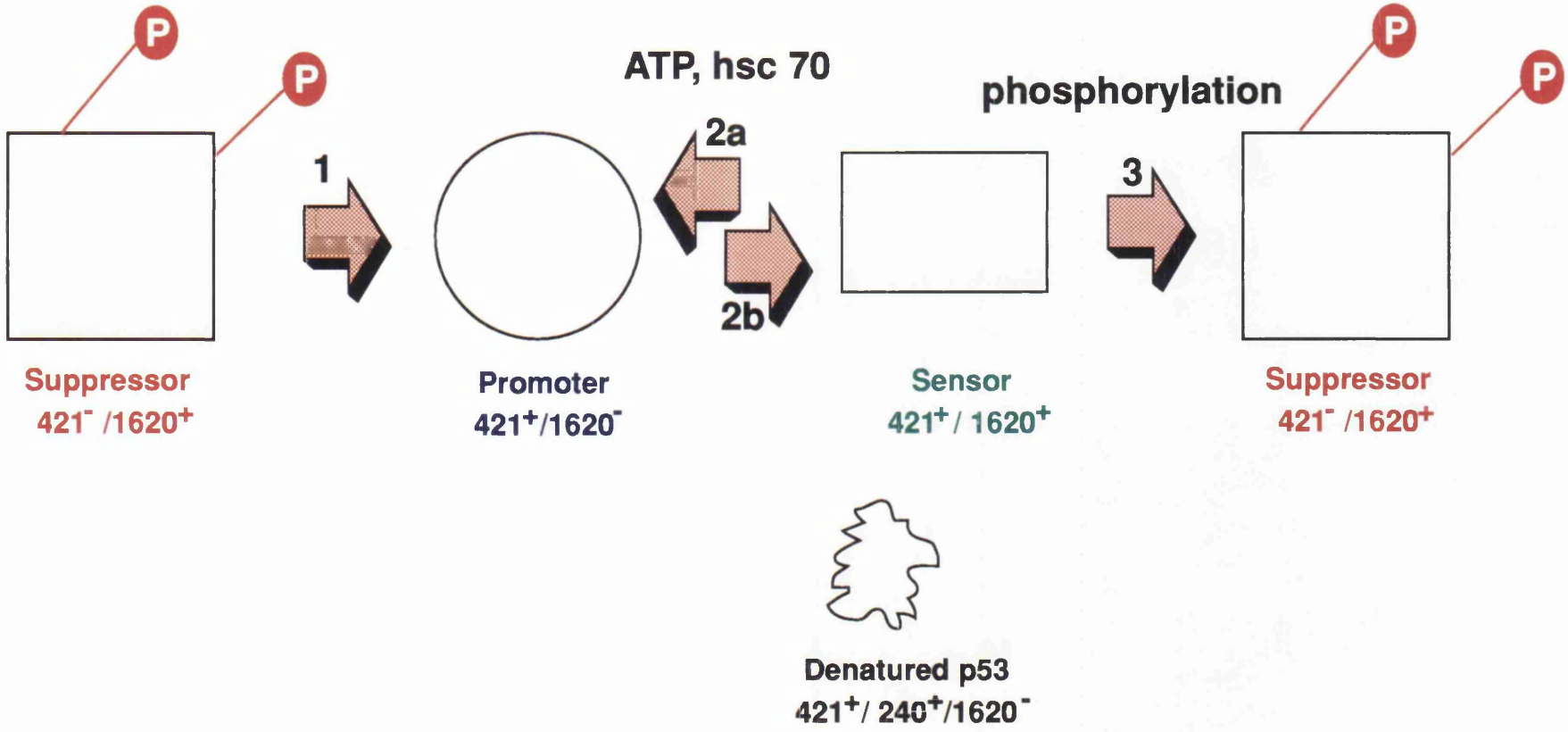
was therefore termed as negative regulator of cell growth and classed as a tumour suppressor gene.

Both p53 mRNA and protein levels are low in normal mouse 3T3 fibroblasts growth arrested in G0 by serum starvation (Reich *et al.*, 1984), or in G1 by isoleucine depletion (Steinmeyer *et al.*, 1990), in resting diploid fibroblasts (Mercer *et al.*, 1984) and in resting peripheral T lymphocytes (Calbretta *et al.*, 1986). When cells are stimulated to enter the cell cycle the level of p53 mRNA and rate of protein synthesis increases reaching a peak near the G1/S boundary just before the onset of DNA replication. Normal p53 protein has a short half-life of 5-20 minutes but this is dependent on the cell type;for example the p53 protein in normal human keratinocytes has a half-life of 3-5 hours (Hubbert *et al.*, 1992 and Delmolino *et al.*, 1993). Many p53 mutations give rise to a highly stable protein with an increased half life. This stabilised protein,unlike the rapidly turned over wild-type form,can be detected by immunohistochemical techniques and this is frequently used as an early and easy indicator for the presence of a mutation.

As well as its antiproliferative function many investigators believe that p53 can also promote growth. It is believed that these opposing functions can be achieved by alternating conformations of the p53 protein (Reviewed by Milner 1994 and Ullrich *et al.*, 1992). It has been observed that p53 can adopt at least three different forms as determined by antibody reactivity. One form suppresses cell growth and maintains quiescence, a second form activates and promotes cell proliferation whilst a third form allows ongoing cell proliferation (see figure 1.5) and acts as a sensor ready to switch in response to positive or negative growth regulatory signals.

As described in the previous section modification of the carboxy terminus of p53 has been shown to be a negative regulator for DNA binding (Hupp *et al.*, 1992). The carboxy terminus was shown to be important during growth suppression by its loss of reactivity with the antibody PAb421 (Ullrich *et al.*, 1992) which recognises an epitope at the carboxy terminus. This loss of reactivity was accompanied by increased phosphorylation possibly by CKII. When cultured in serum free medium quiescent primary lymphocytes continually synthesize the p53 421^{-} form (Milner *et al.*, 1984).

Figure 1.5 **Conformational forms of p53**



Upon growth simulation of quiescent cells, p53 converts from suppressor to promoter (step 1). In proliferating cells p53 is in the sensor form. On growth simulation it changes to the promoter form (step 2a) which requires oxidizing agents. Reverting back to the sensor form (step 2b) requires ATP, reducing conditions and hsc 70 (which is proposed to aid this conformational change). On suppression of growth, p53 converts to the suppressor form (step 3) which requires phosphorylation. Denatured or mutated p53 loses 1620 reactivity and the 240 epitope is exposed.

When these cells were exposed to mitogens there was a conformational switch in synthesis from 421^- to 421^+reactive forms of p53 (figure 1.5). Evidence for the requirement of p53 during mitogenic activation came form early experiments which are frequently overlooked. The micro-injection of anti-p53 antibodies (PAb122) into nuclei of serum starved fibroblasts, early (2-3 hours) after re-stimulation with serum, prevented the entry of the cells into S phase (Mercer *et al.*, 1982). Injection any later and cell cycle progression was not interrupted. The epitope for the antibody PAb122 overlaps with that of PAb421 and therefore this experiment recognises the presence of the 421+ form.

Therefore as shown in figure 1.5 p53 421^- is functional in cell growth suppression and in the maintenance of quiescence. On mitogenic stimulation p53 switches to a 421^+ form which plays a positive role in the early cell growth response. (Step 1). There is also conformation switching of p53 recognised by a different antibody PAb1620. The form of wild-type p53 most commonly detected in proliferating cells is the $421^+/1620^+$ and this form is called the sensor form awaiting modification in order to promote or suppress growth. The above form associated with mitogenic commitment is indistinguishable from the conformation $421^+/1620^-$ which promotes cell growth. In order to function as a suppressor the sensor form looses 421 reactivity and is phosphorylated at its carboxy terminus (step 3) $421^-/1620^+$. This 1620^+ conformation is a prerequisite for DNA binding. Stabilisation of p53 in the sensor form is believed to be dependant on the presence of metal ions (Hainaut and Milner, 1993) . Oxidizing and chelating agents reversibly disrupt the tertiary structure in favour of the promoter form 1620^- (step 2b). Recovery to the sensor form is energy dependant and may require the interaction with hsc70 and reducing agents (step 2a).

Heat shock proteins are a ubiquitous group of proteins often present at low levels in normal cells. Upon physiological stress, the synthesis of these proteins is dramatically increased. Hsc 70 (74kDa) was first shown to bind to mutant but not wild-type p53 (Hinds *et al.*, 1987). Hsc 70 complexes with dimers and possibly monomers of p53 and requires the terminal 28 amino-acids of p53 for this interaction. Using the temperature sensitive mutant $p53^{val135}$, Hainaut and Milner, 1992 showed that hsc70 binds to the 1620^- conformation and is released when p53 is induced to re-fold into the 1620^+

conformation. This observation implies that hsc 70 is involved in regulation of p53 conformation and its ultimate function.

1.4.3.4. Molecular properties of p53 mutants.

Unlike wild-type p53, mutant p53 has lost its growth suppressive properties. (Baker *et al.*, 1990; Chen *et al.*, 1990; Frebourg *et al.*, 1992). Studies have shown that several mutants have lost this normal p53 function by their inability to transactivate genes either by losing their capacity to bind sequence specific DNA (Bargonetti *et al.*, 1992; Zhang *et al.*, 1993) or by loss of their transcriptional activity (Raycroft *et al.*, 1991; Scharer and Iggo, 1992). However, mutational analysis has shown that not all p53 mutants are purely loss of function mutants. Several tumours have been shown to carry heterozygous p53 mutations and in these cases it has been proposed that the mutant p53 inactivates the wild-type function in a trans-dominant negative fashion. In contrast to wild-type p53, mutant $p53^{143}$ when transfected into primary rat embryo fibroblasts can cooperate with *ras* to cause transformation of the cells in culture (Hinds *et al.*, 1990; Slingerland *et al.*, 1993) and this has also been shown for other p53 mutants (Finlay *et al.*, 1988; Halevy *et al.*, 1990). In order to achieve this the mutant p53 must be able to inactivate the endogenous wild-type p53. Sun *et al.*, 1993 showed that the dominant negative effect of mutant p53 is dosage dependant. Wild-type and mutant $p53^{thr280}$ were cotransfected into Saos-2 cells and tested for their ability to transactivate a reporter construct. As more mutant p53 was transcribed the level of transcription decreased and complete inhibition was achieved at a ratio of one wild-type to three mutant p53 molecules. This dose-dependant dominant negative effect was also seen for loss of growth suppression. Expressing the same mutant p53 into JB6 promotion resistant P^-cells that contained endogenous wild-type p53, promoted growth of cells in soft agar in the presence of TPA (Sun *et al.*, 1993). This dominance of the mutant p53 over the endogenous wild-type p53 was also shown to be dose-dependant. Further studies have shown that this effect is only seen in situations where mutant and wild-type p53 proteins are co-translated and does not occur simply by mixing (Milner

and Metcalf, 1991). Other dominant negative mutants identified are $p53^{ser151}$, $p53^{Ile247}$, and $p53^{pro273}$

Wild-type p53 is known to exist as homotetramers and it has been proposed that mutant p53 acts in this dominant negative fashion by either forming mutant homotetramers or wild-type/mutant heterotetramers with one, two or three mutant p53 molecules. Such complexes are thought to have a lower affinity for DNA binding or adopt a conformation with reduced transcriptional activity. This dominant-negative inhibition of wild-type p53 increases as more mutant p53's form the tetramer. Recently the *in vivo* existence of dominant negative mutant p53's has been questioned. In the above transfection experiments mutant p53 is expressed at much higher levels than the endogenous wild-type p53 and therefore may not be physiologically relevant. Frebourg *et al.*, 1994 elegantly used a bicistronic vector to co-express equal amounts of wild-type and several different mutant p53's into Saos-2 cells. It was shown in this case that mutant p53 did not inhibit the transcriptional or growth suppressive activities of wild-type p53.

When mutant p53 alleles are introduced into non-tumorigenic cell lines such as the L12 Abelson murine leukemia virus p53 nonproducer cell line, the tumorigenic potential of these cells is enhanced (Wolf *et al.*, 1984). In such cases the mutant p53 has not acted in a dominant negative manner and therefore seems to have gained a function. The ability of mutants to gain a function was confirmed using the murine fibroblast (10)3 cell line which is devoid of endogenous wild-type p53 (Dittmer *et al.*, 1993). Introduction of p53 mutants 143ala, 175his, 248trp and 273his increased the tumorigenicity of this cell line and furthermore these mutants were shown to stimulate expression from the enhancer promoter region of the MDR gene. These mutant p53's therefore appear to not only have lost their wild-type p53 properties but have also gained a function enabling them to support cellular growth in the absence of endogenous wild-type p53. The p53 mutants 175his and 273his when co-transfected with wild-type p53 were unable to bind to a p53 specific DNA binding region suggesting that these mutants have inactivated the wild-type p53 activity in a dominant negative manner (Bargonetti *et al.*, 1992). In the same experiment the Li-Fraumeni mutant $p53^{248trp}$ was unable to

inactivate the wild-type p53 protein and the complex displayed nearly wild-type levels of DNA binding. Other assays showed the same mutant had lost its growth suppressive property (Frebourg *et al.*, 1992) and was unable to activate transcription (Scharer and Iggo, 1992). This mutant is expressed in a heterozygous state in the normal cells of Li-fraumeni patients (Malkin *et al.*, 1990) and is tolerated because it cannot abolish wild-type p53 activity in a dominant negative manner (Milner and Metcalf, 1991). Instead this mutant appears to predispose the cell to transformation by selecting for loss of the wild-type p53 allele and acquiring a gain of function (Dittmer *et al.*, 1993) that acts to promote transformation in the absence of the wild-type p53 allele.

These observations suggest that the phenotype of mutant p53 is variable depending on the particular mutation and several mechanisms have been identified by which each mutant p53 can overcome the wild-type p53 properties and promote transformation.

1.4.3.5. Molecular functions of wild-type p53

How p53 actually exerts its anti-proliferative effect is currently being investigated in a huge number of laboratories. Two hypotheses have been suggested. The first implicating a direct interaction of p53 on the DNA replication machinery and therefore having a direct effect on the cells DNA replication. The second suggests that p53 may also/ or alternatively regulate directly the expression of other genes involved in negative growth regulation or generally by interacting with transcription factors.

Co-transfection of p53 and plasmids with the SV40 origin of replication into T antigen expressing Cos1 cells inhibited the replication of these plasmids (Braithwaite *et al.*, 1987; Friedman *et al.*, 1990). *In vitro* experiments have shown that murine wild-type p53 can block the binding of DNA polymerase α to the large T antigen (Gannon and Lane, 1987) and also inhibit its helicase activity (Wang *et al.*, 1989) whereas mutant p53 could do neither. Therefore from these experiments it could be envisaged that p53 may directly affect the assembly and function of DNA replication complexes during S

phase. This inhibition was, however, shown to require equimolar concentrations of large T and p53. In a situation (*in vivo*) where large T antigen is in an excess to wild-type p53 (i.e. primary rhesus kidney cells), it was found that wild-type p53 exerted no inhibitory effect on SV40 DNA replication as compared to a mutant p53 that was deficient in binding large T antigen (Von der Weth and Deppert, 1993) Therefore in an *in vitro* system where wild-type p53 is not at physiological concentrations, SV40 DNA replication is probably inhibited by the complexing of all the T antigen to p53, a situation never encountered *in vivo*. Also when cells expressing a temperature-sensitive mutant p53 were released from the temperature block they continued to the next cycle rather than stopping in S phase even when wild-type p53 was expressed (Martinez *et al.*, 1991). If p53 interacts with DNA polymerase it would be expected to act more like a DNA synthesis inhibitor (e.g. aphicolin) which when added to cells, abruptly arrests them in S phase (Mercer *et al.*, 1991).

More recently p53 has been shown to physically interact with and inhibit a cellular DNA replication factor, the ssDNA binding protein complex, RPA (Dutta *et al.*, 1993). RPA is the first cellular factor recruited to the initiation complex and its ability to bind to ssDNA is essential for the unwinding of DNA at the origin of replication. Interaction of p53 with RPA inhibits the ability of RPA to bind to ssDNA *in vitro*. However mutant p53 has also been shown to bind and abolish RPA binding to ssDNA and the *in vivo* relevance of this has yet to be investigated.

The structure of the p53 protein as discussed above has all the hallmarks of a transcription factor. The p53 protein has been shown to regulate the transcription of a variety of genes containing p53 responsive elements. The promoter for the hsc70 protein, shown above to regulate the conformation of p53, is repressed by wild-type p53 (1620^+ form) therefore providing a feedback loop whereby hsc70 is only expressed when the 1620^- form is more abundant. It is believed that this repression may occur via direct interaction of p53 with CBF, the CCAAT binding factor (Agoff *et al.*, 1993). Wild-type p53 has also been shown to repress the promoters of c-*fos* (Kley *et al.*, 1992), IL-6, β-actin and MHC (Santhanam *et al.*, 1991) *in vitro*. As no sequence specific p53 binding

sites have been identified in any of these genes, repression by wild-type p53 may be mediated by its ability to bind to TBP, the TATA box binding subunit of the RNA polymerase II general initiation factor TFIID (Truant *et al.*, 1993; Chen *et al.*, 1993; Mack *et al.*, 1993). However, only monomeric p53 has been shown to bind to TBP although most of p53 *in vivo* exists as tetramers and this interaction is relatively weak. It is possible that what is observed is a consequence of p53 overexpression and has little relevance *in vivo*. It may in fact be that overexpression of p53 in these systems sequesters TBP therefore preventing its use in any specific transcription and a general downregulation in transcription is seen as a consequence. The fact, however, that p53 binds TBP at all is consistent with its role as a transcriptional activator, as it has been suggested that this association is a general characteristic of transactivators (Truant *et al.*, 1993).

Several other target genes for p53 have been identified by the presence of a p53 specific DNA binding site. One such gene GADD45 (see below) has a p53 responsive element located in its third intron and has been shown to be induced by wild-type p53 (Kastan *et al.*, 1992). Another genomic p53 responsive element has been identified in the murine muscle-specific creatine kinase (MCK) gene (Weintraub *et al.*, 1991). This element has been shown to closely match the consensus binding site for p53 (El-Deiry *et al.*, 1992) and is activated *in vitro* by wild-type but not mutant p53. A p53 responsive element has also been identified in the first intron of the gene Thy 1. The biological relevance of p53 regulation of these two genes, MCK and Thy 1, is not yet clear but it is of interest to note that both genes are expressed in differentiation pathways (MCK in muscle differentiation and Thy 1 in T lymphocytes and the brain) and may highlight a possible role for p53 in differentiation (see section 1.4.3.8.).

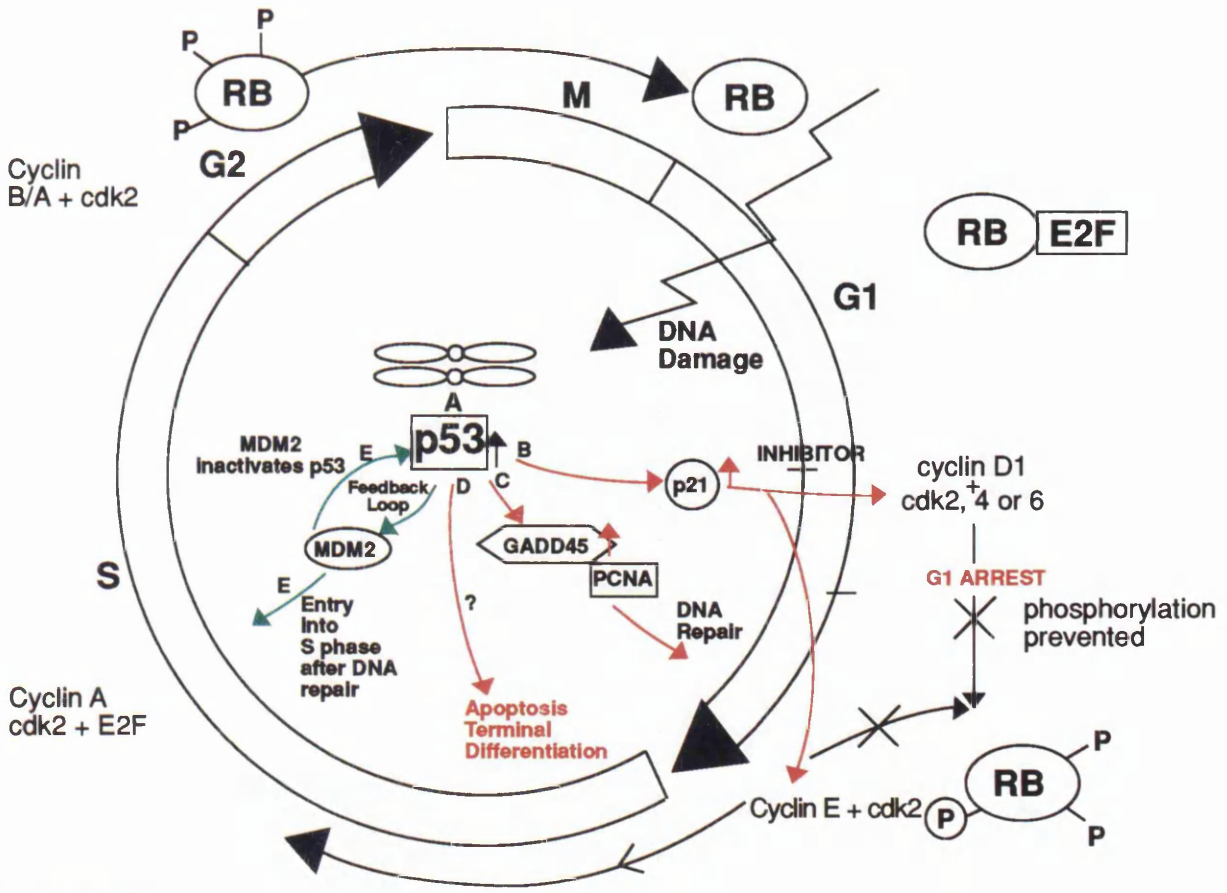
1.4.3.6. p53 recognises DNA damage.

Studies now indicate that p53 may play a role in the cellular response to DNA-damaging agents that specifically induce strand breaks such as actinomycin D, X-rays and γ-irradiation. Irradiation of cells rapidly increases the levels of p53 (Maltzman and

Cyzyk, 1984; Kastan *et al.*, 1991a; Kuerbitz *et al.*, 1992) and initiates a G1 arrest (figure 1.6). This growth arrest may allow cells time to undergo necessary repair to damaged DNA before proceeding to DNA synthesis. Failure to repair damaged DNA may result in point mutations or deletions being incorporated into the genome during DNA replication and division. Cells lacking wild-type p53 or expressing mutant p53 do not undergo radiation induced G1 arrest (Kastan *et al.*, 1991a; Kuerbitz *et al.*, 1992) nor do cells immortalised with the HPV16 E6 oncoprotein (Kessis *et al.*, 1993). The mechanism by which wild-type p53 causes G1 arrest may be by its DNA binding activity as this has been shown to increase in response to even very low levels of radiation (Price and Calderwood, 1993; Lu and Lane, 1993). One such target for p53 is the GADD45 gene (growth arrest and DNA damage inducible protein) which is turned on in cells exposed to stresses that cause DNA damage (figure 1.6). The cell's ability to turn on this protein is dependant on the presence of wild-type p53 (Kastan *et al.*, 1992). GADD45 has been shown to bind to PCNA (proliferating cell nuclear antigen) which is a necessary component of the DNA replication machinery (Smith *et al.*, 1994). PCNA is also needed for the re-synthesis of DNA after damaged portions are removed by the cells nucleotide excision repair system. Removal of GADD45 decreases this excision repair.

The ability of wild-type p53 to apparently monitor genome integrity may be a mechanism by which cells prevent gene amplifications. Whilst undetectable in normal diploid fibroblasts, gene amplification occurs at a high frequency in transformed cells (10^{-3}-10^{-5}). This ability to amplify is a recessive genetic trait (Tlsty *et al.*, 1992) suggesting normal cells contain gene/s that modulate the ability to amplify. By using the resistance to the drug PALA, via the amplification of the CAD gene, to measure gene amplification, it has been shown that cells lacking endogenous p53 show detectable levels of gene amplification (10^{-5}-10^{-4}) and fail to arrest in G1 (Livingstone *et al.*, 1992; Yin *et al.*, 1992). In contrast, cells homozygous for wild-type p53 or LFS cells heterozygous for p53 showed no gene amplification and arrested in the presence of PALA. Transfection of wild-type p53 into tumour cells containing mutant p53 restored G1 arrest and reduced the frequency of gene amplifications (Yin *et al.*, 1992). Although some human tumorigenic cells show detectable gene amplification despite the presence

Figure 1.6



p53 pathways involved in cell cycle regulation

Upon DNA damage p53 levels increase in G1 (A). p53 induces p21 (B) and cells are arrested by preventing cyclin D1 or E phoshorylating Rb and promoting entry into S phase. p53 also transactivates GADD45 (C) which promotes DNA repair. If the DNA is irreparable p53 induces apoptosis or terminal differentiation (D).

If DNA is repaired, p53 stimulates MDM2 transcription which acts via a feedback loop to inhibit p53, releasing cells from the G1 arrest and allowing entry into S phase (E).

of wild-type p53 these observations suggest that p53 can prevent cells becoming aneuploid by monitoring and preventing gene amplifications.

The upregulation of p53 in response to DNA damage, the subsequent growth arrest and activation of the DNA repair machinery by p53 seems to therefore be an important safety step in preventing the cells from accummulating genetic alterations which would result in mutations, aneuploidy and the progression of malignant clones (figure 1.6). If the DNA damage is too severe for the cell to repair, it has been suggested that p53 may trigger cell suicide (see below).

1.4.3.7. The control of apoptotic cell death by p53.

Initial experiments to study the effect of overexpression of wild-type p53 failed due to loss of viability of the cell caused by p53. Experiments where p53 was conditionally expressed under an inducible promoter, or was temperature sensitive, proved to be more successful and in certain cell types showed p53 to be involved in controlled cell death or apoptosis rather than a reversible growth arrest. Myeloid leukaemic M1 cells transfected with the temperature sensitive $p53^{135}$ lost viability rapidly at 32°C when the protein is in the wild-type form (Yonish-Rouach *et al.*, 1991). This process of cell death had the hallmarks of apoptosis, such as chromatin condensation, nuclear fragmentation and DNA laddering. Overexpression of wild-type p53 was also shown to elicit apoptosis in other cell types. Wild-type p53 was transfected into the human colon derived cell line EB under the control of the metallothionein promoter (Shaw *et al.*, 1992). Upon induction of wild-type p53 with $ZnCl_2$, the cells developed morphological features of apoptosis. This cell line was also used to form tumours in nude mice which underwent regression upon induction of wild-type p53. These regressing tumours showed features of apoptosis occurring *in vivo*. However these studies only demonstrated p53 linked apoptosis in arteficial situations where wild-type p53 is forcibly overexpressed . The development of p53 null mice showed direct evidence that p53 was involved in any real apoptotic process. Although the viability and normal development of p53 deficient mice indicated that p53 is not essential for apoptotic processes during

development. A closer examination of thymocytes from such mice showed that they are resistant to the induction of apoptosis by radiation unlike normal controls (Lowe *et al.*, 1993a; Clarke *et al.*, 1993). This agreed with the observations in the previous section that p53 is required for a response to DNA damage whether it is growth arrest or apoptosis.

There are also other pathways that induce apoptosis completely independantly of p53. For example M1 cells will undergo apoptosis without any functional p53 upon serum starvation (Yonish-Rouach *et al.*, 1993) and thymocytes from p53 null mice undergo normal apoptosis in response to glucocorticoids and dexamethasone (Lowe *et al.*, 1993a; Clarke *et al.*, 1993).

Signals other than DNA damage have been suggested to trigger p53-dependant apoptosis, namely the withdrawal of survival factors from the cells enviroment and also cellular response to viral infection. The IL-3 dependent murine lymphoma cell line DA-1 which expresses wild-type p53 dies rapidly upon withdrawal of IL-3 (Gottlieb *et al.*, 1994). However when these cells were infected with retroviruses expressing either a p53 miniprotein encompassing the C-terminus of the protein only, or the $p53^{135}$ temperature-sensitive mutant (both of which act on the wild-type p53 in a dominant-negative manner), the cells showed extended survival without IL-3. Also excess wild-type p53 activity failed to elicit apoptosis as long as IL-3 was present. Wild-type p53 was also required for apoptosis on withdrawal of haemopoietic growth factors from AML blasts. In the presence of antisense p53 oligonucleotides, however, apoptosis was suppressed despite the absence of growth factors (Zhu *et al.*, 1994). These findings therefore suggest that p53 plays a role in mediating the dependence of cells on hematopoietic survival factors at least. However, unlike the p53 response to DNA-damage, the upregulation of p53 is probably not the trigger for apoptosis on survival factor withdrawal as excess wild-type p53 activity failed to elicit any apoptotic response as long as IL-3 was available (Gottlieb *et al.*, 1994). It is probably more likely that the presence of p53 provides the necessary pathway for the apoptotic response and loss of this activity allows cell survival without the appropriate survival factors signals facilitating the survival of neoplastic cell clones.

Apoptotic death in response to viral infection would be an effective host defense mechanism for early protection against an infection. Infection of rodent fibroblasts with adenovirus E1A protein causes the cells to upregulate p53 in response and to lose viability by apoptosis (Lowe *et al.*, 1993b). The virus however appears to counteract this defense by expressing another early protein E1B. E1B binds and inactivates p53 thereby preventing p53-dependant death of the infected cell. The expression of a dominant-negative mutant p53 inhibits this E1A-mediated cell death by blocking the action of wild-type p53 (Debbas *et al.*, 1993). In the same way as adenoviruses other viruses have evolved mechanisms for inactivating p53 i.e. HPV E6 and SV40 large T antigen all bind and inactivate p53 thereby aiding cellular transformation by the virus.

1.4.3.8. Does p53 play a role in normal cellular differentiation ?

In vitro and *in vivo* experiments have suggested that wild-type p53 plays an important role in normal development and in several differentiation pathways. The investigation of the normal function of p53 is complicated by the fact that p53 is expressed at low levels in the cell and is obviously tightly regulated. The expression of wild-type p53 from an exogenous promoter leads to high levels of p53 and tends to result in either growth arrest or apoptosis as described in the previous sections. However, when p53 was transfected into a p53 non-producer early pre-B cell line (L12), stable cell lines were produced (Shaulsky *et al.*, 1991) in which the effects of p53 could be studied. These cell lines showed an altered cell cycle in which more cells were found in the G0/G1 phase. When injected into syngeneic mice they induced a lower incidence of tumours which were less aggressive than the parental controls. Interestingly, the cell lines had also appeared to progress to a more differentiated phenotype with expression of the cytoplasmic immunoglobulin μ heavy chain and increased levels of the B cell specific surface marker B220. A separate study showed that treatment of a pre-B cell line that had retained expression of wild-type p53 (70Z/3) with a differentiation agent such as LPS resulted in the expression of the rearranged κ light chain gene. This differentiation step was preceded by increased expression of p53 (Aloni-Grinstein *et al.*, 1993).

Furthermore introduction of mutant p53 interfered with this differentiation process. It can be concluded from these findings that wild-type p53 is involved in the normal maturation and differentiation of pre-B cells. This was further substantiated by showing increased transcription from a CAT gene driven by a p53 promoter upon differentiation of the 70Z/3 cell line. Furthermore, wild-type p53 was shown to transactivate the promoter control sequences of the κ light chain gene (Aloni-Grinstein *et al.*, 1993).

Other investigators have shown a similar involvement of p53 in differentiation. Induced differentiation of ML-1 cells showed increased levels of p53. Low levels of p53 protein were also detected in the nonproliferative mature lymphoid, granulocytic and monocytic cell lineages but not in the more immature proliferating counterparts (Kastan *et al.*, 1991b). An acute phase CML cell line (K562) was induced to express 50 fold more haemoglobin upon transfection with wild-type p53 (Feinstein *et al.*, 1992). Infection of primary human foreskin keratinocytes with a retrovirus encoding wild-type p53 induced premature cell flattening and increased involucrin expression, a marker of terminal differentiation, although other differentiation proteins were not affected (Woodworth *et al.*, 1993).

Taken together all these experiments suggest that p53 plays a vital role in the decision to embark on a terminal differentiation programme. It is interesting to note that the B cell differentiation pathway involves several events of DNA rearrangements. During this process it is likely that repair to the DNA will be required due to strand breakage and faulty pairing of DNA fragments. This provides a connection to the observation that p53 is required for repair to DNA damage (as discussed above) and it is likely that the trigger for p53 intervention is identical in both cases.

Very little is known of the function of p53 during development. The viability of mice null for p53 suggests that p53 is redundant in this process and is only required for repair later in life. However such p53 null mice develop tumours by the age of six months and the most frequent are of a lymphoma type (Donehower *et al.*, 1992; Jacks *et al.*, 1994). As pointed out above development of the lymphoid lineage involves gene rearrangements and further supports the role for p53 in this development pathway.

In order to examine the normal expression pattern of p53 during development, transgenic mice were used that carried a construct in which p53 promoter sequences in amplified copy number regulated the expression of the CAT gene. Such mice were found to express CAT activity predominantly in the testes (Almon *et al.*, 1993). In situ hybridization indicated that the p53 gene is expressed during spermatogenesis and is confined to the tetraploid primary spermatocytes at the meiotic pachytene stage during the first round of spermatogenesis (Schwartz *et al.*, 1993). Primary spermatocytes double their DNA contents immediately after the last mitotic division and embark on a long meiotic prophase before two further meiotic divisions of the tetraploid spermatocytes generates four haploid spermatids. The pachytene stage is the longest meiotic phase which involves cell replication not coupled with DNA duplication. The expression of p53 at this stage and the observations that p53 can arrest DNA replication (discussed above) suggests that p53 functions in spermatogenesis by halting DNA replication and allowing successful meiosis which involves pairing of chromosomes, recombination and repair of DNA to be completed. The conclusion that p53 plays a role in halting DNA replication and allowing DNA repair is further substantiated by the observation that transgenic mice carrying the multiple p53 promoter CAT transgenes, exhibit the giant cell degenerative syndrome (Rotter *et al.*, 1993). These giant cells arise due to the inability of tetraploid spermatocytes to undergo meiosis generating haploid sperm cells. Instead these cells undergo additional DNA replications giving rise to multinucleated giant cells. Such cells are shown to have a reduction in the endogenous level of p53 brought about by a squelching effect whereby the extra promoter binding sites lead to reduced activity and therefore the pachytene stage which requires p53 malfunctions. P53 null mice however do not develop such giant cells and it is generally assumed that in the case of mice null for p53 and also other vital genes, for example Myo-D (Rudnicki *et al.*, 1992), which appear to develop normally, that these pathways have become functionally redundant and other parallel pathways are utilised.

Investigations into the functions of p53 in normal development and differentiation have uncovered an interesting connection between the already established roles of p53 in inhibition of DNA replication and DNA repair triggered by strand breaks. It is therefore

likely that p53 plays an important role in many cellular pathways in which such events are vital.

1.4.2.9. Identification of p53 associated proteins.

The MDM-2 gene was originally identified by virtue of its amplification in a spontaneously transformed derivative of mouse BalB/c cells (3T3DM) (Fakharzadeh *et al.*, 1991) and was found to bind to p53 in rat cells transfected with p53 (Hinds *et al.*, 1990; Momand *et al.*, 1992). Human MDM-2 was also found to bind to both wild-type and mutant human p53 *in vitro*. The human MDM-2 gene was localised to chromosome 12q13-14 and this gene was amplified in over a third of sarcomas studied (Oliner *et al.*, 1992) offering another mechanism by which the cell can overcome p53 regulated growth control.

MDM-2 is a putative transcription factor and has been shown to inhibit the transactivating abililty of p53 (Momand *et al.*, 1992) by binding to a region coinciding with the N-terminal p53 acidic activation domain (Oliner *et al.*, 1993; Picksley *et al.*, 1994). The MDM-2 protein has a short half-life of approximately 20 minutes and is localised to the nucleus. In resting cells stimulated with serum, MDM-2 levels and MDM-2/p53 levels increase in late G1 (Olson *et al.*, 1993) and as MDM-2 negatively regulates p53, the binding of MDM-2 and p53 may signal entry into S phase.

Irradiation of mammalian cells with UV light, as discussed previously, results in a dose dependant accumulation of p53 within 2 hours. There is also a dramatic increase in p53 specific transcriptional transactivation activity and an increase in expression of MDM-2 (Perry *et al.*, 1993). However this UV stimulated MDM-2 expression does not directly correlate with the level of stimulated p53 protein. MDM-2 induction is delayed, occurring along with the recovery of normal rates of DNA synthesis presumably after DNA repair, whereas p53 levels rise immediately. Cells in which p53 is mutated or deleted have been shown not to respond to UV light by increasing their expression of MDM-2 (Price *et al.*, 1994). Wild-type p53 has been shown to bind sequence specifically to DNA residing downstream of exon 1 on the MDM-2 gene and activates

transcription (Juven *et al.*, 1993). Taken together these experiments suggest that MDM-2 is part of a p53 negative feedback loop as shown in figure 1.6. On exposure to UV irradiation, p53 protein levels increase and the cell becomes arrested in G1. Once the DNA damage is repaired p53 stimulates expression of MDM-2 which in turn binds and inactivates p53 allowing the cells to continue into S phase.

Activation of MDM-2 by p53 involves the promoter (P_2) located within the first intron of the murine MDM-2 gene This promoter gives rise to distinct transcripts which lack the entire first exon and a few nucleotides from the second exon of murine MDM-2 (Barak *et al*, 1994). P_2 activation is strongly dependant on p53 both *in vitro* and *in vivo*. In comparison,the transcription from the upstream, constitutive MDM-2 promoter (P1) is only mildly, if at all, induced by wild-type p53. The sequence of these two MDM-2 transcripts varies in their 5' non-coding regions but suggests that they should encode identical products. However,in vitro translation gives rise to distinct proteins varying in their p53 binding abilities. This therefore implies that p53,through activation of an alternative promoter,can potentially modulate the amount and nature of the MDM-2 proteins.

MDM-2 may not modulate p53 function merely by binding to p53. Otto *et al.*, 1993, showed that after expression of wild-type p53 into Meth A tumour cells a few cells survived that expressed wild-type p53. These cells were also shown to overexpress MDM-2 mRNA and protein but the majority of MDM-2 and p53 were not in a complex together. A possible explanation yet to be substantiated is that MDM-2 as a transcription factor itself may regulate the transcription of other growth promoting or p53 inhibiting genes.

In an effort to identify wild-type p53 responsive genes one has recently been identified independantly by a number of groups. El-Deiry *et al.*, 1993 described a wild-type p53 induced gene, wild-type p53 activated fragment (WAF-1). WAF-1 was shown to be induced by wild-type but not mutant p53 and can itself suppress tumour cell growth in culture. The gene is localised on chromosome 6p21.2 and encodes a 21kDa protein with a structure suggesting it to be a potential transcription factor. At the same time an

identical 21kDa protein, CIP-1 was identified when looking for potential cdk2 regulators (Harper *et al*, 1993) and also published by other investigators as p21 (Xiong *et al.*, 1993). CIP-1/p21 was shown to be a potent tight-binding inhibitor of cdks and inhibits the phosphorylation of Rb by cyclin A-cdk2, cyclin E-cdk2, cyclin D1-cdk4 and cyclin D2-cdk4 complexes (figure 1.6). CIP-1/p21 mRNA is ubiquitously expressed in all adult human tissues and levels do not appear to vary throughout the cell cycle. In cells lacking functional p53, CIP-1/p21 is absent from cyclin-cdk complexes and mRNA levels in fibroblasts derived from null p53 mice showed 50 fold lower levels than normal embryo fibroblasts,agreeing with other groups that this protein is regulated by the p53 pathway. Rapid induction of CIP-1/p21 has been shown to occur by treating a variety of cells with differentiating agents such as TPA, IL-6 and G-CSF (Steinman *et al.*, 1994). Differentiating cells have a prolonged G1 arrest and Rb is hypophosphorylated suggesting that such agents may function by inducing p21 expression which causes growth arrest in response to p53 and inhibits the cyclin-cdk complexes from phosphorylating Rb and progression of the cell cycle.

This same protein was identified by two other groups whilst looking for a melanoma differentiating gene (mda-6) (Jiang *et al.*, 1994) and as an inhibitor of DNA synthesis expressed at high levels in senescent non-dividing human cells (SDI) (Johnson *et al.*, 1994). Mda-6 was shown to be induced in terminally differentiating human melanoma cells and also in HL60 cells within 1-3 hours of treatment with TPA, retinoic acid or DMSO.

As HL60 cells and other cells shown to induce p21 on differentiation are null for p53, and p53 levels are low in senecent cells,it has been suggested that there are possibly two kinetic profiles for the induction of mda-6/SDI/p21/CIP-1/WAF-1. The first is p53 dependant occurring early enough to cause cell cycle arrest. The second method is independant of p53 and may occur during the normal course of terminal differentiation.

1.5. Aims

The initial aim of this thesis was to identify the genetic differences between cell lines SCC12B and SCC12F which could be responsible for their different phenotypes. Such genetic alterations could ultimately be important in the progression of squamous cell carcinomas of the head and neck. A novel heterozygous p53 mutation at codon 216, substituting a glycine for a valine, was detected in SCC12B and SCC12F. On closer examination, the two cell lines appear to express different mutant and wild-type p53 gene dosages. The $p53^{216}$ mutant protein was characterised at a biological and molecular level to distinguish any different properties from wild-type protein.

The subsequent aims of this thesis were then to study the affect of this mutant p53 on keratinocyte terminal differentiation by expressing the mutant in a clone of SCC12F that expressed little endogenous p53 protein in order to assess directly whether it could affect the progression of SCC12F to the less differentiated and more tumorigenic phenotype displayed by SCC12B.

Chapter 2

Materials and Methods

2.1. MATERIALS

2.1.1. Tissue Culture

Cell Lines

Saos	ATCC HTB
Swiss 3T3 feeders	ATCC CCL92
3T3 M1 Neo	Beatson Institute, R9 stocks. Produced by infection of Lesch-Nyhan human diploid fibroblast strain 5-BR with the amphotropic retrovirus pmos^{-3} neo (Berry *et al*, 1994)
SCC12F.AC3	Rheinwald and Beckett, 1981
SCC12B	Rheinwald and Beckett, 1981
SCC4	Rheinwald and Beckett, 1981
SVK14	Dr. D. Lane.
HFF 9	Beatson Institute, R9 stocks.
BICR3	Dr. E.K. Parkinson.

Aldrich Chemical Company, U.K.

Methylcellulose
Poly(2-hydroxyethylmethacrylate) (Polyhaem A)

Beatson Institute Central Services.

Amphotericin B
Penicillin
Streptomycin

Becton Dickinson U.K. Ltd.

Sterile plastic flasks and plates

Boehringer-Mannheim, Germany

DOTAP lipofection reagent

BDH Analar, U.K.

High vacuum silicone grease

Clonetics, U.S.A.

Bovine pituitary extract

Costar Corporation, U.S.A.

Transwells

Flow Laboratories, U.K.

Mycoplasma removal agent

Donor calf serum

Gibco Europe Life Technologies Ltd., U.K.

Dulbecco's modified Eagles medium

Epidermal growth factor (recombinant)

Fetal calf serum

Glutamine

G418 Sulphate

Keratinocyte SFM

Sodium bicarbonate

Sodium pyruvate

Harlan Olac Ltd., U.K.

MF-1 NuNu mice

Hughes and Hughes Ltd., U.K.

Histomount

Nitta Gelatin Inc., Japan.

Cellmatrix

Nunc, Denmark.

Cryotubes

Chamber slides (glass and permanox)

Sigma Chemical Company Ltd., U.K.

Cholera toxin

Hydrocortisone

Rhodamine B

Surgipath Medical Industries Inc., U.S.A.

Eosin

Harris' Haematoxylin

Scotts tap water

Unipath Ltd., U.K.

Phosphate buffered saline (PBS)

Worthington Biochemical Company, U.K.

Trypsin

2.1.2. Immunocytochemistry

Antibodies

p53 antibodies PAb1620, PAb421, PAb240, CM-1 were a gift from Prof. D. Lane when stated. p53 antibodies PAb1801, PAb1620, PAb240 were obtained from Oncogene Science when stated.

Rabbit anti-involucrin antibody was a gift from Dr. F. Watt.

BDH Analar, U.K.

Aquamount

Formaldehyde

Chance Propper Ltd., U.K.

Coverslips

Sigma Chemical Company Ltd., U.K.

Acrydine Orange

Bovine serum albumin fraction V

Diaminobenzine tablets

Hydrogen peroxide

Tween 80

Vector Laboratories, U.K.

Alkaline phosphate substrate kit 1 (red)

Vectastain ABC peroxidase and phosphatase kits

Vectashield mountant for fluorescence staining

2.1.3. Protein Biochemistry

Antibodies

Cyclin D antibody 287-3 was a gift from Dr. G. Peters.
p53 antibodies as above.

Amersham International plc., U.K.

Anti rabbit Ig, horseradish peroxidase linked F(ab')2 fragment (from donkey)
Anti mouse Ig, horseradish peroxidase linked whole ab (from sheep)
ECL western detection agent
Rainbow colour markers (14,300-200,000 Da)

BDH, Analar, U.K.

40% Acrylogel 3 solution (Acrylamide 29.1 : 0.9 N N'Methylene bisacrylamide) used for protein gels.
Ammonium peroxodisulphate (APS)

Bio-rad Laboratories Ltd., U.K.

Protein Assay DC

Oncogene Science Inc., U.S.A.

Protein G plus agarose
p53 antibodys PAb1801, PAb1620 and PAb240 used when stated.

Millipore Corporation, U.S.A.

Immobolin P nitrocellulose

Sigma chemical Company Ltd., U.K.

Aprotinin
Bromophenol blue
β-mercaptoethanol
Hydrogen peroxide
Leupeptin
Nonidet P-40 (NP40)
Phenylmethylsulfonyl fluoride (PMSF)
Ponceau's solution
Sodium Fluoride
TEMED (N,N,N',N'-tetramethylethylenediamine)
Xylene cyanol

2.1.4. Molecular Biology

Vectors

pCMV-Neo, pC53-SN3 and pC53-SCX3 were all a kind gift from Dr. B. Vogelstein.

Aldrich Chemical Company, U.K.

Ammonium chloride
Potassium carbonate

Amersham International PLC, U.K.

^{35}S dCTPαS 400Ci/mmol
α ^{32}P dCTP 3000Ci/mmol
γ ^{32}P dATP
Hybond nylon filter

Beatson Institute Central services

L-Broth

Beta Laboratories, U.K.

Yeast Extract

Bethesda Research Laboratories, U.S.A.

LMP agarose
Φ X 174 Hae III-digested DNA
Agarose
Protease K
1kb Ladder

BDH Analar, U.K.

Acetone
Butan-1-ol
Chloroform
Repelcote silicone treatment
Sodium dodecyl sulphate (SDS)
Sodium dihydrogen phosphate

Bibby-Sterilin Ltd., U.K.

Sterilin bacteriological plates

Bio 101, Inc., U.K.

Geneclean 2® kit

Biolabs, New England, U.S.A.

Bio-rad Laboratories Ltd., U.K.

Muta-gene® M13 *In vitro* mutagenesis kit, version 2

Boehringer-Mannheim, Germany.

Caesium chloride

DNAse-free RNAse

IPTG (Isopropyl-β- D- thiogalactopyranosid)

Protease K

Tris-HCL

Tris base

Xgal (5-bromo-4-chloro-3-indolyl-β-D galactopyranosid)

Cinna/Biotecx Laboratories Inc., U.S.A.

RNAzol B

Cruachem Ltd., U.S.A.

Oligonucleotide purification cartridges

Triethylamine Acetate (TEAA)

Trifluoroacetic acid (TFA)

DIFCO Laboratories, U.S.A.

Agar

Bactotryptone

Bactoagar

Dynal Ltd., U.K.

Dynabeads® M-280 Streptavidin

Dynal MPC®

Eastman Kodak Company, U.S.A.

X-OMAT AR X-ray film

X-OMAT S X-ray film

DUP1 duplicating

Flowgen Instruments Ltd., U.K.

Nusieve® agarose

Fluka Chemika-Biochemika AG, Switzerland

Tetramethyl ammonium chloride (TMAC)

Fisons Scientific Equipment, U.K.

Acetic acid
EDTA (sodium salt)
Isopropanol
Magnesium sulphate
Methanol
Sodium acetate
Sodium chloride
Sodium citrate
Sodium hydroxide
Urea

Gibco Europe Life Technologies Ltd., U.K.

E.coli DH5α competent cells
Bam H1
Eco R1
T4 DNA ligase

James Burrough Ltd., U.K.

Ethanol

Lab-Scan Ltd., Ireland, U.K.

Acetonitrile

Northumbria Biologicals Ltd., U.K.

Alkaline phosphatase
Pvu II
T4 polynucleotide kinase
Xba 1

Perkin-Elmer Cetus, U.S.A.

GeneAmp® DNA PCR kit
GeneAmp® RNA PCR kit

GeneAmp® thin walled reaction tubes
AmpliTaq DNA polymerase

Pharmacia AB, Sweden

Ficoll 400
Sephadex G-25 NICK columns
dNTP set

Rathburn Chemicals Ltd., U.K.

Phenol

Severn Biotech Ltd., U.K.

30% (w/v) acrylamide : 0.8% bisacrylamide

Sigma Chemical Company, U.K.

Ampicillin
Ammonium chloride
Bovine serum albumin (fraction V)
Chloramphenicol
Ethidium Bromide
Lysozyme
Polyvinylpyrrolidone
Thiamine

Technical Photo Systems, U.K.

Fuji RX medical X-ray film

United States Biochemical Corporation, U.S.A.

Sequenase® version 2 kit

Whatman International Ltd., U.K.

Whatman 3MM filter paper

2.1.5. Cytogenetics.

BDH, Analar, U.K.

Methanol

Boehringer-Mannheim, Germany.

Sheep antidigoxigenin antibody

Jackson Immunoresearch Lab. Inc., U.S.A.

FITC conjugated donkey anti-sheep antibody.

Oncor Inc., U.S.A.

Chromosome 17 centromere probe (αCHAD 9)

p53 DNA probe (P5106-DIG)

Sigma Chemical Company, U.K.

Acetic acid

Demecolcine (cat no. D7385)

Streck Lab. Inc., U.S.A.

Streck tissue fixative.

2.2. Methods

2.2.1. Tissue culture

2.2.1.1. Culture of Swiss mouse 3T3 feeder cells

Swiss 3T3 fibroblast cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) donor calf serum, 0.23% sodium bicarbonate, 2mM glutamine, 37.5μg/ml penicillin and 10μg/ml streptomycin. Cells were seeded at 10^4/9cm dish, incubated in a moist atmosphere at 37°C and gassed with air containing 5% (v/v) CO_2. Medium was changed on day 11 and cells used for feeders on day 14.

For use as feeders, cells were trypsinized with 0.1% (w/v) trypsin in phosphate buffered saline (PBS, 0.14M NaCl, 27mM KCl, 10mM Na_2HPO_4, 15mM K_2HPO_4) and resuspended in fresh growth medium. Cells were then lethally irradiated by exposure to 60Gy of γ- irradiation from a ^{60}Co source. This prevents further cell divisions. The cells were then either used immediately or stored for up to 48 hours at 4°C without loss of feeding capacity. Feeder cells are usually plated at a density of $1x10^6$/ 9cm dish.

Feeder cells were removed from keratinocyte cultures as required by vigorously washing the dish with 0.02% (w/v) EDTA in PBS, followed by rinsing with PBS.

2.2.1.2 Derivation and culture of Human Epidermal Keratinocytes (HEK)

Normal HEKs were prepared from new-born human foreskins obtained from Yorkhill hospital, Glasgow. The tissue was washed in PBS, cut into thin strips and trypsinized overnight at 4°C using 0.125% (w/v) trypsin, 0.01% EDTA in PBS. Epidermal cells were separated from mesenchyme by scraping a scalpel along the epidermal layer. Cells were centrifuged at 1000rpm for 5 minutes and resuspended as a single cell suspension in growth medium.

HEKs were grown in serum-free Keratinocyte-SFM supplemented with bovine pituitary extract (50μg/ml) and recombinant epidermal growth factor (5ng/ml) which stimulates migration of the growing keratinocytes out of the centre of the colonies (Barrandon and Green, 1987) and has also been shown to increase the culture lifetime of epidermal cells (Rheinwald and Green, 1977).

Alternatively, HEKs were grown in serum-containing medium in the presence of a 3T3 layer as described above. The 3T3 layer is necessary for keratinocytes to initiate colony formation (Rheinwald and Green, 1975a). The medium consists of DMEM supplemented with 20% (v/v) foetal calf serum, 0.3% (w/v) sodium bicarbonate, 2mM glutamine, 37.5μg/ml penicillin, 10μg/ml streptomycin, 0.4μg/ml hydrocortisone and 10ng/ml cholera toxin. Hydrocortisone is required to make colony morphology more orderly and maintains an increased proliferation rate (Rheinwald and Green, 1975b). Cholera toxin, by raising cyclic AMP levels, seems to oppose the tendency of keratinocytes to increase in size (Green, 1978) and may therefore oppose the onset of terminal differentiation (Sun and Green, 1976).

Squamous cell carcinoma (SCC) cell lines were also grown on a 3T3 feeder layer in the above medium supplemented with 10% foetal calf serum and without cholera toxin. Epidermal growth factor and cholera toxin which are potent mitogens for normal keratinocytes, can be inhibitory to SCC. The medium was changed every 3-4 days.

All cells were incubated in a moist atmosphere at 37°C and gassed with air containing 5% (v/v) CO_2.

Cells were passaged by firstly removing the feeder layer and rinsing with PBS. The cells were removed from flasks or plates with trypsin (0.1% (w/v) trypsin, 0.01% (w/v) EDTA in PBS). Trypsin was inactivated by adding ten volumes of serum-containing medium and removed by centrifugation at 1000rpm for 5 minutes at room temperature. The supernatant was removed and cells resuspended in fresh growth medium and replated as required.

Stocks of all cells were kept frozen in liquid nitrogen. Cells trypsinized as above were resuspended at a concentration of 10^6 cells/ml in ice-cold medium containing 10% serum (above) plus 10% (v/v) dimethyl sulphoxide (DMSO). Care was taken to ensure

all solutions were ice-cold as DMSO is toxic to cells at room temperature. Cells were aliquoted into cryotubes and the ampoules wrapped in cotton wool and placed in a plastic box. Cells were frozen slowly, firstly at -20°C for 30 minutes and then at -70°C overnight. Cells were then stored in liquid nitrogen. The cotton wool insulation ensures gradual cooling of the cells and therefore increases the viability of the cells on thawing.

Cells were thawed by transferring the ampoule directly from liquid nitrogen to water at 37°C. Once thawed cells were added to a large volume of pre-warmed growth medium, centrifuged, resuspended in fresh growth medium and plated onto an irradiated feeder layer.

2.2.1.3. Growth of keratinocytes on raft cultures

Collagen gel type I (rat tail, pH3 acetate) was prepared by adding 20mM Hepes, 5mM NaOH, 1x SF12 medium 2mM glutamine and 0.2% (w/v) sodium bicarbonate . The gel was seeded with $3x10^5$ 3T3 fibroblasts/ml, mixed well, trying to avoid air bubbles and 2mls were placed into transwell inserts (3.0μm pore membrane). The gel was incubated at 37°C for 30 minutes, then overlaid and underlaid with growth medium and incubated for a further 24 hours. The growth medium was changed and the gel seeded with $1x10^6$ keratinocytes/well. The cells were grown submerged for at least 24 hours at 37°C in a humid incubator. The wells were then transferred to a sterile beaker and underlaid with growth medium. Any medium above the confluent keratinocytes was removed, the rafts were covered and cells grown for a further 14 days. Cryostat sections of the raft cultures were prepared by Dr. I Macmillan (Veterinary department, Glasgow University).

2.2.1.4 Transfection of cells.

Keratinocytes were plated on an irradiated feeder layer at $2x10^5$/5cm dish and incubated at 37°C until cells were 80% confluent. 30μl DOTAP (1mg/ml) was diluted to 100μl with HBS (20mM Hepes, 150mM NaCl pH7.4). This was mixed with an

equivolume of DNA (5μg) in HBS and incubated at room temperature for 10 minutes. This lipofection mixture was added to 5mls fresh growth medium containing serum, placed on the cells and incubated overnight at 37°C. The medium was replaced with fresh growth medium and cells incubated again overnight. The cells were trypsinized as described above and plated at low density on neomycin resistant feeders (3T3 M1 Neo). The cells were incubated until small colonies were visible and then selected with 400ug/ml G418. Resistant colonies were visible within 14 days.

Single resistant colonies were marked using a microscope ring marker attachment. The medium was aspirated and feeders gently removed as described above. Cloning rings were lightly greased and placed over the marked colonies. Pre-warmed trypsin/EDTA was gently dropped into the cloning rings and incubated for 10 minutes. Using a pasteur pipette, medium was added to the trypsin and gently mixed to resuspend the cells. The trypsin/medium mixture was transferred to pre-warmed medium and spun at 1000rpm. The cells were resuspended and plated onto a fresh feeder layer. Cells were kept under selection until checked for the required protein expression. Prior to any further experiment transfectants were removed from selection, washed thoroughly with PBS and grown without selection for at least a week.

Saos cells were grown in growth medium supplemented with 10% serum without a feeder layer. They were transfected as described above using 30μl DOTAP and 10μg DNA. Cells were trypsinised 24 hours after transfection and plated at $4x10^5$cells / 10cm dish. The cells were incubated at 37°C for 2 days and then selected with 500μg/ml G418. Resistant colonies were visible after 21 days. Single colonies from each transfection were ring cloned and checked for protein expression as described above. In order to analyse the growth suppressive function of some p53 mutants whole plates of transformed cells were selected and stained for p53 expression as described in section 2.2.2.1.

2.2.1.5 Growth of keratinocytes in suspension culture.

Keratinocytes can be induced to terminally differentiate *in vitro* by culturing them in methylcellulose. This prevents cell to cell contact, causes the cells to round up and become unresponsive to mitogen stimulation (Green, 1977).

Methylcellulose (1.3% w/v) was dissolved in unsupplemented medium (DMEM containing 1mM sodium pyruvate, 0.3% (w/v) sodium bicarbonate, pH7.2) pre-heated to 60°C. Once cooled to room temperature 10% (v/v) fetal calf serum, 37.5μg/ml penicillin, 10μg/ml streptomycin 2mM glutamine and 0.4μg/ml hydrocortisone were added and stirred for 30 minutes at room temperature and at 4°C overnight. This was then centrifuged at 15000g at 4°C for 30 minutes. The supernatant was decanted into fresh, sterile bottles and frozen at -20°C.

Sterilin bacteriological dishes were treated with polyhaem A to prevent cells from attaching to the dish. Polyhaem A (10% in 95% ethanol) was diluted to a working stock of 0.4% in acetone (50%), 95% ethanol (50%) and used to wash dishes which were then air-dried. Before use dishes were rinsed in DMEM.

Cells were seeded at either $2x10^6$ /5cm dish (high density) or $3x10^5$ /5cm dish (low density) without feeders and were incubated at 37°C for four days. Cells were trypsinised and diluted to $1x10^6$/ml. 1 part cell suspension was mixed with 9 parts pre-warmed methylcellulose (final cell concentration was 10^5/ml methylcellulose). Cells were suspended in methylcellulose evenly taking care not to produce air bubbles. The cell suspension was then pipetted into polyhaem A coated petri dishes and incubated for the required length of time in a humid incubator at 37°C.

Cells were recovered from methylcellulose by washing the cells and methylcellulose off the plates with cold PBS. The mixture was diluted 20 fold with PBS and centrifuged at 2000rpm for 10 minutes. The supernatant was aspirated off, the cells were re-washed in PBS and counted.

2.2.1.6 Cornified envelope formation assay.

Cornified envelopes are formed as keratinocytes terminally differentiate (Sun and Green, 1976). Cells were induced to differentiate by placing them in suspension culture as described above for up to 5 days. Cells were removed from the methylcellulose and diluted to $3x10^5$/ml in 5% SDS. Cells were solubilised by adding 1% β-mercaptoethanol and boiling for 5 minutes. Cornified envelopes were visualised and counted on a haemacytometer under a light microscope. Each count was an average of 16 large squares on a haemacytometer $x10^4$/ml. Experiments were carried out at least in triplicate for each cell line.

2.2.1.7 Cloning efficiency.

Cells were induced to differentiate by placing them in suspension culture as described above for up to 24 hours. Cells were removed from the methylcellulose and counted. Cells that had not been placed in suspension culture were plated at 500 cells/ T25 on an irradiated feeder layer. Cells removed from the methylcellulose after 24 hours were plated on an irradiated feeder layer at 10^3/T25. Cells were incubated at 37°C until colonies were large but still individual (approximately 20 days). Cells were then washed in PBS and fixed in 10% formalin (10% formaldehyde in PBS) for 30 minutes in a fumehood. The formalin was removed and the cells stained with 1% (w/v) rhodamine B for 30 minutes. The cells were then washed, air-dried and the colonies counted by eye. Cells from each time point were plated onto feeders in duplicate for each experiment. Experiments were repeated at least in triplicate for each cell line. Colony counts are given per 500 cells plated.

2.2.1.8 Tumorigenicity in nude mice.

Cells were removed from selection and expanded at identical cell densities. The cells were then trypsinised and washed twice in serum-free DMEM. $5x10^6$ and $1.6x10^6$

cells were resuspended in 200µl serum-free DMEM and subcutaneously injected into the left or right flank respectively of four week old nude mice (MF1 NuNu mice). Three mice were used per cell line tested. Mice were examined by animal house staff weekly, for tumour presence and the tumour volumes were recorded. A progressively growing tumour which remained for 3 months was scored as positive. After this time or when the tumour became $1cm^3$ the mice were sacrificed and cryostat sections prepared from the tumour.

2.2.1.9. Haematoxylin and Eosin staining

Cells or tissue sections where counterstained with haematoxylin, which stains the nucleus blue and eosin, which stains the cytoplasm pink, to enhance cellular morphology for examination and photography.

Tissue sections were fixed twice in 100% ethanol, once in 70% ethanol, then placed in water. Individual cells were either trypsinised or removed from suspension culture, washed in PBS and resuspended in 100% ethanol. Cells were then dropped onto glass slides and air-dried. The slides were placed into 100% ethanol, then 70% ethanol, followed by water.

Slides were placed in Harris' Haematoxylin for 10 seconds, washed in running tap water and developed in Scott's tap water until the nucleus was sufficiently dyed. The intensity of the stain can be adjusted by washing in 1% HCl in 70% ethanol. The slides were washed in tap water and dipped briefly in Eosin stain followed by further washing in tap water. Once the required dye intensities had been achieved the cells were dehydrated once in 70% ethanol, twice in 100% ethanol, then cleared in xylene and mounted in histomount.

2.2.1.10. Acrydine orange staining

Nuclear DNA was stained with acrydine orange. Cells were removed from methylcellulose and stained with 5µg/ml acrydine orange in PBS for 10 minutes. Cells

were pelleted, resuspended in H_2O and air-dried onto slides. Cells were mounted in anti-fade medium (Vectashield) and sealed with nail varnish. Nuclear staining was visualised under a narrow band FITC filter in fluorescent light. Cells were photographed using 400 ASA daylight film, pushed to 1600 on camera and developed twice in initial developing solution.

2.2.2. Immunocytochemistry

2.2.2.1 p53 staining

Cells were seeded in an 8 well chamber slide at 10^4cells and $2x10^4$ 3T3 feeders per well. Cells were incubated in a humid incubator at 37°C until 60% confluent.

The plastic wells were removed from the slides taking care to retain the plastic gasket which defines the boundaries between wells. The slides were then washed briefly in PBS and drained. The cells were fixed for 20 minutes in -20°C methanol on ice and air-dried for 40 minutes or until completely dry.

Normal horse blocking serum (1:10 dilution in 0.1% BSA in PBS, pH7.6) from the mouse vectastain peroxidase kit was added to each well and the slide incubated in a humidified box at room temperature for 20 minutes. The slides were drained and 100µl diluted p53 antibody added to each well. PAb 1801 (Banks *et al*, 1986) which recognises the wild-type conformation of p53 was used at a final concentration of 1µ g/ml. PAb 240 (Gannon *et al*, 1990) which recognises the mutant conformation of p53 was used at a final concentration of 3µg/ml. The slides were then incubated for 1 hour in a humidified box. Excess antibody was removed by washing in a high salt buffer (0.15M Nacl, 0.05% Tween 80 in PBS, pH7.6) for 30 minutes, changing the buffer every 10 minutes. The secondary antibody, anti-mouse IgG biotinylated antibody, was diluted 1:200 in 0.1% BSA/PBS buffer and 100µl added to each well. The slides were again incubated in a humidified box for 1 hour at room temperature and then washed as above. The biotinylated antibody was detected using a complex between avidin and a further biotinylated enzyme (ABC reagent). This complex is formed at least 30 minutes before

required. One drop (50µl) of reagent A (Avidin) and one drop reagent B (Biotinylated enzyme) was added to 5mls 0.1% BSA/PBS and 100µl added to each well. The slides were incubated for another hour in a humidified box and again washed as before. The substrate diaminobenzene was dissolve in PBS (0.6mg/ml) containing 0.06% H_2O_2 and added to each slide, in the dark, for 7.5 minutes or until a brown stain was visible. The slides were then washed in water, mounted in aquamount and sealed with nail varnish.

The cells were photographed with panotomic X film under phase contrast or bright field with a green filter.

2.2.2.2 Involucrin staining

Cells were either grown on chamber slides as above or cells pre-washed in PBS were air-dried onto slides. Cells were fixed in -20°C methanol on ice for 20 minutes and then air-dried. Goat blocking serum from the rabbit alkaline phosphatase Vectastain kit was diluted 1:10 in 0.1% BSA/PBS and added to the cells for 25 minutes in a humidified chamber at room temperature. The excess blocking serum was removed and the rabbit anti-involucrin antibody (diluted 1:5000) added to the cells overnight in a humidified box at 4°C. The slides were then washed in NaCl/PBS/Tween as above for 30 minutes. The slides were drained and the goat anti-rabbit secondary antibody from the kit (diluted 1:200) was added for 1 hour at room temperature. The slides were again washed and drained. The ABC reagent diluted as above was added for 1 hour in a humidified box and then the excess removed by washing in NaCl/PBS/Tween buffer as before. The alkaline phosphatase substrate kit I (red) was used to visualise antibody reactivity. Reagent 1, 2 and 3 were mixed in equal amounts, as directed, in 100mM Tris-HCL pH8.2. This was added to the cells for 5 minutes or until the required staining intensity was achieved. Slides were washed in water, mounted in aquamount and sealed with nail varnish.

Cells were photographed with colour slide film under phase contrast or bright field optics.

2.2.3. Protein Biochemistry

2.2.3.1 Western Blot

a) p53

Cells were trypsinised when 50% confluent and washed twice in PBS. $1x10^6$ cells were lysed in 100µl ice cold lysis buffer (1% NP40, 20mM Tris, 2mM EDTA, 100mM Nacl, pH8.0) containing protease inhibitors (0.01% PMSF, 1µg/ml Aprotinin, 1µg/ml Leupeptin, 5mM NaF). Note, sodium orthovanadate has been shown to alter the conformation of p53 (Landesman *et al*, 1994) and was therefore not used in the lysis buffer. Cells were lysed on ice for 30 minutes and then centrifuged for 15 minutes at 4°C. The supernatant was transferred to a fresh eppendorf tube. The protein concentration was determined using the Bio-rad DC protein assay. SDS-sample buffer (50mM Tris, pH6.8, 10% glycerol, 2.5% SDS, 0.1% bromophenol blue) and 2.5% β-mercaptoethanol were added to 50 or 250ug protein and boiled at 100°C for 30 minutes. The samples were centrifuged briefly and run on a 10% SDS-polyacrylamide gel along with rainbow markers as described in section 2.2.3.2.

The protein was transferred onto Immobilin-P nitrocellulose filter, also described in section 2.2.3.2. After washing briefly in TBS (10mM Tris, 150mM Nacl, pH8.0), the filter was stained for 5 minutes in Ponceau's stain, then de-stained for 5 minutes in 5% (v/v) acetic acid to check for consistency of protein transfer and loading. The filter was rinsed again in TBS and blocked overnight at 4°C in TBS-T (0.1% Tween 20 in TBS) containing 5% Marvel (milk fat) and 0.025% sodium azide.

The filter was incubated with the primary p53 mouse monoclonal antibody PAb1801 (1µg/ml in TBS-T plus 5% Marvel, Oncogene science) for 2 hours at 4°C, then washed 3 times, each for 15 minutes in TBS-T plus marvel. The secondary antibody, anti-mouse Ig horse-radish peroxidase linked whole antibody, was incubated with the filter at a 1:5000 dilution in TBS-T/Marvel for 20 minutes. The filter was again washed as above and rinsed twice more in TBS for 15 minutes each. The antibody was

detected by immersing the filter in ECL detection reagent for 1 minute, wrapping in SaranWrap and exposing it to Kodak X-Omat fast film for up to a minute.

b) Cyclin D.

Semi-confluent dishes of cells were washed twice with PBS and the cells lysed by adding 1ml ice-cold lysis buffer (50mM Tris pH7.4, 150mM NaCl, 20mM EDTA, 0.5% NP40) containing protease inhibitors (1mM PMSF, 25μg/ml leupeptin, 25μg/ml aprotinin, 1mM benzamidine, 10μg/ml trypsin inhibitor, 1mM sodium fluoride) to each dish for 30 minutes on ice. The cells were scraped from the dish and centrifuged for 5 minutes at 4°C. The supernatant was removed to a fresh eppendorf tube. The protein concentration was measured using the Bio-rad DC protein assay kit. 30μg protein was run on a 10% SDS-polyacrylamide gel and blotted onto immobilin-P nitrocellulose as described in section 2.2.3.2. The filter was washed briefly in PBS-T (PBS plus 0.1% Tween 80) and the protein visualised in Ponceaus' stain as described above. The filter was blocked in PBS-T plus 5% marvel overnight. The primary antibody, rabbit polyclonal cyclin-D (287-3), was added to the filter at 1:500 dilution in PBS-T/Marvel for 3 hours at 4°C, then the filter was washed three times in PBS-T/Marvel each for 15 minutes. The secondary antibody, anti-rabbit Ig, horseradish peroxidase linked $F(ab')_2$ fragment, was diluted 1:3000 and added to the filter for 1 hour at room temperature. The filter was again washed as before and rinsed in PBS-T for 15 minutes. The filter was immersed in ECL detection reagent for 1 minute, wrapped in SaranWrap and exposed to Kodak X-Omat film for up to 1 minute.

2.2.3.2. SDS-polyacrylamide gel electrophoresis and blotting.

10% SDS-polyacrylamide gel (10% acrylogel, 375mM Tris, pH 8.8, 0.1% SDS, 0.1% ammonium persulphate, 0.03% TEMED) was poured between gel plates, covered with H_2O-saturated butan-2-ol, and allowed to polymerise for 1 hour. The gel front was washed with H_2O and blotted dry. The stacking gel (4.8% Acrylogel, 125mM Tris,

pH6.8, 0.1% SDS, 0.1% ammonium persulphate, 0.03% TEMED) was poured on top of the resolving gel, a comb inserted and allowed to polymerise. Due to the difference in pH between the stacking and resolving gel, the stacking gel should only be poured up to 1 hour before use to minimise merging of the pH's. The gel was run at 30mA in running buffer (25mM Tris, 191mM glycine, 3.5mM SDS) for 3-4 hours.

The gel was carefully removed from the glass plates, discarding the resolving gel and soaked in transfer buffer (25mM Tris, pH8.3, 191mM glycine, 20% (v/v) methanol) for 30 minutes. The protein was transferred in the above buffer onto immobilin-P nitrocellulose using a bio-rad wet trans-blot apparatus run at 30 volts overnight.

2.2.3.3. Immunoprecipitaton of p53.

Semi-confluent plates of cells were washed twice in PBS and 1ml ice-cold lysis buffer (150mM NaCl, 50mM Tris, pH8.0, 5mM EDTA, 1% NP40) containing protease inhibitors (1mM PMSF, 5mM sodium fluoride, 1μg/ml aprotinin, 1μg/ml leupeptin) was added to each dish for 30 minutes on ice. The cells were scraped from the dish, centrifuged for 30 minutes and the supernatant transferred to a fresh eppendorf. The protein concentration was measured using the Bio-rad DC protein assay kit. 20μl protein G plus agarose beads were added to the lysate and rotamixed for 40 minutes at 4 °C. The supernatant was removed and split equally between antibodies used. 1ul antibody (either DO-1, PAb1620 or PAb240 all a kind gift from Dr. D. Lane) was incubated with the cell lysate overnight at 4°C. 20μl protein G agarose beads were added and the samples rotamixed for 40 minutes at 4°C. After brief centrifugation, the supernatant was removed and the beads washed 3 times with ice-cold lysis buffer taking care to remove all the buffer. The beads were resuspended in 20μl sample buffer (50mM Tris, pH6.8, 10% glycerol, 2.5% SDS, 0.1% bromophenol blue) and 2.5% β-mercaptoethanol and boiled for 10 minutes at 100°C. Samples were run on a 10% SDS-polyacrylamide gel as described above. The protein was transferred onto Immobilin-P nitrocellulose and probed as described in the western protocol (section 2.2.3.1a.) using the rabbit polyclonal p53 antibody CM-1 (1:250 dilution, a gift from Dr.D.Lane) as the

primary antibody and anti-rabbit Ig, horseradish peroxidase linked whole antibody (1:5000 dilution) as the secondary antibody.

2.2.4. Molecular Biology

2.2.4.1. Preparation of genomic DNA from SCC cell lines.

DNA was prepared using the salting out method as described by Miller *et al*, 1988. Cells were lysed in 3ml lysis buffer (10mM Tris-HCl, 400mM NaCl, 2mM EDTA, pH8.0) and digested overnight at 37°C with 0.2ml 10% (w/v) SDS and 0.5ml protease K solution (1mg protease K in 1% SDS, 2mM EDTA). After digestion 1ml saturated NaCl (approximately 6M) was added and the tube shaken vigorously for 15 seconds, followed by centrifugation at 3000g for 5 minutes to pellet the protein. The DNA was precipitated from the supernatant with 2 volumes of room temperature absolute ethanol. The DNA strands were spooled out on a glass pipette, air-dried and dissolved in 100-200 µl TE (10mM Tris-HCl, 0.2mM EDTA, pH7.5). DNA concentrations were determined by measuring absorbance at 260nm and using the conversion of 1 OD unit at 260nm is equivalent to a concentration of 50µg/ml. The absorbance ratios 260nm/280nm should be 1.8-2.0 showing good deproteinization of the DNA sample. Genomic DNA was stored at 4°C.

2.2.4.2. Preparation of RNA from SCC cell lines.

Cells were lysed directly in the culture dish by the addition of RNAzol B (1ml/3.5cm dish). The RNA was solubilized by passing the lysate a few times through the pipette. 0.2ml chloroform was added per 2ml lysate, the tubes were covered with parafilm, vigorously shaken for 15 seconds then incubated on ice for 5 minutes. The samples were centrifuged at 12,000g, 4°C for 15 minutes after which the upper colourless aqueous phase was transferred to a fresh tube and an equal volume of isopropanol added. The samples were stored at 4°C for 15 minutes and the RNA

precipitate pelleted by centrifugation at 12,000g, 4°C, for 15 minutes. The RNA pellet was washed once with 1ml 75% ethanol by vortexing and centrifugation at 7,500g, 4°C, for 8 minutes. The RNA was re-dissolved in 200-300µl diethylpyrocarbonate (DEPC) treated RNase-free H_2O. The RNA was re-precipitated by adding 1/10 volume 3M NaAc, pH5.2 and 2 volumes ethanol for 1 hour at -20°C. Note, all solutions and tubes should be pre-treated with DEPC to prevent RNase contamination. The RNA concentration was determined by measuring the absorbance at 260nm and using the conversion of 1 OD unit being equivalent to 40µg/ml. RNA was stored in aliquots at -70°C.

2.2.4.3. Minipreps of plasmid DNA

Small amounts of plasmid DNA were prepared using the alkaline lysis method as described by Maniatis *et al*, 1989.

Single colonies of bacteria carrying the required plasmid were picked using a sterile toothpick and grown in 2ml L-Broth (1% w/v bactotryptone, 0.5% w/v yeast extract, NaCl) containing antibiotic (50µg/ml ampicillin or 30µg/ml chloramphenicol) at 37°C in a shaking incubator. Bacteria were pelleted from 1.5ml of overnight culture by brief microcentrifugation and resuspended in 100µl lysozyme solution (50mM glucose, 25mM Tris-HCl, pH8.0, 10mM EDTA containing 2mg/ml lysozyme) for 5 minutes. 200µl freshly made solution 2 was added (0.2M NaOH, 1% w/v SDS), mixed gently and left at room temperature for 5 minutes. 150µl ice-cold 3M KAc pH 4.8 was then added, mixed thoroughly and stored on ice for 5 minutes. The precipitated cell debris was pelleted by microcentrifugation for 2 minutes and the supernatant removed to a fresh eppendorf tube. The nucleic acids were precipitated by adding 2 volumes of cold ethanol to the supernatant, standing for 5 minutes at room temperature and microcentrifugation for 10 minutes. The pellet was washed in 1ml -20°C 70% ethanol, microcentrifuged for 5 minutes and all ethanol removed using a drawn out pippette. The pellet was air-dried and dissolved in 20µl TE containing DNase-free RNase A (20µg/ml) and incubated at 37°C for 30 minutes. The DNA solution was made up to 200µl with TE, extracted with

an equal volume of phenol/chloroform (1:1) and re-precipitated with 1/10 volume 3M NaAc pH 5.0 and 2 volumes ethanol at -70°C for 1 hour. The DNA was pelleted by microcentrifugation, washed in 70% ethanol and dissolved in 20µl TE. Plasmid DNA was stored at -20°C.

2.2.4.4. Large scale preparation of plasmid DNA

10 ml overnight culture was transferred to 500ml L broth containing antibiotic (50µg/ml ampicillin) and shaken at 37°C overnight.

If the plasmid requires amplification then the culture was grown at 37°C until the absorbance at 600nm reached 0.4-0.6. Chloramphenicol was then added (170µg/ml) and the culture shaken at 37°C for a further 16-20 hours.

Cells were pelleted from the overnight culture at 5000rpm, 4°C for 10 minutes, rinsed with 50mM Tris-HCl pH 8.0 and resuspended in 25ml lysozyme solution (25mM Tris-HCl pH 8.0, 10mM EDTA, 50mM glucose containing 5mg/ml lysozyme) for 30 minutes at room temperature. 40 ml solution 2 (0.2M NaOH, 1% SDS) was added, mixed well and placed on ice for 15 minutes. 20 ml 3M KAc pH4.8 was then added, mixed by inversion and left on ice for another 15 minutes. The flocculates were centrifuged at 10,810g for 5 minutes at 0°C. The supernatant was filtered through gauze and the DNA precipitated by adding 0.6 volumes of -20°C isopropanol and immediately centrifuged at 10,810g, room temperature for 5 minutes. After discarding the supernatant, the pellet was left to drain for 10 minutes and then resuspended in 5ml TE, pH8.0.

Plasmid DNA was purified on a CsCl gradient. 7.5g CsCl was dissolved in the 5ml DNA solution and 5mg ethidium bromide added. The refractory index was adjusted to 1.3860-1.3900 with TE pH 8.0. The CsCl solution was balanced in oakridge tubes and centrifuged in a sorvall ultracentrifuge (rotor T1270) at 146,600g for 40 hours at 20°C.

The plasmid band was removed from the gradient using a syringe with a 19 gauge needle and extracted with an equal volume water-saturated isobutanol until all the ethidium bromide had been removed. The DNA was precipitated by adding an equal

volume H_2O and 2 volumes room temperature ethanol and standing at room temperature for 15 minutes. The DNA was pelleted in glass corex tubes by centrifugation at 11,950g, 4°C for 15 minutes. The pellet was resuspended in 1ml deionised water and reprecipitated with 0.1 volumes 3M NaOAc pH 5.0 and 2 volumes ethanol at -20°C for 1 hour. DNA which was used for transfection of cells in culture was purified further by phenol/chloroform extraction before precipitation. The DNA was pelleted as above, rinsed with 70% ethanol, freeze-dried and dissolved in TE. The DNA concentration was measured as described in section 2.2.4.1.

2.2.4.5. Restriction enzyme digestion of DNA.

Plasmid DNA was incubated with 5-10 units enzyme/μg DNA in a buffered solution as specified and supplied by the manufacturer for 1-2 hours at 37°C. The digestion fragments were analysed by agarose gel electrophoresis as described below.

2.2.4.6. Agarose gel electrophoresis.

Flat bed electrophoresis apparatus from Pharmacia was used. Agarose gels (0.7%-4% (w/v) agarose) were cast in 1xTAE buffer (40mM Tris base, 16mM acetic acid, 1mM EDTA, pH8.0) containing 0.5μg/ml ethidium bromide. Low melting point agarose was used in order to isolate and purify required DNA restriction fragments. 4% Nusieve agarose gels were used for greater separation of PCR products. Stop and dye buffer (0.45% (w/v) bromophenol blue, 1% (w/v) SDS, 100mM EDTA, 2.5% w/v Ficoll 400 in TE) was added to each sample. The gel was submerged in 1xTAE buffer containing 0.5μg/ml ethidium bromide, the samples were loaded into each well along with an appropriate size marker (i.e. either ΦX174 Hae-III digested DNA or 1Kb ladder) and run at 100V for 30-60 minutes. The separated DNA was visualised by illumination with short wave (312nm) UV light and photographed through a red filter using polaroid type 57 high speed film or using an Appligene Imager.

2.2.4.7. Isolation of specific restriction fragments from digested DNA

a) Using a low melting point agarose gel and Geneclean 2®kit.

Digested DNA was run on a low melting agarose gel at 4°C as described above. The required DNA fragment was excised from the gel and the agarose block containing the fragment was weighed and volume calculated (1g = 1ml). 2.5 volumes of 6M NaI was added and the agarose melted at 55°C for 5 minutes. 5µl glassmilk®, a suspension of silica matrix in water which binds single and double-stranded DNA without binding contaminants, was added for every 5µg or less DNA. The mixture was vortexed and left on ice for 5 minutes to allow binding. The glassmilk was pelleted by brief centrifugation and washed 3 times by mixing with 300µl NEW wash (a Tris and EDTA-buffered solution of NaCl, ethanol and water), pelleting the glass milk and removing the supernatant. Finally half the desired volume of TE was added to the cleaned pellet and heated to 55°C for 2 minutes. The glassmilk was pelleted and the TE containing DNA was removed to a fresh eppendorf. This was repeated to elute all the DNA.

b) Directly from an agarose gel using filter paper.

The restricted DNA was run on a 1-2% agarose gel as described in section 2.2.4.6. An incision was made just below the required DNA fragment in the gel. Filter paper was backed with dialysis tubing and cut to the size of the incision. This was placed carefully into the gel with the filter paper nearest the DNA fragment and the capillary tubing facing away. The gel was re-submerged into the buffer and run until the DNA fragment was collected on the filter paper. The capillary tubing prevents the DNA from running straight through the filter paper. The filter paper was then removed from the gel and placed in an eppendorf with a hole in the bottom standing in another eppendorf tube. The tubes were briefly centrifuged to elute the DNA. 30µl TE was added to the filter paper, allowed to stand for a few minutes and the centrifuged again. The DNA solution collected in the bottom eppendorf tube was made up to 80µl with TE and was either

collapsed for double-stranded sequencing as described in section 2.2.4.12 or ethanol precipitated.

2.2.4.8. Ligation of DNA fragments.

Vector DNA was digested as described in section 2.2.4.5. The DNA fragment to be inserted was also digested as above and then isolated by gel electrophoresis as described in section 2.2.4.7.

To prevent the vector DNA from ligating back to itself without an insert it was firstly dephosphorylated. The vector DNA was digested with the required enzyme as directed by the manufacturer and an aliquot removed to check that digestion was complete. The reaction mixture was adjusted by adding dephosphorylation buffer (50mM Tris-HCl, 0.1mM EDTA, pH 8.5) and 1 unit alkaline phosphatase. This was incubated at 37°C for 1 hour, after which the reaction was stopped by adding 50mM EDTA pH8.0 followed by phenol/chloroform extraction. The DNA was re-precipitated by adding 0.5x volume 7.5M NH_4Ac and 2x volume ethanol at -70°C for 30 minutes. The DNA precipitate was microcentrifuged for 10 minutes, washed with 70% ethanol and redisssolved in TE.

The insert was ligated to the dephosphorylated vector (100ng) at a ratio 3:1 respectively. The vector and insert DNA were incubated together in a reaction containing 50mM Tris-HCl pH 7.6, 10mM $MgCl_2$, 1mM ATP, 1mM DTT, 5% (w/v) polyethylene glycol-8000 and 1 unit T4 DNA ligase at 4°C overnight.

2.2.4.9. Transformation of competent bacterial cells

Ligation mixtures were firstly diluted 5 fold and 1µl of this (containing 1-10ng of ligated DNA) was added to 20µl DH5α competent cells in chilled microcentrifuge tubes, shaken gently and placed on ice for 30 minutes. The cells were heat shocked by placing in a 42°C waterbath for 40 seconds and then placed directly on ice. 80µl SOC medium (2% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 10mM NaCl, 2.5mM KCl, 10mM

$MgCl_2$, 10mM $MgSO_4$, 20mM glucose) was added to the cells and shaken at 225 rpm for 1 hour at 37°C. All the cells were spread onto an L-Broth agar plate (15g Bacto-Agar/litre L-Broth) containing antibiotic and incubated overnight at 37°C. Plasmid DNA was prepared from transformed colonies as deescribed in section 2.2.4.3.

2.2.4.10. Oligonucleotide synthesis and purification.

Oligonucleotides were either ordered from Research genetics or synthesised on an Applied Biosystems 381A DNA Synthesiser or 392 DNA/RNA Synthesiser using the manufacturers protocols and Cruachem reagents. Primers from Research Genetics arrived ready for use at 20μM in TE pH8.0. Other primers were synthesised with or without trityl group protection. All these primers were firstly deprotected by incubating overnight at 55°C.

"Trityl on" primers were detritylated using an Applied Biosystems oligonucleotide purification cartridge. 5ml acetonitrile was passed through the column to waste at a rate of 1 drop/sec using a syringe. This was followed by 5ml 2M triethylammonium acetate. The deprotected oligonucleotide ammonia solution was diluted with an equal volume of distilled water and passed through the column the same way. The eluate was collected and passed through a second time. The cartridge was then flushed through with 5ml 10% (v/v) ammonia and 10ml distilled water. The oligonucleotide was detritylated while bound to the support by passing 2.5ml 2% (v/v) trifluoroacetic acid through the column, allowing it to stand for 5 minutes and repeating. The cartridge was then flushed with 10ml distilled water. The oligonucleotide was eluted drop-by-drop with 3ml 20% (v/v) acetonitrile, freeze-dried overnight and dissolved in 250ml TE pH8.0. Primer concentration was determined by measuring absorbance at 260nm and using the conversion that $1OD_{260nm}$ unit is equivalent to 24μg/ml. Primers were stored at -20°C.

"Trityl off" primers were either stored after deprotection in ammonia at -20°C and purified as needed, or kept at 4°C and deprotected and purified as needed. The primers were purified by desalting them by precipitation with butan-1-ol. 1ml butan-1-ol was added per 150μl primer solution and microcentrifuged at 13,000g for 20 minutes at room

temperature. Excess butanol was removed by centrifugation under vacuum and the primer dissolved in 150µl TE pH8.0. Primer concentrations were calculated as above.

2.2.4.11. Polymerase chain reaction (PCR)

a) Amplification from RNA.

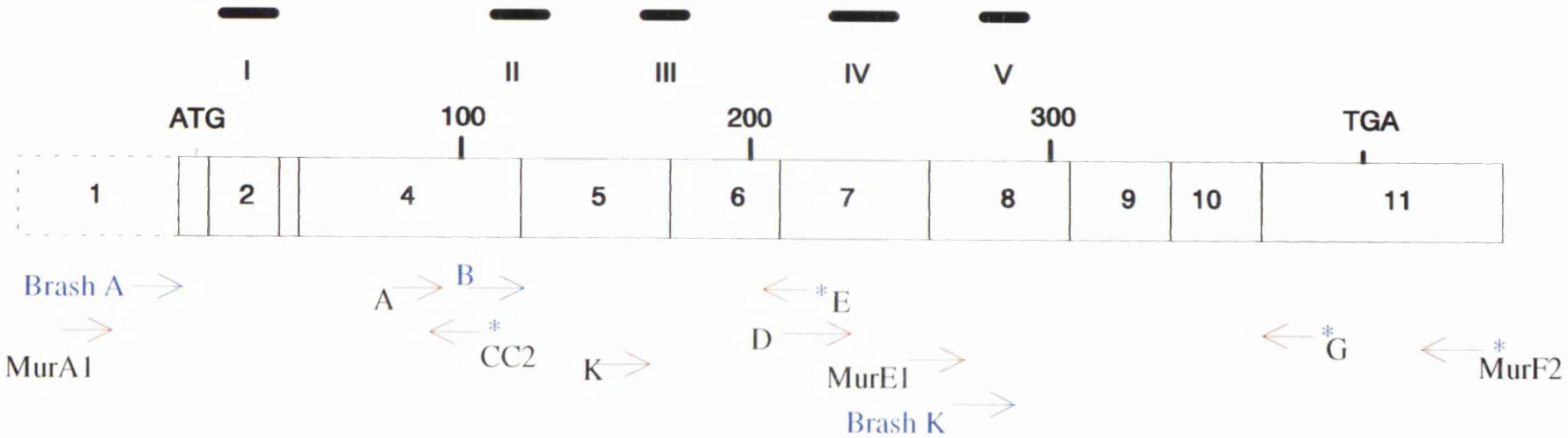
Firstly cDNA was synthesised from RNA by reverse transcription using the Perkin-Elmer Cetus RNA PCR kit. The reaction was carried out in a final volume of 20µl comprising of 5mM $MgCl_2$, 1x PCR kit buffer II (50mM KCl, 10mM Tris-HCl), 1mM of each of dATP, dGTP, dTTP, dCTP, 1 unit RNase inhibitor, ≤ 1µg RNA, 2.5µM random hexamers and 2.5 units reverse transcriptase. This was incubated at room temperature for 10 minutes allowing the extension of the hexameric primers by reverse transcriptase. All samples were placed in the thermocycler and further incubated at 42°C for 15 minutes, 99°C for 5 minutes and soaked at 4°C for 5 minutes.The cDNA was then amplified by using specific primers as detailed in table 1. The above reaction volume was increased to 100µl by adding 2mM $MgCl_2$, 1x kit PCR buffer II, 0.15µM of each of the upstream and downstream p53 primers (detailed in table 1 and figure 2.1) and 2.5 units *Taq* polymerase. Amplification proceeded for 35 cycles of 95°C for 30 seconds and 60°C for 30 seconds plus a 1 second extension per cycle followed by a final extension at 60°C for 7 minutes using the Perkin-Elmer cetus 9600 thermocycler. The samples were then analysed by agarose gel electrophoresis as described in section 2.2.4.6 to ensure correct amplification.

b) Amplification of DNA.

All reagents were provided in the Perkin-Elmer Cetus DNA PCR kit. Primer sequences are detailed in table 2.

The reaction mixture comprising of 200µM of each dATP, dCTP, dTTP, and dGTP, buffer J (50mM Tris pH 9.0, 50mM KCl, 1.5mM $MgCl_2$, 0.01% gelatin),

Figure 2.1 **Primer positions used for PCR amplification and sequencing of human p53 cDNA.**



conserved regions

PCR primer

Sequencing primers

* Biotinylated primer

1-11 p53 exons

Table 1

PCR primers for amplification of p53 from cDNA

Primer pairs	Fragment amplified	Primer sequence	Reference
A E	447-894bp	5'CAGCTCCTACACCGGCGGCCCCTGCACCAG3' 5'Biotin-GAGCCAACCTCAAGGCGGCTCTCATAGGGCACC3'	Rodrigues *et al*, 1990 Rodrigues *et al*, 1990
D G	855-1434bp	5'TAGTGTGGTGGTGCCCTATGAGCCG3' 5'Biotin-GTGGGAGGCTGTCAGTGGGGAACAA3'	Rodrigues *et al*, 1990 Rodrigues *et al*, 1990
MUR E1 G	970-1434bp	5'CACCATCATCACACTGGAAG3' as above	Murakami *et al*, 1991
D MUR F2	855-1506bp	as above 5'Biotin-CTGACGCACACCTATTGCAA3'	 Murakami *et al*, 1991
MUR A1 CC2	155-509bp	5'TCCACGACGGTGACACGCTT3' 5'Biotin-AAGGGACAGAAGATGACAGG3'	Murakami *et al*, 1991 Burns *et al*, 1993
K G	700-1434bp	5'CTACAAGCAGTCACAGCACAT3' as above	Burns *et al*, 1993

Table 2

PCR primers for amplification of p53 from genomic DNA

Primer pair	Primer position	Primer sequence	Reference
6U 6D	Exon 6	5'CTGATTGCTCTTAGGTCTGG3' 5'Biotin-AGTTGCAAACCAGACCTCAG3'	Brash *et al*, 1991 Brash *et al*, 1991

0.15μM of each primer and 200ng DNA, was aliquoted into GeneAmp PCR reaction tubes in a final volume of 100μl. The tubes were placed into the PCR machine (Perkin-Elmer Cetus type 9600) and heated to 94°C for 5 minutes to inactivate DNases and ensure all DNA duplexes were melted. The samples were cooled to 85°C and 2.5 units *Taq* polymerase (a thermolabile DNA polymerase from *Thermus aquaticus*) was added. The DNA was then amplified (using Perkin-Elmer cetus 9600 thermocycler) for 35 cycles at 94°C for 30 seconds, 55°C for 1 minute, to allow the primers to anneal to the template DNA, followed by 72°C for 1 minute, to allow extension of the amplimer sequences. After completion of the cycles, the reaction was incubated at 72°C for a further 7 minutes to ensure full extension and then cooled to 4°C. 5μl of each sample was analysed by agarose gel electrophoresis (as described in section 2.2.4.6.) to check the correct product was amplified.

c) PCR of DNA containing microsatellite sequences.

Amplification of all microsatellite sequences were carried out the same way and not as specified in the original reference. Primers used are described further in table 3.

Reactions were carried out in 25μl final volumes containing 40ng DNA, 10mM Tris-HCl pH8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.001% (w/v) gelatin, 10% (v/v) DMSO, 140ng of each amplimer, 200μM of each of dATP, dTTP, dGTP, dCTP, and 1μl $\alpha(^{32}P)$ dCTP (diluted 1:30). This was heated to 94°C for 5 minutes then cooled to 85°C before 2.5 units *Taq* polymerase was added. Each reaction tube was placed in the thermocycler (Perkin-Elmer Cetus 9600) and subjected to 6 cycles of 94°C for 30 seconds, 60°C for 30 seconds, followed by 28 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. Extensions were completed by incubating finally at 72°C for 7 minutes and then cooling to 4°C.

5μl of each reaction was analysed by electrophoresis as described in section 2.2.4.6. on a nusieve agarose gel to check the product size.

Loss of heterozygosity at microsatellite positions were analysed by polyacrylamide gel electrophoresis as described in section 2.2.4.13.

Table 3

Microsatellite primers

Chromosome 9

Primer	Primer position	Reference
D9S 156	9p23-p22	Weissenbach *et al*, 1992
D9S 157	9p23-p22	Weissenbach *et al*, 1992
D9S 199	9p23	Graw and Kwiathowski, 1993
D9S 168	9p23-p22	Weissenbach *et al*, 1992
D9S 162	9p21	Weissenbach *et al*, 1992
Interferon α	9p21	Weissenbach *et al*, 1992
D9S 171	9p21	Weissenbach *et al*, 1992
D9S 55	9p11-p12	Sharma *et al*, 1991
D9S 127	9q31	Lyall *et al*, 1992
D9S 109	9q31	Furlong *et al*, 1992
D9S 116	9q31-q34	Kwiatkowski and Gusella, 1992
GSN	9q33	Kwiatkowski *et al*, 1993
ABL	9q34.1	Kwiatkowski, 1991
D9S 66	9q34-qter	Kwiatkowski *et al*, 1992

Chromosome 3

Primer	Primer position	Reference
D3S 1038	3p26.1-p25.2	Jones *et al*, 1992
D3S 1478	3p21.3-p21.2	Human genome mapping project
D3S 1076	3p21.2-p21.1	Jones *et al*, 1992
D3S 1067	3p21.1-p14.3	Jones *et al*, 1992
D3S 1477	3p14	Human genome mapping project

2.2.4.12. Sequencing.

a) Sample preparation from PCR reaction.

Amplification of DNA or cDNA was carried out as described in section 2.2.4.11. using a biotinylated "downstream primer" as detailed in tables 1 and 2

For each PCR reaction 20µl (200µg) of washed Dynabeads M-280 Streptavidin® was used. The beads were pre-washed by placing 20µl beads (10µg/µl) into an eppendorf and placing in a Dynal Magnetic Particle Concentrator (MPC). The beads are collected on one side of the tube and the supernatant removed. The beads were resuspended in 20µl TES (10mM Tris-HCl pH8.0, 1mM EDTA, 100mM NaCl), placed in the MCP and the supernatant again removed. The beads were finally resuspended in 40µl TES to a final concentration 5µg/µl. The prewashed beads were added to an equal volume of PCR reaction and incubated for 15 minutes keeping the beads resuspended. The tube was placed in the MCP and the supernatant removed. 100µl 0.15M NaOH was added, the beads resuspended and left at room temperature for 5-10 minutes. This converted the DNA to single-stranded form, the biotinylated strand being held onto the beads and the unbiotinylated strand was removed by placing the tube in the MCP and removing the NaOH solution (the unbiotinylated strand could be ethanol precipitated from this and used directly for sequencing). The beads were washed once with 100µl TES, once with 100µl H_2O and resuspended in 7µl H_2O for use in the sequencing reaction as outlined below.

b) Preparation of double-stranded plasmid DNA for sequencing.

Supercoiled DNA was separated from open-circular and linear DNA, RNA and other contaminants by separation on and purification from a 1% agarose gel as described in section 2.2.4.7b. The DNA spun from the filter paper was made up to 80µl with TE, denatured by adding 200mM NaOH, 0.2mM EDTA for 5 minutes at room temperature and precipitated by adding 0.5x volume 4M NH_4Ac and 2x volume ethanol at -20°C

overnight. The DNA precipitate was microcentrifuged for 10 minutes, washed with 70% ethanol, dried and redissolved in 7µl H_2O ready for sequencing as described below.

c) Sequencing reaction.

Sequencing was carried out essentially as described in the Sequenase® kit supplied by United State Biochemicals. 7µl DNA prepared as above was added to a solution containing 40mM Tris-HCl pH7.5, 20mM $MgCl_2$, 50mM NaCl and 20ng primer (detailed in table 4). The primer was annealed by incubation at 65°C for 2 minutes and slowly cooling to room temperature. The annealed DNA was labelled by adding 1µl DTT (0.1M), 1µl labeling mix (diluted 5 fold) and 0.5µCi (^{35}S) dATP. 2µl Sequenase® version 2 enzyme (diluted 8 fold) was added to the mixture and incubated at room temperature for 5 minutes. 3.5µl of this labelling reaction was added to each termination mixture (2.5µl) pre-heated to 37°C and incubated at 37°C for a further 5 minutes. The reactions were stopped by adding 4µl stop solution. The samples were heated to 75°C for 2 minutes immediately prior to loading onto a polyacrylamide gel as described in section 2.2.4.13.

2.2.4.13. Polyacrylamide gel electrophoresis.

a) Analysis of amplified microsatellite PCR samples.

Polyacrylamide gels were used to separate radiolabelled amplified DNAs differing in size by approximately 4-20 base-pairs. 5-11% polyacrylamide gels (ratio 30% acrylamide : 0.8% bisacrylamide) were cast in 1x TBE (90mM Tris, 90mM boric acid, 2mM EDTA) buffer and polymerised with 420µl APS and 42µl TEMED. The gel was poured between glass plates, pre-treated with repelcote, separated by 0.4mm spacers and a comb was inserted. After polymerisation the gel was placed in a vertical tank (Flowgen) with each end submerged in a reservoir of 1xTBE.

Table 4

Primers used for sequencing p53 from amplified cDNA.

Sequencing primer	Primer position	Primer sequence	Reference
Brash A	194bp	5'ACTGCCTTCCGGGTCACTGC3'	Brash *et al*, 1991
B	500bp	5'TCTGTCCCTTCCCAGAAAACC3'	Burns *et al*, 1993
Brash K	995bp	5'AGTGGTAATCTACTGGGACG3'	Brash *et al*, 1991

Note, the unbiotinylated primer from each PCR primer pair shown in table 1 and 2 were also used as sequencing primers.

Table 5

Primers used for site-directed mutagenesis.

Mutagenesis primer	Primer sequence
216 val→gly	5'ACATAGTGGGGTGGTGCCCT3'
248 arg→trp	5'GCATGAACTGGAGGCCCATC3'

DNA samples (5μl) were loaded into each well in loading buffer (30% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol) and run at 25W until the bromophenol blue dye (lower dye front) reached the bottom of the gel. Gels were of a percentage such that the microsatellite band ran halfway between the bromophenol blue and xylene cyanol bands in order to achieve maximum separation. The gel was then removed from the apparatus and transferred onto Whatman 3MM paper. It was covered in SaranWrap and exposed to Kodak X-OMAT S or X-OMAT AR film with intensifying screens at -70 °C.

Loss of heterozygosity (LOH) was scored by comparing the number of allele bands between cell lines (SCC12B, SCC12F and swiss 3T3 fibroblast). Where two bands were clearly visible a cell line was marked as informative. If one of these bands has been lost in another cell line then this was regarded as LOH. If all the cell lines showed only one band then these were scored as either uninformative or loss in all cell lines. Microsatellite analysis at each locus was repeated at least twice.

b) Analysis of sequencing reactions.

Sequencing reactions were analysed by denaturing gel electrophoresis. An 8% polyacrylamide gel (ratio acrylamide to bisacrylamide 29.1:0.9) in TBE containing 50% (w/v) urea was polymerised with 0.04% APS and 0.05% TEMED between gel plates pretreated with repelcote. The gel was then placed in a vertical apparatus (Flowgen) with both ends submerged in 1xTBE buffer. Samples were heated to 75°C for 2 minutes and loaded onto the gel using a sharks-tooth comb. The gel was run at 40W for approximately 3 hours. The gel was transferred onto 3MM Whatman paper, covered with SaranWrap and dried at 80°C for 2 hours. The gel was exposed directly onto Kodak X-OMAT AR or S film at room temperature. Autoradiographs were read from the bottom (5' end of DNA) upwards.

2.2.4.14. Dot Blot analysis

DNA or cDNA was amplified by PCR as above and serial dilutions of each sample made. 20µl of diluted DNA was denatured by adding 100µl 0.4M NaOH, 25mM EDTA and heating to 95°C for 2 minutes. The samples were then placed on ice and 100µl 10x SSC (1.5M NaCl, 0.15M sodium citrate pH7.0) was added. The Hybond nylon filter was moistened in 20x SSC (3M NaCl, 0.3M sodium citrate, pH 7.0) and fitted into a dot blot manifold attached to a vacuum. Each denatured DNA solution was added to the appropriate well followed by 100µl 10x SSC and allowed to dry under a vacuum for a further 10 minutes. The filter was removed from the manifold and allowed to dry overnight.

Oligonucleotides (synthesised as described in section 2.2.4.10) specific to either wild-type (5' ACATAG**TGT**GGTGGTGCCCT 3') or the 216 p53 mutant (5' ACATAG**TGGGGT**GGTGCCCT 3') were labelled with γ ^{32}P dATP. 100ng of each oligonucleotide was incubated with 50mM Tris-HCl pH7.5, 10mM $MgCl_2$, 5mM DTT, 0.1mM spermidine, 0.1mM EDTA, 50µCi γ ^{32}P dATP and 20 units T4 polynucleotide kinase for 45 minutes at 37°C. The labelled probe was purified on a sephadex G-25 nick column. Prior to use the column was washed with 1ml TE and the probe added and eluted in the second volume of 400µl TE added to the column. Radioisotope incorporation in the probe was measured using a scintillation counter.

The filter was cut into strips and crosslinked on a UV lightbox, DNA side down, for 5 minutes and the other side for 1 minute. The filters were pre-hybridised in hybridisation buffer containing 5x SSPE (0.75M NaCl, 44mM NaH_2PO_4, 5mM EDTA), 5x Denhardts solution (0.1% (w/v) ficoll 400, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) BSA), 0.5% SDS, and 100mM sodium pyrophosphate pH 7.5, for 1-2 hours at 37°C. $5x10^6$ cpm/ml of labelled probe was incubated with the filter in hybridisation buffer for at least 2 hours at 37°C. The filters were then washed in 6x SSC twice, each time for 10 minutes at room temperature. Non-specifically bound probe was removed by washing the filters at exactly 61°C in 3M tetramethylammonium chloride (TMAC), 50mM Tris, pH 8.0, 2mM EDTA and 0.1% SDS for 20 minutes. This removed any

oligonucleotide mismatched by even 1 base pair. Monitoring the filter whilst washing ensured adequate washing. The filters were then washed again in 6x SSC at room temperature for 10 minutes, sealed in plastic and autoradiographed at -70°C for 2-16 hours. Each dot blot was repeated at least twice.

2.2.4.15. Oligonucleotide site-directed mutagenesis

Mutagenesis was carried out as described in the Muta-gene® M13 mutagenesis kit from Bio-rad and outlined briefly below. All bacterial strains and vector DNAs were supplied in the kit. Oligonucleotides were synthesised as described in section 2.2.4.10. and are detailed in table 5.

a) Cloning of DNA into M13 vector

Wild-type p53 was removed from the vector pC53-SN3 by a Bam H1 digest and was inserted into the Bam H1 site in the vector M13mp18 as described in sections 2.2.4.5 to 2.2.4.8.

b) Preparation of competent MV1190 cells and transformation with M13mp18/p53

E.coli MV1190 is unable to utilise lactose or biosynthesis proline. The F' plasmid carries the proline synthesis gene and a truncated β-galactosidase gene which can be complimented by a fragment coded for by M13 phage. If this fragment in M13 is intact (i.e. no insert) then cells carrying this phage will produce blue plaques in the presence of the inducer IPTG and the dye indicator x-gal. If DNA (to be mutated) has been successfully inserted into M13 then colourless plaques will result. The F' plasmid necessary for pili formation via which M13 phage enters the cell is selected for as it allows growth in the absence of proline.

MV1190 cells were streaked onto a glucose-minimal plate (77mM KH_2PO_4, 25mM K_2HPO_4, 8mM NaCl, 19mM NH_4Cl,1mM $MgSO_4.7H_2O$, 0.001% thiamine, 0.2% glucose, 1.5% bactoagar) and grown at 37°C until colonies appear. This selects for cells containing the F' plasmid. A colony was used to inoculate 10 ml L broth and grown overnight. 250ml L broth was inoculated with enough overnight culture to give an OD_{600} 0.1 and grown to an OD 0.8-0.9. The cells were then pelleted at 5000 rpm for 5 minutes at 4°C and resuspended in 50ml ice-cold 100mM $MgCl_2$ and left on ice for 30 minutes. The cells were again pelleted and resuspended gently in 10ml ice-cold 100mM $CaCl_2$. A further 100ml $CaCl_2$ was added and incubated on ice for 30-90 minutes. The cells were pelleted and resuspended in 12.5ml 85mM $CaCl_2$, 15 % glycerol. The cells were aliquoted into 0.5mls and frozen in a dry ice/ethanol bath and kept at -70°C.

0.3ml competent MV1190 cells were transformed with 1-10ng ligation mixture on ice for 30-90 minutes. The cells were heat shocked at 42°C for 3 minutes and returned to ice. 10 or 50µl of transformed cells were added to 0.3 ml MV1190 overnight culture. 50µl 2% X-gal and 20µl 100mM IPTG were added to 2.5ml molten top agar (medium containing 0.7% bactoagar). This was cooled, added to the cells and immediately poured onto H agar plates (1% bactotryptone, 8mM NaCl, 1.5% bactoagar). The top agar was allowed to solidify and inverted overnight at 37°C. Clear plaques were picked and placed into 1ml TE and kept at 4°C.

Stocks of recombinant phage were prepared as described in Maniatis *et al*, 1989. Briefly, 50µl of MV1190 overnight culture was added to 2ml L broth. 100µl of phage suspension was added and incubated for 5-6 hours at 37°C. 1ml was pelleted at 12,000g for 5 minutes and the supernatant containing the phage was stored at 4°C. The remaining culture was used to make a DNA miniprep from phage infected cells as described in Maniatis *et al*, 1989 and the presence of a correct insert verified by digestion as described in section 2.2.4.5.

c) Titering phage.

The above M13 phage was titered on MV1190 cells. A colony of MV1190 cells was picked from a glucose minimal plate and used to inoculate 20ml L broth. This was grown overnight at 37°C. 3ml molten top agar was aliquoted into sterile tubes and kept at 55°C. The above phage stock was diluted in 4 serial, 100-fold dilutions in L broth. 0.2ml of the MV1190 overnight culture was then added to 100µl of the last 3 dilutions and incubated at room temperature for 5 minutes. One tube of top agar was mixed with each phage/culture mix and poured immediately onto an L broth agar plate. This was allowed to harden and then incubated at 37°C overnight. The number of plaques per plate were counted and the titer calculated using the formula:

titer = (no. plaques x 10 x dilution factor) pfu/ml.

d) Growth of Uracil-containing phage.

E.coli CJ236 has a *dut*-1, *ung*-1 phenotype which results in occasional uracils being substituted for thymine in all DNA synthesised in the bacterium. The F' plasmid which enables pili formation and phage infection was selected for by growing in the presence of chloramphenicol.

50ml 2xYT (1.6% bactotryptone, 1% yeast extract, 8mM NaCl) medium plus 30µg/ml chloramphenicol was inoculated with 1ml CJ236 overnight culture and grown until OD_{600} was 0.3 which corresponds to $1x10^8$ cfu/ml. The titered phage was added to a multiplicity of infection of 0.2 and the culture further incubated at 37°C for 4-6 hours. The cells were pelleted at 17,000g for 15 minutes. 150µg RNase A was added to the supernatant, containing the uracil containing phage, and incubated at room temperature for 30 minutes. 0.25x volume of 3.5M NH_4Ac, 20% PEG 8000 was added to the supernatant and held on ice for 30 minutes. The precipitate was collected by centrifugation at 17,000g for 15 minutes at 4°C. The pellet was drained and resuspended in 200µl high salt buffer (300mM NaCl, 100mM Tris pH8.0, 1mM EDTA). This was

incubated for 30 minutes on ice, centrifuged briefly and the supernatant stored at 4°C. The efficiency of infection of the phage on CJ236 cells was calculated by titering the uracil-containing phage stock on CJ236 and MV1190 as described in part c) of this section. As uracil-containing phage are inactive on MV1190, the titer on MV1190 was 10^4 or less lower than that on CJ236 cells indicating successful uracil incorporation into the M13 phage DNA.

Single-stranded DNA was extracted from the phage by phenol/chloroform extraction and precipitation with 0.1x volume 7.8M NH_4Ac and 2.5x volume ethanol at -70°C for 30 minutes.The DNA was pelleted by centrifugation, washed and re-dissolved in TE.

e) Synthesis of mutant strand.

The oligonucleotide containing the required mutation was firstly phosphorylated by incubating 200pmol oligonucleotide with 100mM Tris pH 8.0, 0.2M $MgCl_2$, 0.1M DTT, 1mM ATP, 4.5 units T4 polynucleotide kinase and incubating at 37°C for 45 minutes. The reaction was stopped by heating to 65°C for 10 minutes.

2-3pmol oligonucleotide was annealed to 200ng uracil-containing DNA in 20mM Tris-HCl pH7.4, 2mM $MgCl_2$, and 50mM NaCl. The reaction was heated at 70°C for 5 minutes, cooled slowly to 30°C, then placed on ice.

The complementary DNA strand was synthesised by adding 0.4mM of each of dATP, dTTP, dCTP, dGTP, 0.75mM ATP, 17.5mM Tris-HCl pH7.4, 3.75mM $MgCl_2$, 21.5mM DTT, 2-5 units T4 DNA ligase and 0.5 units T7 DNA polymerase to the above and leaving on ice for a further 5 minutes. The reaction was continued at 25°C for 5 minutes followed by 37°C for 30 minutes and stopped by adding 90µl 10mM Tris pH8.0, 10mM EDTA and freezing.

The mutant phage DNA was then transformed into MV1190 (which will only replicate the non-uracil containing template i.e. mutant sequence) as described in part b) of this section. Single-stranded DNA was prepared as in part d) and sequenced as described in section 2.2.4.12. to identify phage carrying the required mutation.

2.2.5. Cytogenetics

2.2.5.1. Preparation of metaphase chromosome spreads from human SCC for FISH

Glass slides were pre-soaked in a solution of 3% conc. HCl in 70% methanol : 30% H_2O for a minimum of 1 hour but not more than 24 hours. Slides were then rinsed in distilled water and stored at 4°C until required.

0.03μg/ml colcemid was added to 50% confluent monolayers of SCC cell lines in 5cm dishes and incubated for 2.5-3 hours. The cells were then trypsinised and pelleted at 1000rpm for 5 minutes. The pellet was flicked up and 1.5ml hypotonic solution (0.075M KCl pre-warmed to 37°C) was added drop-wise, using a pasteur pipette, with constant agitation. A further 5ml was added, the tube inverted and incubated at 37°C for 18 to 20 minutes. The swelling was stopped by adding 10 drops of freshly prepared Carnoy's fixative (3:1 methanol: glacial acetic acid), inverting to mix and pelleting the cells as above. The pellet was again gently flicked up and resuspended in 1.5ml freshly prepared fixative with constant agitation to prevent clumping. A further 5ml fixative was added, mixed by inverting and incubated at room temperature for 10 minutes. The cells were again pelleted and resuspended in fixative as above and incubated for 30 minutes at room temperature. The cells were pelleted and resuspended in sufficient fixative to give correct density for dropping slides.

50μl aliquots of the above metaphase preparations were dropped onto the prepared drained slides. The slides were allowed to air dry before checking the spreads.

Slides used for FISH analysis were aged for 1-2 weeks before use. This was found to lower background.

2.2.5.2. Fluorescence In situ hybridisation (FISH).

The slides carrying the chromosome spreads were fixed in freshly prepared Carnoy's fixative for 1 hour and rinsed in 2x SSC. They were then treated with

100μg/ml RNase in 2x SSC for 1 hour at 37°C and rinsed in 2x SSC. Protein was digested by incubating the slides in 0.01% pepsin in 0.01M HCl for 10 minutes at 37°C, then rinsed in PBS. The spreads were fixed again by placing in Streck tissue fixative for 10 minutes at room temperature. The slides were then rinsed in PBS and dehydrated twice in 70% ethanol for 2 minutes and twice in 100% ethanol for 2 minutes and allowed to air dry.

The chromosomes were denatured on the slides in 70% formamide in 2x SSC at 70 °C for 5 minutes. The slides were then washed in a large volume of 70% ethanol and dehydrated in 70% ethanol and 100% ethanol as before.

The p53 cosmid probe (p5106-DIG) was denatured immediately before use, at 37° C for 5 minutes. The chromosome 17 centromere probe (αCHAD 9) was diluted 1:25 and denatured at 70°C for 5 minutes. 10μl of each probe was dropped onto the spreads and covered with a coverslip. Cowgum was used to seal around the edges of each coverslip and the slides were placed in a dark, humidified box at 37°C overnight.

The coverslips were removed in 2x SSC and the slides washed twice in 50% formamide, 1x SSC at 42°C for 20 minutes. The slides were then washed in 2xSSC at 42°C for 20 minutes. The probe was detected by firstly blocking in PN-TB (0.1M NaH_2PO_4, 0.1M Na_2HPO_4, 0.05% Tween) buffer for 15 minutes at room temperature. 100μl primary antibody (sheep anti-digoxigenin, diluted 1:200 in PN-TB) was placed onto each slide and covered with parafilm. The slides were incubated for 1 hour, in the dark, at room temperature. The slides were then washed for 10 minutes in PN-TB at room temperature. The secondary antibody, FITC conjugated donkey anti-sheep, was diluted 1:500 in PN-TB and 100μl placed on each slide as described above. The slides were again incubated for 1 hour at room temperature in the dark. The slides were finally washed in PN-TB for 20 minutes at room temperature, dehydrated in 70% and 100% ethanol as before and mounted in antifade medium (Vectashield).

Hybridisations were analysed using a Biorad confocal microscope MRC600 with a krypton argon laser.

Chapter 3

Genetic analysis of SCC12B and SCC12F

3.1. Characterisation of SCC12F and SCC12B.

SCC12B and SCC12F were originally derived from the same tumour but appear to differ in their ability to terminally differentiate and undergo controlled cell death when detached from the culture dish and also differ in their degree of tumorigenicity as discussed in section 1.1.2. The two cell lines were therefore analysed for genetic differences in order to try and explain their different phenotypes.

3.1.1. Chromosome 9.

Microsatellite analysis was carried out on both arms of chromosome 9 using a wide range of primers as detailed in table 3 (section 2.2.4.11). These microsatellites were investigated in SCC12F and SCC12B as they have previously been reported to show a high degree of LOH in SCC-HN (Loughran *et al.*, 1994; Edington *et al.*, 1995). There is also an 84% chance of LOH at 9p21 in both lines based on the fact that the chance of all six microsatellites in this region being non-informative is less than 16% (Loughran *et al.*, 1994; Latif *et al.*, 1992).

Loss of heterozygosity (LOH) at a certain locus is usually scored by comparing the number of allele bands between tumour DNA and normal DNA from the same individual. In this case normal DNA was not available for analysis but it was sufficient to compare SCC12F with SCC12B to highlight any differences in LOH between the two cell lines. Examples of PCR of microsatellite allele bands GSN and D9S157 are illustrated in figure 3.1 and the complete data is summarised in figure 3.2. Clones 19 is a single cell clone of SCC12F (see section 5.1.1 for further details). Swiss 3T3 fibroblast DNA was included as a control in order to eliminate any microsatellite amplification due to contamination of the cell line DNA with feeder DNA. No microsatellite amplification from the 3T3 DNA was observed at any loci. Where two bands were visible in all cell lines, this was marked as informative and no loss of heterozygosity. If one allele band had disappeared but was clearly visible in the other cell lines then this was recorded as loss of heterozygosity. If only one band was apparent in all cell lines then these were scored as either

Figure 3.1

Microsatellite analysis of chromosome 9

Microsatellite amplification at two loci, GSN (top) and D9S157 (bottom) on chromosome 9 are shown for examples. The remaining microsatellite data is summarised in figure 3.2.

Cell lines SCC12B, SCC12F and two clones of SCC12F clone 19 and clone 24 are compared. 3T3 DNA was incorporated to eliminate the possibility of contamination from feeder cells. No microsatellite amplification from 3T3 DNA was observed at any loci.

GSN (top) Amplified microsatellite DNA was electrophoresed on a 9% polyacrylamide gel and the product size was approximately 89-117bp. The microsatellite band is shown by the lower arrow and the shadow band above.

D9S157 (bottom) Amplified microsatellite DNA was electrophoresed on a 10% gel and the product size was 133-157bp. The microsatellite and shadow bands are again shown by the arrows.

Both loci show amplification of only one allele band in all four lanes.

Figure 3.1

Microsatellite analysis of chromosome 9

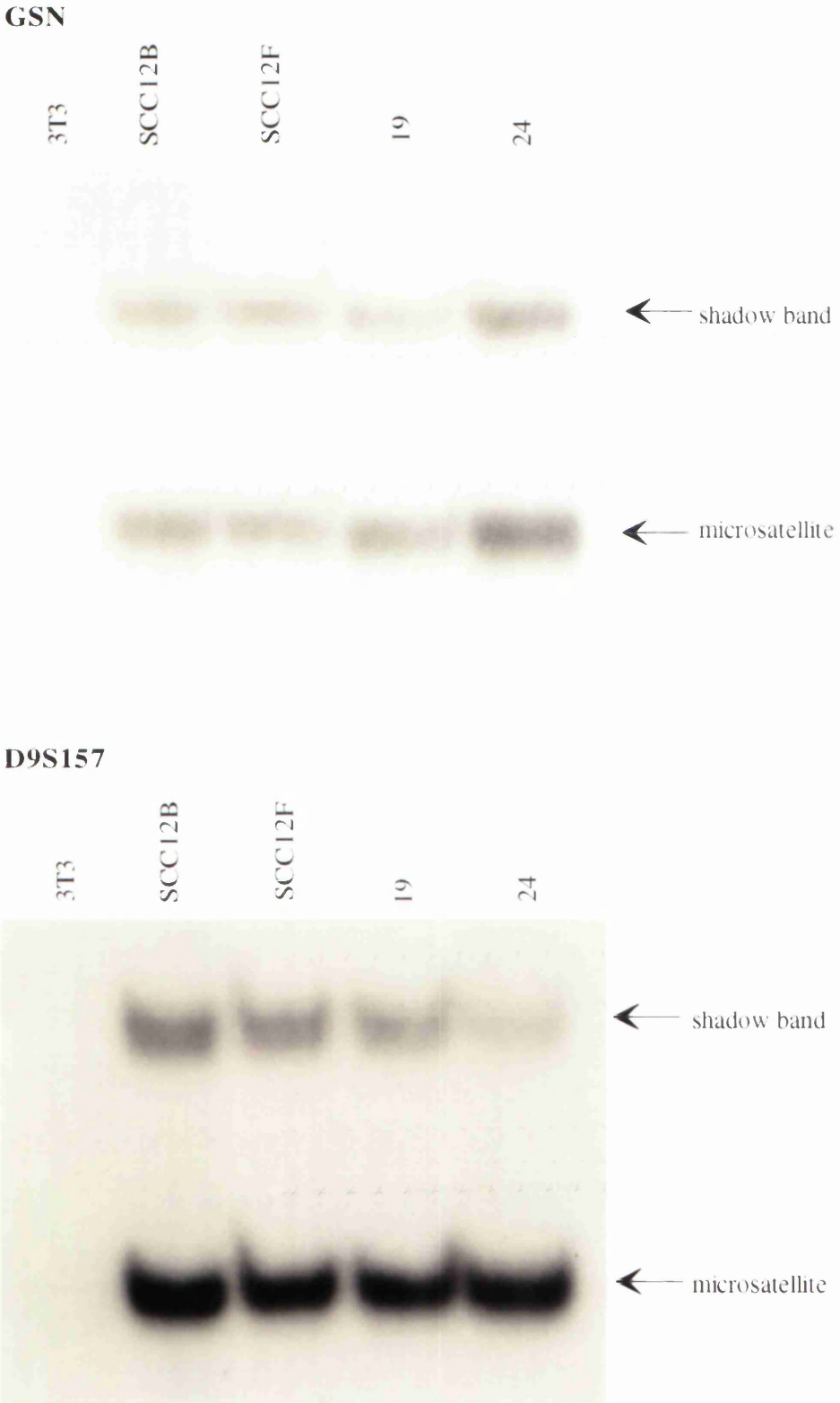
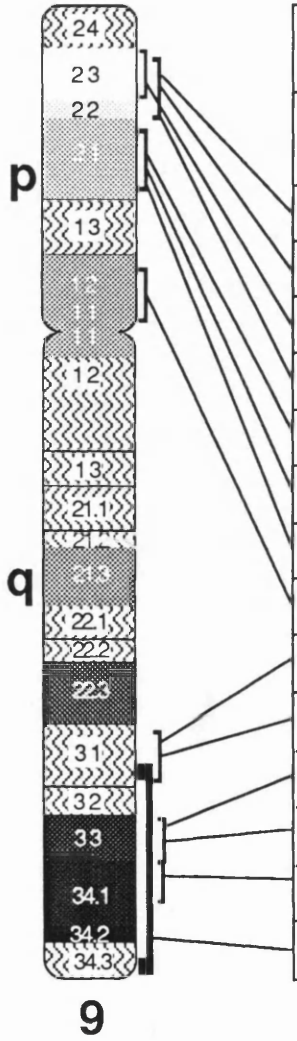


FIGURE 3.2 SUMMARY OF MICROSATELLITE DATA FOR CHROMOSOME 9



PRIMER	CELL LINE			
	SWISS 3T 3	SCC12B	SCC12F	CLONE19
D9S156	N.A.	●	●	●
D9S157	N.A.	●	●	●
D9S199	N.A.	●	●	●
D9S168	N.A.	●	●	●
D9S162	N.A.	●	●	●
INF α	N.A.	●	●	●
D9S171	N.A.	●	●	●
D9S55	N.A.	●	●	●
D9S127	N.A.	●	●	●
D9S109	N.A.	●	●	●
D9S116	N.A.	●	●	●
GSN	N.A.	●	●	●
ABL	N.A.	●	●	●
D9S66	N.A.	●	●	●

KEY

● 1 ALLELE

◐ 2 ALLELES

N.A. NO AMPLIFICATION OF MICROSATELLITE

noninformative or loss in all cases but as no normal DNA was available to make comparisons this result was inconclusive. Results are expressed as amplification of one allele or two alleles (figure 3.2) and SCC12F, SCC12B and clone 19 were compared.

The "shadow" bands (shown by the arrows on figure 3.1) appearing at larger increments are common in PCR of microsatellites . It is unclear as to what they represent or how they can be eliminated, but it has been suggested that where there is a vast excess of primers, the shadows are caused by amplified, partially denatured product with extra primer bound at the denatured ends (D.Black, personal communication).

As summarised in figure 3.2, no differences in the amplification of microsatellites investigated on chromosome 9 between SCC12B, SCC12F and clones 19 was apparent.

3.1.2. Chromosome 3p

Microsatellite analysis was also carried out on the short arm of chromosome 3 using the primers as detailed in table 3 (section 2.2.4.11). These loci have previously been shown to exhibit a high degree of LOH in SCC-HN (Latif *et al.*, 1992; Edington *et al.*, 1995) and therefore were good candidates on which to find differences in LOH in SCC12B and SCC12F.

An illustration of amplified microsatellite allele bands obtained from primers D31067 and D31038 are shown in figure 3.3 and the complete micosatellite data for chromosome 3p is reported in figure 3.4. Results were scored as for chromosome 9 described in section 3.1.1.

No differences in the microsatellite amplification pattern on the short arm of chromosome 3 were observed between SCC12B, SCC12F and clones 19.

3.1.3. Cyclin D1

Cyclin D1 protein levels were compared between SCC12B, SCC12F and clone 19 by western analysis. Results are shown in figure 3.5. BICR 6 was used as a positive control as it has been shown to overexpress cyclin D1 (Nikolic *et al*, submitted for

Figure 3.3.

Microsatellite analysis of chromosome 3

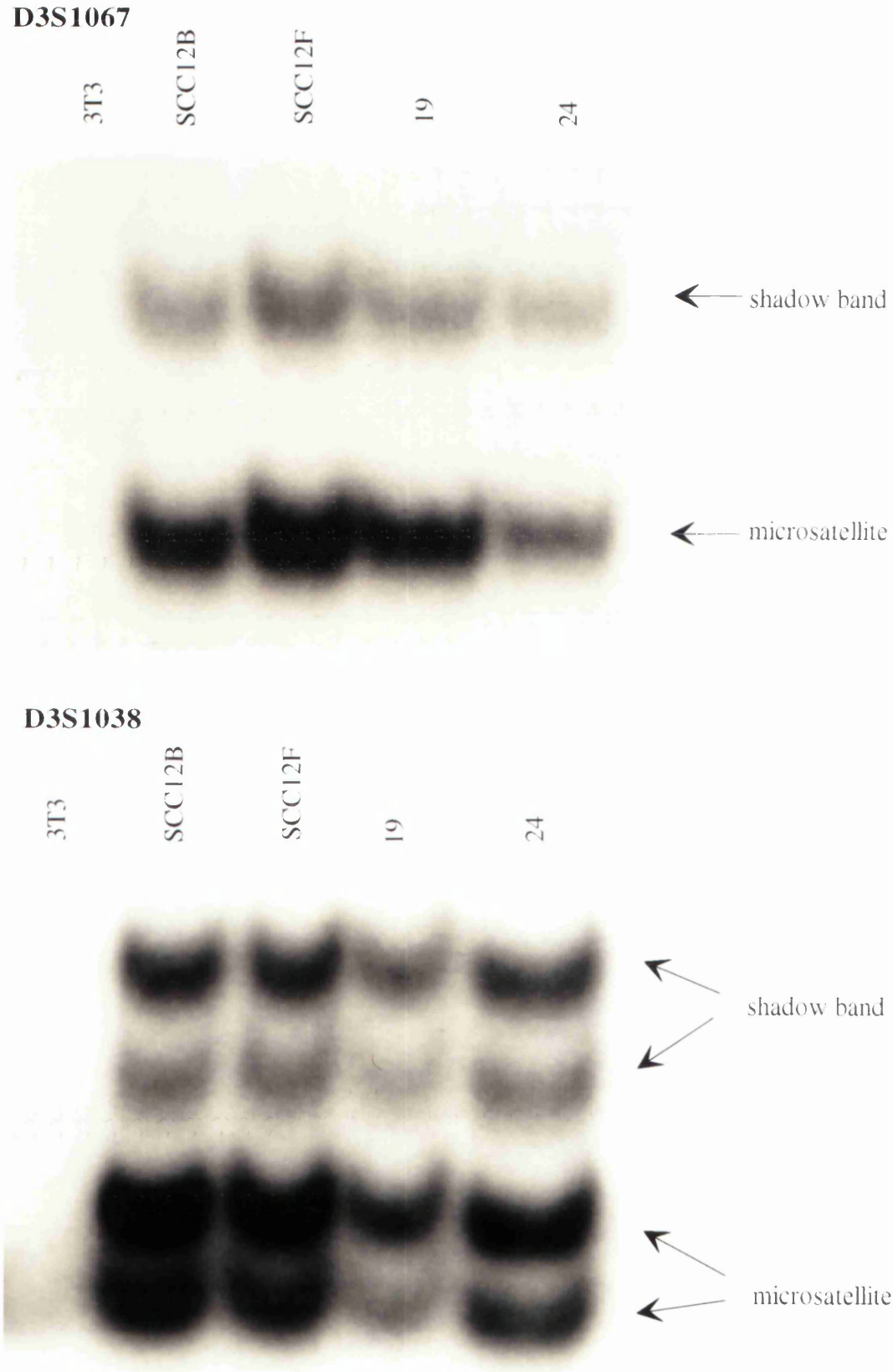
Microsatellite amplification at two loci, D3S1067 (top) and D3S1038 (bottom), on chromosome 3 are shown for examples. The remaining data is summarised in figure 3.4

Cell lines SCC12B, SCC12F and two clones of SCC12F clone 19 and clone 24 are compared. 3T3 DNA was incorporated to eliminate the possibility of contamination from feeder cells. No microsatellite amplification from 3T3 DNA was observed at any loci.

D3S1067 (top) Amplified microsatellite DNA was electrophoresed on a 10% polyacrylamide gel and the product size was approximately 95bp. The microsatellite and shadow band are shown by the arrows.
This microsatellite showed amplification of only one allele band in all four cell lines.

D3S1038 (bottom) Amplified microsatellite DNA was electrophoresed on a 10% polyacrylamide gel and the product size was approximately 115bp. The microsatellite and shadow bands are shown by the arrows.
All cell lines were heterozygous at this microsatellite showing amplification of both allele bands in all four cell lines.

Figure 3.3 Microsatellite analysis of chromosome 3



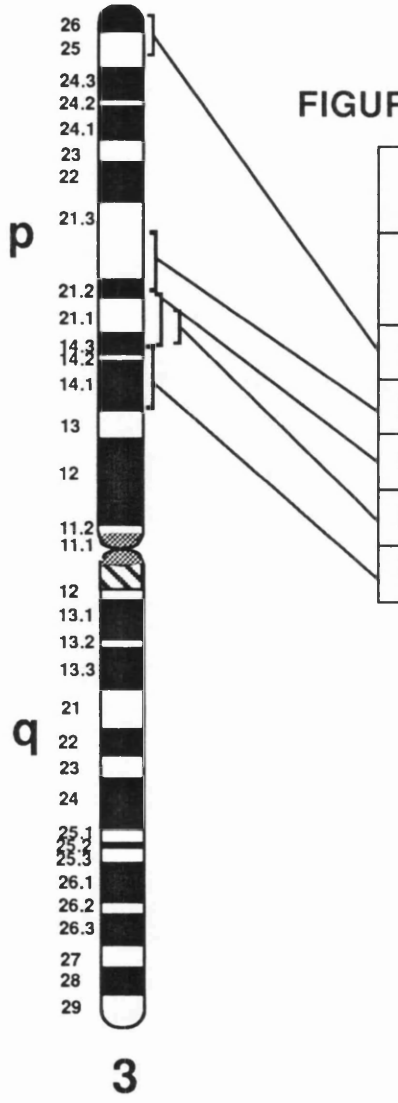


FIGURE 3.4 SUMMARY OF MICROSATELLITE DATA FOR CHROMOSOME 3

PRIMER	CELL LINE			
	SWISS 3T 3	SCC12B	SCC12F	CLONE19
D3S1038	N.A.	◐	◐	◐
D3S1478	N.A.	◐	◐	◐
D3S1076	N.A.	●	●	●
D3S1067	N.A.	●	●	●
D3S1477	N.A.	◐	◐	◐

KEY

● 1 ALLELE

◐ 2 ALLELES

N.A. NO AMPLIFICATION OF MICROSATELLITE

publication). Human cyclin D1 was seen as a band at approximately 34kDa and a mouse specific peptide was seen as a faint band approximately 100kDa. This 100kDa band was used as an indicator of the extent of contamination of mouse 3T3 feeders in the protein extract, and as can be seen from the western this contamination was very low.

The western blot shows that there is no difference in the levels of cyclin D1 protein expression between cell lines SCC12B, SCC12F and clone 19.

3.2 Analysis of p53 in SCC12B and SCC12F

The p53 tumour suppressor gene is frequently mutated in a wide variety of cancers (discussed in section 1.4.3.1.) and has been shown to be important in the progression of tumours in the oral cavity (Gusterson *et al.*, 1991; Burns *et al.*, 1993; Burns *et al.*, 1994a and b). It is therefore of interest to analyse and compare the p53 status in SCC12F and SCC12B in order to assess the role that p53 plays in the progression of this particular tumour.

The level of p53 protein is very low in normal cells due to its short half-life and is therefore difficult to detect by conventional protein detection methods such as western blotting and immunoprecipitation. Mutation of p53 however leads to a highly stabilised protein with a much increased half-life that is readily detected by these methods. The detection of high levels of p53 is therefore used as an early indication that the protein may have acquired a mutation and indeed in SCC-HN overexpression of the protein has been shown to always correlate with a p53 missense mutation or an in-frame deletion (Burns *et al.*, 1993; Burns *et al.*, 1994b).

3.2.1. p53 protein levels

p53 protein levels were analysed in SCC12B, SCC12F and single-cell clones of SCC12F clone 19 and clone 24 as shown in figure 3.6. SCC4 was used as a positive control as it has been shown to have a stabilised p53 protein and a point mutation at codon 151 (Burns *et al.*, 1993). HFF9 cells (human foetal fibroblasts) express wild-type

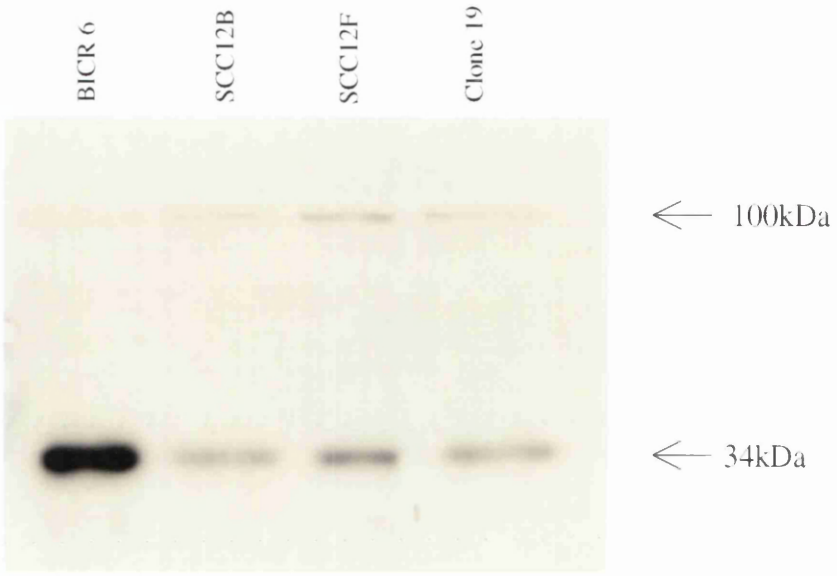


Figure 3.5 Western blot analysis of Cyclin D1 in SCC cell lines

50ug of protein extracts were electrophoresed through a 10% denaturing polyacrylamide gel and blotted, as described in materials and methods. The rabbit polyclonal antibody (287-3) was used at a 1:500 dilution. BICR 6 cell line overexpresses human p34 cyclin D1 and wasused as a positive control. Arrows point to a murine specific peptide ~ 100kD and the cyclin D1 specific 34kD protein.

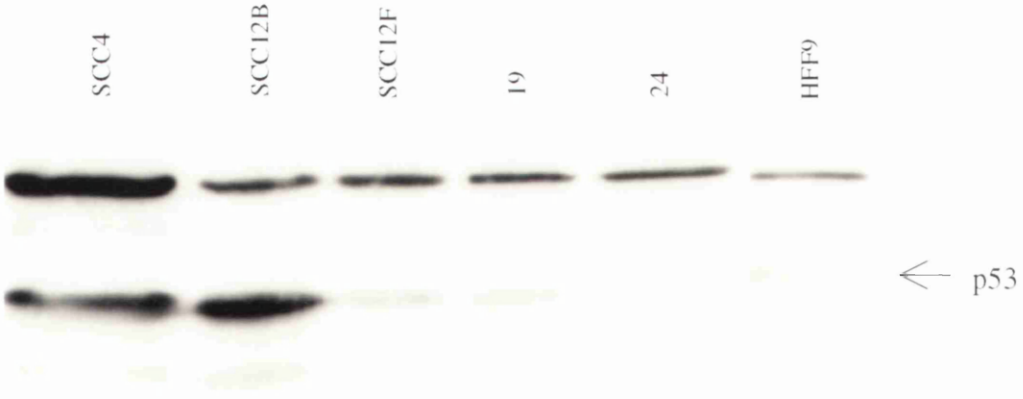


Figure 3.6 Western blot analysis of p53 in SCC cell lines

250ug of protein extracts were electrophoresed through a 10% denaturing polyacrylamide gel.as described in materials and methods. The antibody PAb1801 was used at a final concentration of 1ug/ml. SCC4 expresses mutant p53 containing a mutation at codon 151. HFF9 cells express wild-type p53 which can hardly be detected. Arrow points to p53 protein.

p53 therefore giving a very faint p53 signal in this assay. This cell line also enabled the p53 protein band to be identified as the lower of the two bands shown in figure 3.6 migrating at approximately 53kDa and often seen as a doublet (Harlow *et al*, 1985).

Interestingly, SCC12B appears to have very high levels of p53 compared with SCC12F. Clones of SCC12F have variable levels of p53 protein (see also figure 5.1) and clone 19 was used as a target cell in subsequent experiments (see section 5.1.).

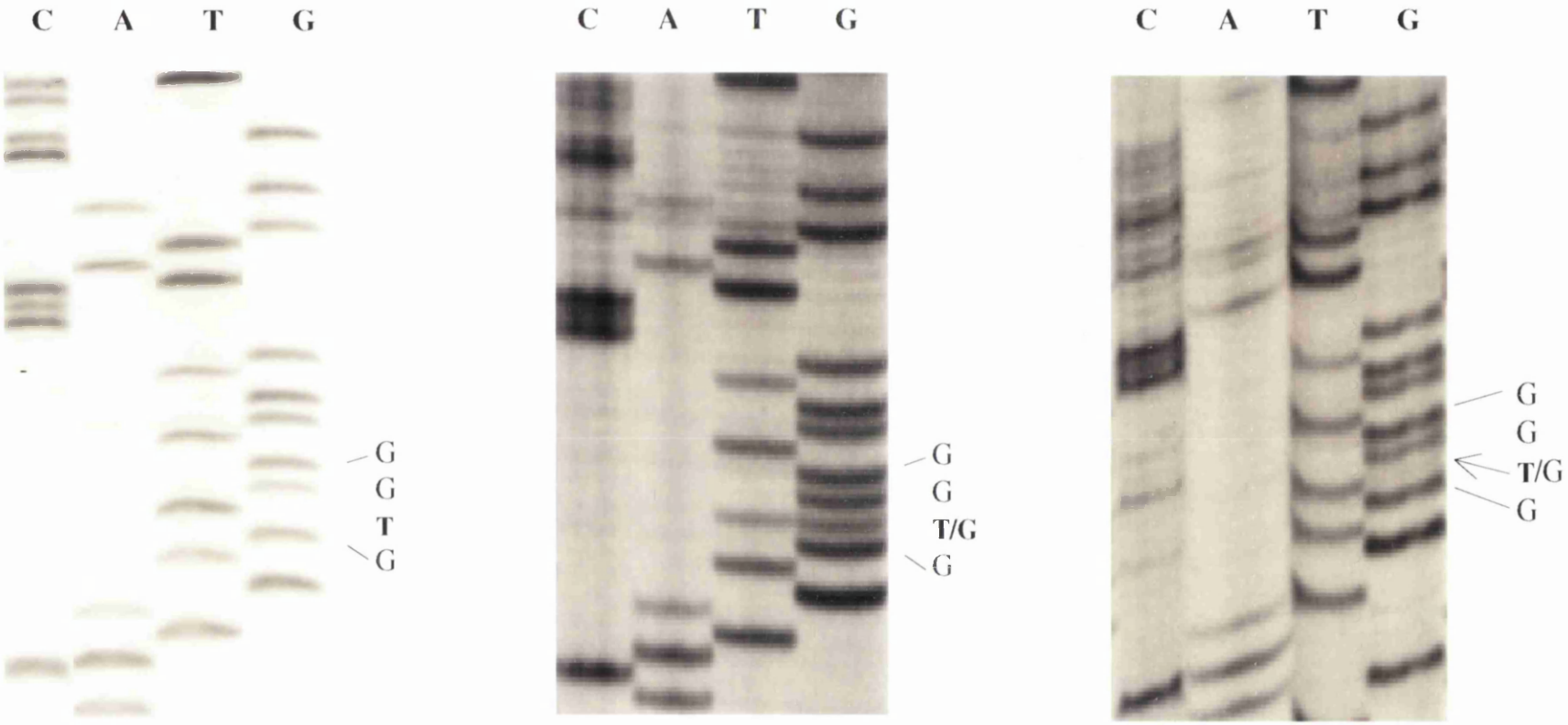
3.2.2 Identification of a p53 mutation in SCC12B and SCC12F

Detection of high levels of p53 protein indicates the presence of a stabilised p53 protein and suggests the possibility of a p53 mutation. SCC12B and SCC12F appear to have different levels of p53 protein (as shown in the above section) and were therefore sequenced for a p53 mutation. Total RNA was reverse transcribed into cDNA and various fragments of p53 were amplified by PCR using the p53 specific primers which are detailed in table 1. The location of the PCR primers and also the sequencing primers within the p53 cDNA are illustrated in figure 2.1. The complete coding sequence, codons 1-393 of p53 cDNA was sequenced in both cell lines. SCC12B was found to contain a novel p53 mutation at codon 216, a T→G transversion causing a valine to be substituted for a glycine, as shown in figure 3.7. Interestingly, the mutation appears to be a heterozygous one with the wild-type p53 allele unusually still being expressed. SCC12F expresses much less p53 protein (figure 3.6) and accordingly appears to express very little $p53^{216}$ mutant transcript as shown by the very faint additional G base highlighted by the arrow on the sequence in figure 3.7. SCC12F appears to mostly express the wild-type p53 allele. The normal wild-type sequence at codon 216 from BICR 3 (which has been shown to have an alternative mutation at codon 282 (Burns *et al*, 1993) is shown in figure 3.7 for comparison.

Exon 6 amplified DNA from both cell lines was sequenced directly with primer 6U (table 2) and identical results were obtained as from RNA

Whilst searching for a mutation in p53 a polymorphism was identified at codon 72 exchanging a proline for an arginine in both cell lines. Differences in the sequence of

Figure 3.7 **Sequence analysis of p53 cDNA from SCC12B and SCC12F**



Total RNA was reverse transcribed into cDNA and then amplified using p53 primers K->G. The fragment was then sequenced using primer K as described in materials and methods. Codon 216 is shown by the letters above. BICR3 shows the normal p53 sequence GTG; SCC12B shows a heterozygous mutation where both the wild-type GTG and mutant GGG sequences are seen. SCC12F mostly expresses the wild-type transcript but a faint G base can be seen highlighted by the arrow suggesting that SCC12F expresses some mutant p53 but not as much as SCC12B.

codon 72 have been observed by other investigators analysing cDNA and genomic DNA from various sources (Buchman *et al*, 1988; Lamb and Crawford, 1986). This amino acid change has been shown to cause a difference in protein mobility on SDS-polyacrylamide gels. It is a fairly frequent polymorphism and both forms of p53 have been shown to be functional. The presence of this polymorphism in both cell lines is further proof that SCC12F and SCC12B originated from the same tumour.

3.2.3. p53 gene dosage in SCC12F and SCC12B

Both western blot analysis and sequencing of p53 as shown previously, showed that SCC12F and SCC12B express different dosages of wild-type and mutant $p53^{216}$ protein. The dosage of each were quantified further by dot blot analysis probing both cDNA amplified with primers K and G and DNA amplified with 6U and 6D. Equal amounts of amplified cDNA or DNA (as judged by agarose gel electrophoresis) were used for each cell line and 2 fold dilutions were added to the filter vertically. Normal human keratinocytes (HEK) was used as a control for background hybridisation.

The cDNA dot blot is shown in figure 3.8a. The density of each spot was measured by molecular dynamics laser densitometry using PDI image analysis software and is summarised in figure 3.8b. The dot blot confirms that SCC12B expresses more mutant p53 transcript than SCC12F and the PDI measurements suggests that there is a two fold difference if the density of each spot at varying dilutions are compared between the two cell lines. The background hybridisation is very low as shown by the lack of hybridisation of the mutant probe to HEK cDNA.

The blot probed with the wild-type oligonucleotide shows that SCC12F and SCC12B appear to express lower levels of wild-type p53 than HEK cells. This is not surprising in the case of SCC12B which has been shown to express mutant and wild-type sequences and it is therefore likely that the level of wild-type p53 would be lower than in normal HEK cells. However sequence analysis suggests that SCC12F expresses mostly wild-type DNA and RNA. The difference in the wild-type spot intensities on the cDNA dot blot in figure 3.8 and also on the DNA dot blot in figure 3.9 between SCC12F and

Figure 3.8a

cDNA dot blot analysis

Total RNA from HEK, SCC12B and SCC12F was reverse transcribed and amplified by PCR using primers K→G. Equal amounts of DNA were hybridised to each filter with two fold serial dilutions added vertically. Either wild-type p53 (right) or $p53^{216}$ mutant (left) oligonucleotide was used as a probe and any non-specific binding was removed by washing in TMAC at 61°C as described in section 2.2.4.14.

Figure 3.8b

Densitometry measurements

The density of each spot in the dot blots in figure 3.8a above were measured by molecular dynamics laser densitometry using PDI image analysis software. Each measurement illustrated in the table corresponds to the dot in the identical position on the blots in figure 3.8a. Density is measured in pixel density units ($ODxMM^{2)}$ which is a function of spot intensity and the number of pixels covered. Only those dots that can be seen by eye are included in the tables.

Figure 3.8a

cDNA dot blot probed with wild-type or p53^{216} mutant oligonucleotides.

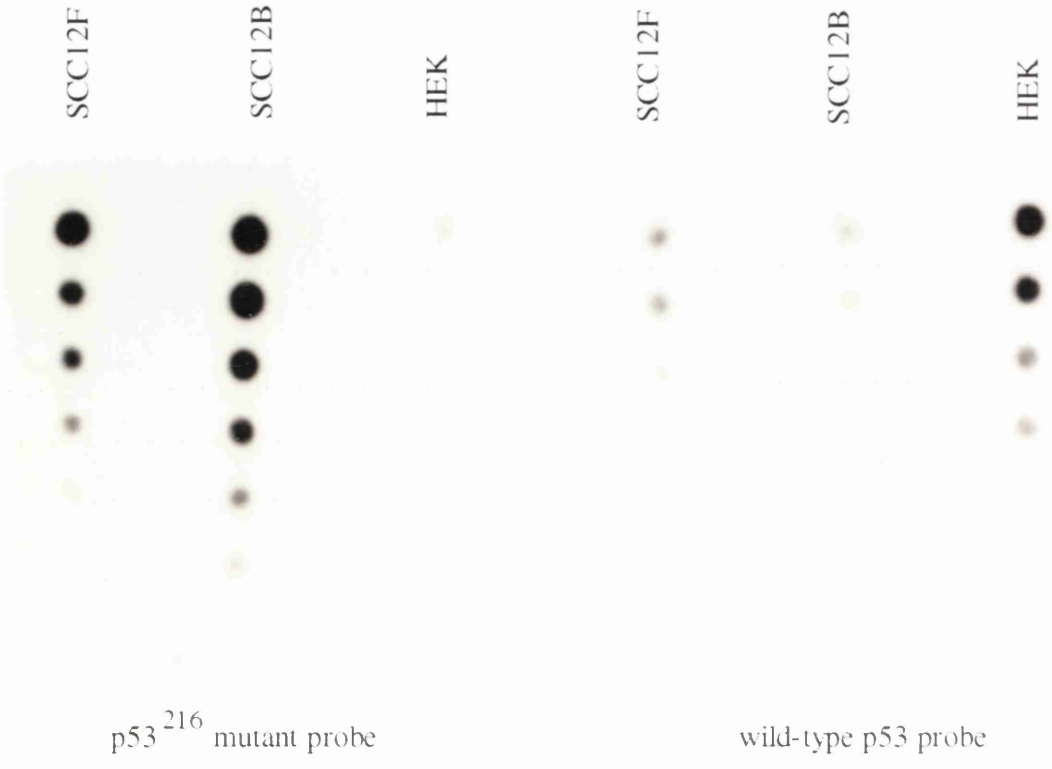


Figure 3.8b **Densitometry measurements**

p53^{216} mutant probe

SCC12F	SCC12B	HEK
37.6	45.1	1.2
18.2	40.4	0.4
11.2	26.3	0.2
5.1	15.8	
1.0	5.4	
0.3	1.5	

wild-type p53 probe

SCC12F	SCC12B	HEK
3.9	1.9	26.5
3.2	1.4	16.8
1.0	0.4	5.7
0.4	0.3	3.5
		0.4

Figure 3.9a

DNA dot blot analysis

Exon 6 of p53 DNA was amplified using primers 6U and 6D from HEK, SCC12B, SCC12F and a clone of SCC12F, clone 19. Equal amounts of DNA were hybridised to each filter with two fold serial dilutions added vertically. Either wild-type p53 (left) or $p53^{216}$ mutant (right) oligonucleotide was used as a probe and any non-specific binding was removed by washing in TMAC at 61°C as described in section 2.2.4.14.

Figure 3.9b

Densitometry measurements

The density of each spot in the dot blots in figure 3.9a above were measured by molecular dynamics laser densitometry using PDI image analysis software. Each measurement in the tables corresponds to the dot in the identical position on the blots in figure 3.9a. Density is measured in pixel density units ($ODxMM^2$) which is a function of spot intensity and the number of pixels covered. Only those dots that could be seen by eye are included in the tables.

Figure 3.9a

DNA dot blot probed with wild-type or p53^{216} mutant oligonucleotide.

wild-type p53 probe

p53^{216} mutant probe

Figure 3.9b

Densitometry measurements (OD/MM^2)

wild-type p53 probe

HEK	SCC12B	SCC12F	clone 19
51.4	9.9	11.1	12.6
41.1	3.5	2.8	7.6
12.2	1.6	1.1	4.7
6.0	0.5	0.8	2.2
1.7	0.3	0.4	0.6

p53^{216} mutant probe

HEK	SCC12B	SCC12F	clone 19
2.7	8.1	1.4	2.7
1.2	1.6	0.7	1.4
0.4	0.6	0.3	0.5

HEK are therefore hard to explain. It may be that SCC12F does have less wild-type sequence as a result of aneuploidy or it may be simply that HEK DNA and RNA is more easily and cleanly amplified than tumour DNA. More importantly,comparison of the hybridisation of the wild-type probe to SCC12F and SCC12B (figure 3.8a) suggests that SCC12F may have a two fold increase of wild-type transcript as compared to SCC12B. This was repeated and the results confirmed.

The dot blot was repeated using DNA as shown in figure 3.9a and the density measurements summarised in figure 3.9b. Although this blot was difficult to reproduce identically, it does again show that SCC12B contains more mutant p53 than SCC12F. The blot probed with wild-type p53 oligonucleotide does not indicate any difference between the level of wild-type p53 in the two cell lines unlike the cDNA dot blot in figure 3.8a. It does however show that a single cell clone of SCC12F, clone 19, contains more wild-type p53 than SCC12B and SCC12F. Hybridisation of the $p53^{216}$ mutant p53 oligonucleotide to DNA from each cell line confirms that SCC12B also contains more mutant p53 DNA than any other cell line.

Dot blot analysis of SCC12F and SCC12B at both the DNA and RNA levels together with the sequencing data has shown therefore that SCC12B expresses more mutant $p53^{216}$ than SCC12F, and the densitometry measurements suggest this to be by a 2:1 ratio respectively. Also SCC12F and clones of SCC12F may express more wild-type p53 than SCC12B.

3.2.4. Analysis of chromosome 17 number in SCC12F and SCC12B by fluorescent in situ hybridisation (FISH).

Taken together the analysis of p53 by western blot, sequencing and dot blot in SCC12B and SCC12F have indicated a difference in the wild-type to mutant $p53^{216}$ dosage between the two cell lines. It is therefore important to establish the number of p53 genes and as the p53 gene maps to chromosome 17p13.1, the number of copies of chromosome 17 in these two cell lines, so that a clearer picture of the p53 status in these two cell lines can be established.

The number of chromosome 17 copies in SCC12F, SCC12B and clone 19 was analysed by FISH using a chromosome 17 centromere probe as shown in figure 3.10. Fluorescence was analysed on a Bio-rad MRC-600 laser scanning confocal microscope equipped with a krypton/argon ion laser. Hybridisations of the probe were visualised with FITC (pseudocoloured green) using 488/568nm line excitation and dual channel 522 and 585nm emission filters.

Metaphase chromosomes from normal human blood lymphocytes were used as a control for hybridisation frequency. Two signals were visualised on most normal chromosome spreads as shown in figure 3.10 and an average hybridisation frequency (i.e. average number of signals / spread) of 1.93 was achieved. In comparison, three signals were observed on the metaphase spreads of clone 19, SCC12B and SCC12F as indicated by the arrows. These signals were localised to the centre of each chromosome and only one signal was obtained per chromosome as expected with a centromeric probe. The hybridisation frequencies for each cell line ranged between 2.9 and 3.1. and no gross structural rearrangements of chromosome 17 was observed.

Clone 19, SCC12B and SCC12F therefore all carry three copies of chromosome 17.

3.2.4. Analysis of chromosome 17 number in SCC12F and SCC12B by fluorescent in situ hybridisation (FISH).

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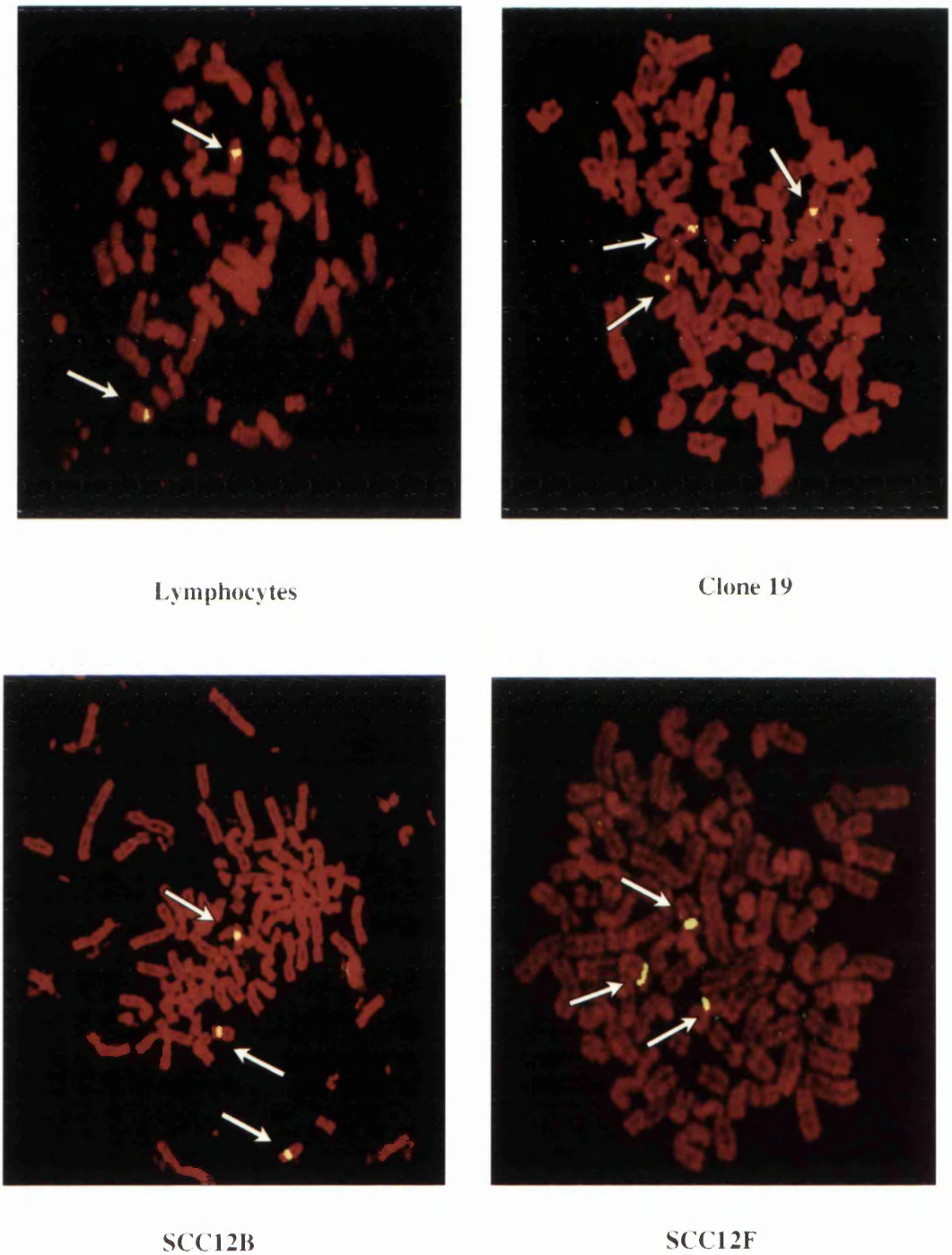
Clone 19, SCC12B and SCC12F therefore all carry three copies of chromosome 17.

Figure 3.10 **FISH analysis of chromosome 17**



Chromosome 17 number was analysed in Clone 19. SCC12B andSCC12F using the centromeric probe alpha-CHAD 9. Metaphase spreads are shown for each cell line. Chromosomes are counterstained red and the hybridisations are shown in green.

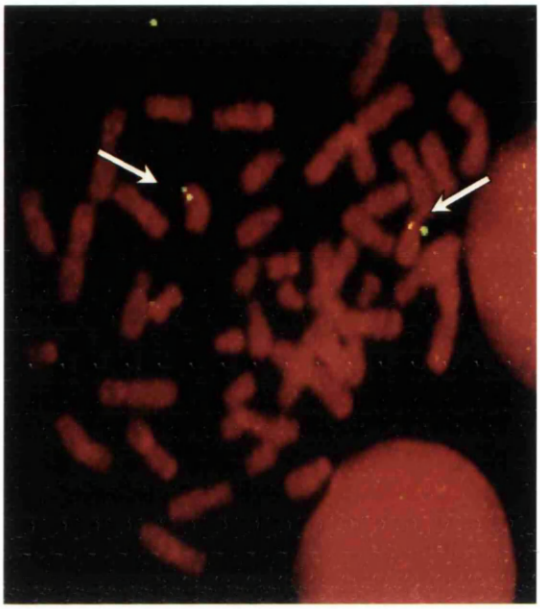
3.2.5. Analysis of p53 gene amplification in SCC12F and SCC12B by fluorescent in situ hybridisation (FISH).

As the p53 gene is located on chromosome 17p and we have observed an amplification of chromosome 17 in clone 19, SCC12F and SCC12B as well as a difference in the mutant to wild-type p53 gene dosage it was of interest to analyse the p53 gene copy number in these cell lines and to see if this correlates with the numbers of chromosome 17

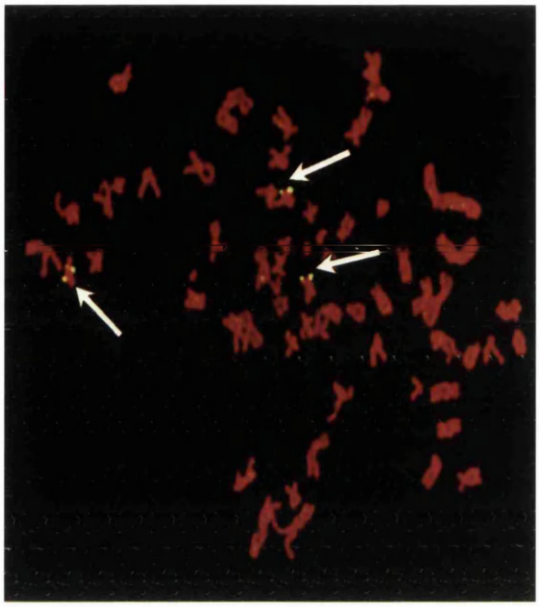
FISH was carried out as described in section 2.2.5. and the fluorescence was observed as described in section 3.2.4. Metaphase spreads from normal human lymphocytes were again used as a control and as shown in figure 3.11. As expected four signals were observed, one on each chromatid consistant with the presence of two copies of the p53 gene. The observed hybridisation frequency was again 1.9. Six signals were however visualised on each metaphase spread from Clone 19, SCC12F and SCC12B as shown in figure 3.11. The hybridisation frequency ranged from 2.8-3.0 for each cell line. Two signals were visualised per chromosome and these were located at the ends of the short arms of the chromosomes consistent with the position of the p53 gene.

Therefore clone 19, SCC12F and SCC12B all carry three copies of the p53 gene and although double hybridisations were not carried out it is assumed that as there are also three copies of chromosome 17 that each p53 gene lies on a the short arm of chromosome 17and has not been translocated to another chromosome.

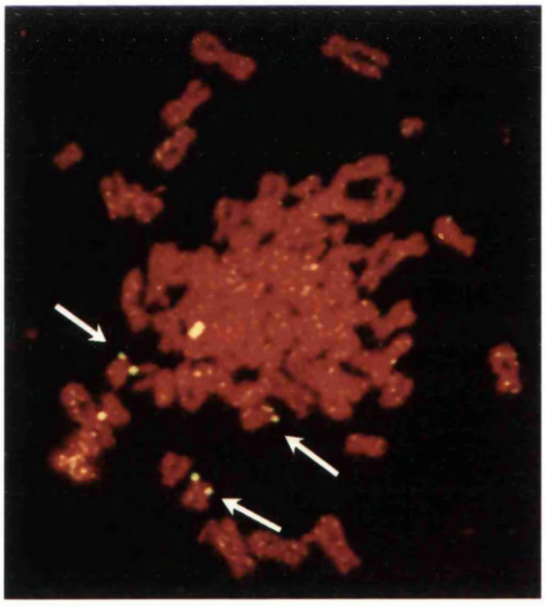
Figure 3.11 **Detection of p53 gene copies by FISH.**



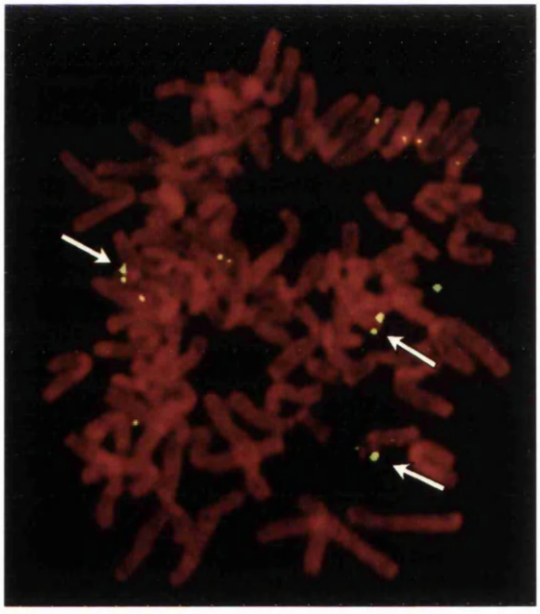
Lymphocytes



Clone 19



SCC12B



SCC12F

p53 copy number was analysed in clone 19, SCC12F and SCC12B using the p53 probe p5106-DIG. Metaphase spreads are shown for each cell line. Chromosomes are counterstained red, and p53 hybridisations green.

Chapter 4

Characterisation of $p53^{216}$ mutant.

4.1 Characterisation of the $p53^{216}$ Val →Gly mutant.

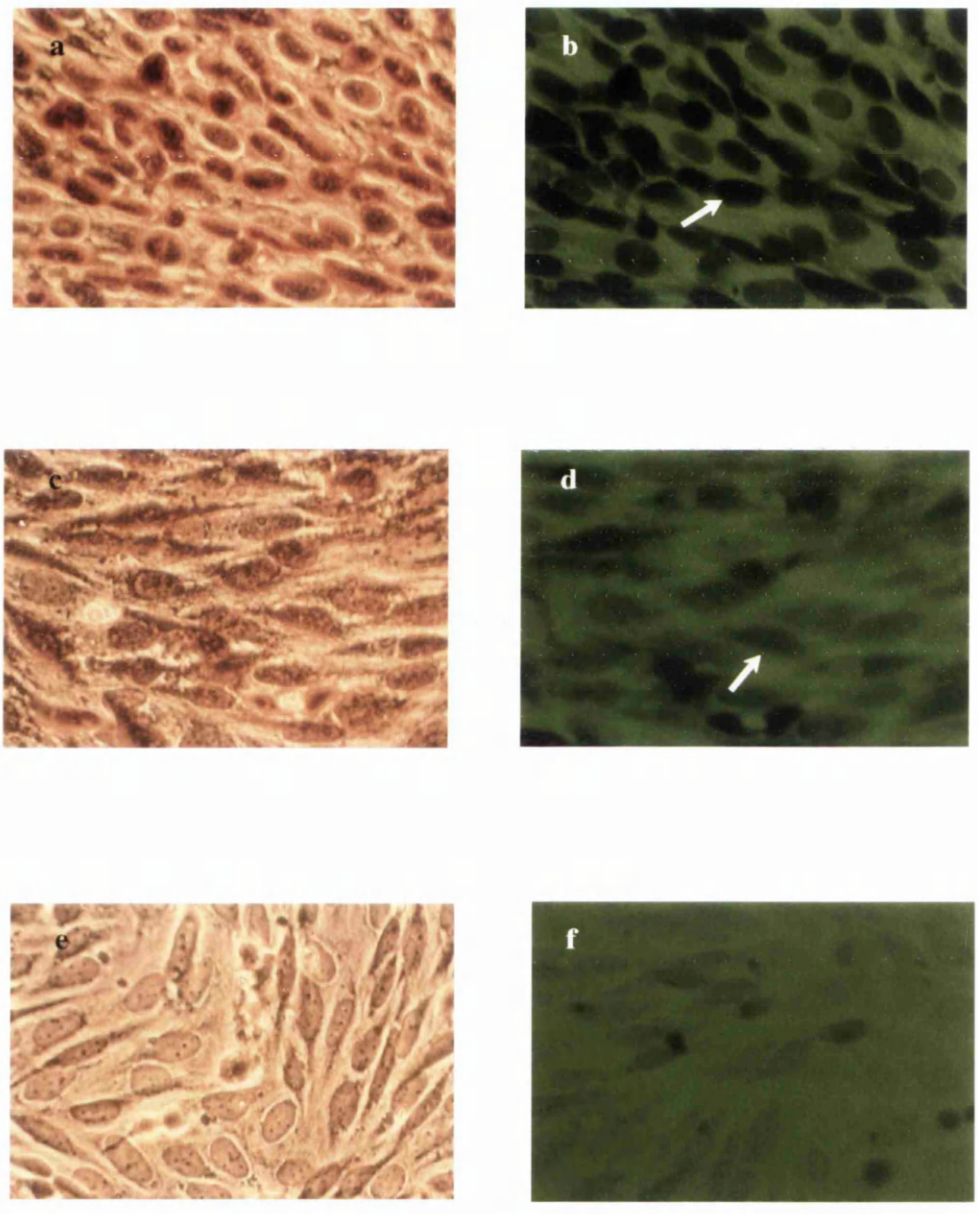
4.1.1. Synthesis of recombinant p53 mutants.

p53 mutants, $p53^{216}$ val→gly and $p53^{248}$ arg →trp were synthesised using the Biorad M13 mutagenesis kit as described in section 2.2.4.15. Wild-type p53 removed from the vector pC53-SN3 was used as the template and was placed into M13mp18 for mutagenesis. Once synthesised each p53 mutant was completely sequenced to check that only the required mutation had been incorporated. To further ensure any phenotype produced by the $p53^{216}$ mutant was actually due to the acquisition of this mutation alone and not by some other spontaneous mutation which frequently occurs in culture, two $p53^{216}$ mutant clones were selected and used in subsequent experiments, namely 216 (1) and 216 (2). The mutants were returned to the pCMV-Neo-Bam vector where the p53 cDNA is under the transcriptional control of the cytomegalovirus (CMV) promoter enhancer and the neomycin resistance gene is under the control of the simian virus 40 (SV40) promoter enhancer.

4.1.2. Loss of growth suppressive function of $p53^{216}$ mutant.

The introduction of wild-type p53 into a cell causes the arrest of cellular proliferation in G1 (Baker *et al.*, 1990; Casey *et al.*, 1991; Diller *et al.*, 1990; Mercer *et al.*, 1990). Indeed as discussed in section 1.4.3.6., one of the known functions of wild-type p53 is to induce a G1 arrest after DNA damage to allow the cell to undergo any necessary repair before proceeding further through the cell cycle. Escape from this cell cycle checkpoint is an important step in the progression of oncogenesis, and many different types of tumours have been shown to inactivate p53 by acquiring a p53 mutation in one allele followed by loss of the other wild-type p53 allele during the progression of the cancer. In order to investigate whether the $p53^{216}$ mutant observed in SCC12B is an important player in the progression of this tumour it is important to

Figure 4.1 Expression of p53 mutant constructs in Saos-2 cells



Saos-2 cells were transfected with $p53^{216}$ val $\longrightarrow$ gly (a and b), wild-type p53 (c and d) and CMV vector alone (e and f) and stained with PAb1801 to show p53 expression pattern. Phase contrast (left) and green filter (right) photographs are shown. Arrows indicate nuclear staining in b. and cytoplasmic staining in d.
Bar = 10um

establish that this mutant has lost its ability to suppress cell growth and is not a silent mutation or a polymorphism.

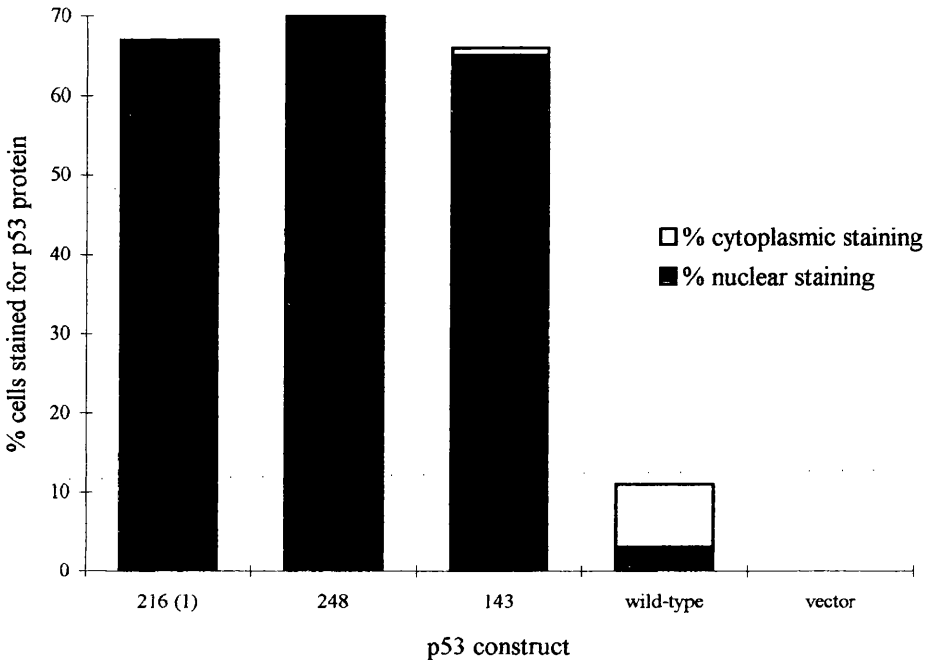
Saos-2 is a human osteosarcoma cell line with no endogenous p53 due to the deletion of both p53 alleles. Previous experiments have shown that Saos-2 cells cannot tolerate the expression of wild-type p53 (Diller *et al*,1990) and other investigators (Frebourg *et al*, 1992; Chen *et al*, 1990) have used Saos-2 cells to reveal various properties of p53 mutants. This therefore provided an ideal target on which to test the growth suppressive ability of the $p53^{216}$ mutant. It also allowed the expression of p53 from the CMV promoter to be analysed before its use in subsequent experiments.

The $p53^{216}$ val→gly mutant was transfected into Saos-2 cells along with $p53^{248}$arg→trp, $p53^{143}$ala→val mutant (pC53-SCX3), wild-type p53 (pC53-SN3) and the vector containing no p53 insert (pCMV-Neo) as described in section 2.2.1.4.

Phase contrast photographs of the expression of each p53 protein in Saos-2 cells is shown in figure 4.1. Figure 4.1a shows the expression of $p53^{216}$ protein. This mutant protein is expressed at high levels in the nucleus of the cells as shown by the arrows. Visualising the staining through a green filter enhances this nuclear expression pattern as shown in figure 4.1b. Expression of $p53^{248}$ and $p53^{143}$ were identical to figure 4.1a and b (data not shown). No p53 protein expression was seen in cells transfected with vector alone (figure 4.1e and f) as expected. Slight background staining appears when visualised through a green filter (figure 4.1f). Few colonies were expected from cells transfected with wild-type p53 due to its growth suppressive properties (see also figure 4.2). Those that arose mostly showed abnormal expression of the p53 protein in the cytoplasm (figure 4.1c) which is seen clearer in the photograph taken with a green filter (4.1d) and identified as cytoplasmic by comparison with the nuclear pattern seen in figure 4.1b. The ability of these colonies to survive the expression of wild-type p53 and its abnormal cytoplasmic expression suggests that the protein is inactive and although the protein in these cells was not sequenced it is likely that they have accummulated a mutation which has prevented nuclear targetting.

Figure 4.2

Expression of various p53 mutants in Saos-2 cells.



Various p53 constructs were transfected into Saos-2 cells as described in methods section 2.2.1.4. Transfected cells were plated at $4x10^5$ cells /10cm dish.
Colonies were selected with 500ug/ml G418 and were visible within 3 weeks.
The percentage of total cells plated that gave rise to resistant colonies for each construct was 216(1) 0.4%; 248 0.6%; 143 0.9%; wild-type 0.3% and vector 0.6%.
Whole plates were stained for p53 expression using PAb1801.
Positively stained cells were counted under a light microscope for p53 expression and it was noted whether this expression was the usual nuclear pattern or whether expression was mostly cytoplasmic, indicating a non-functional protein.
p53 constructs used were : 216 gly - val, 248 arg - trp, 143 ala - val or wild-type p53 inserted into the pCMV-Neo vector or vector alone.

Saos-2 colonies positive for p53 staining were counted in each transfection and the positionof the protein expression, either nuclear or cytoplasmic, was noted. Results are shown in the graph in figure 4.2. 66-70% of Saos-2 cells transfected with either the $p53^{216}$, $p53^{248}$ or $p53^{143}$ mutants tolerated the expression of the mutant p53 protein and this expression was almost entirely nuclear (shown by the solid shading in figure 4.2). In comparison only 11% of the Saos-2 cells transfected expressed wild-type p53 and of these, 8% expressed the p53 protein abnormally in their cytoplasm. This therefore shows that the $p53^{216}$ mutant identified in SCC12B as well as has $p53^{248}$ and $p53^{143}$ mutants have lost their growth suppressive properties and cells are able to tolerate the expression of these mutant proteins. The $p53^{216}$ mutant therefore actively contributes to the oncogenic phenotype of SCC12B by preventing the cells from growth arresting.

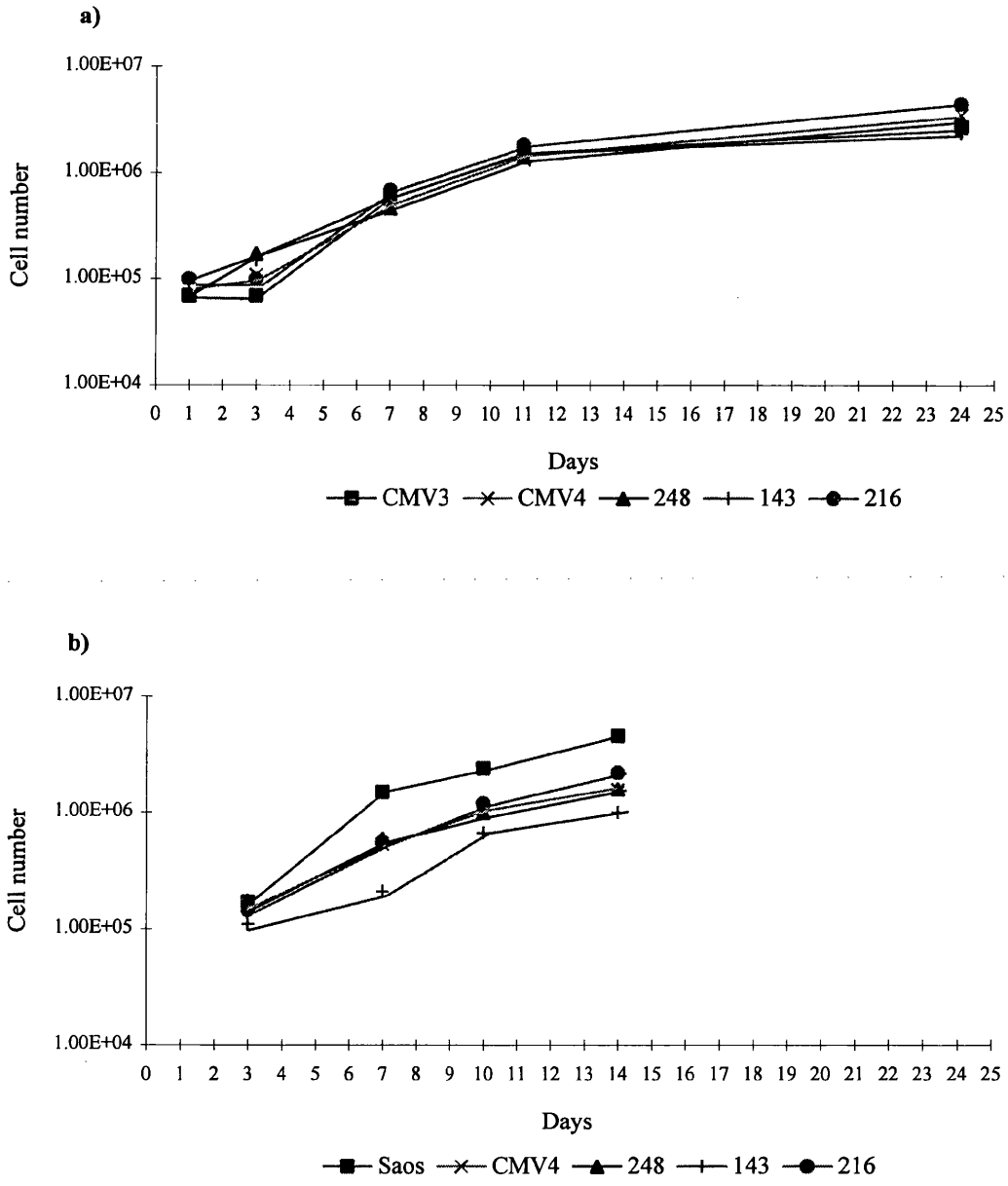
4.1.3. Saturation density of Saos-2 cells expressing mutant $p53^{216}$.

As determined in the previous section, the mutant $p53^{216}$ val→gly mutant has lost its ability to suppress cellular growth. It is not clear from this however whether this mutant has purely lost its wild-type function or whether it can also promote growth on an oncogenic background. Several groups have shown that some p53 mutants have gained a function (Dittmer *et al*, 1993 and Chen *et al*, 1990) enabling them to promote the oncogenic phenotype of the cell.

Saos-2 cells transfected with either $p53^{216}$,$p53^{248}$ or $p53^{143}$ mutants were seeded at a density of $1x10^5$ cells/5cm dish. Cells were trypsinised in duplicate at various time points and cell numbers recorded as shown in figure 4.3. The experiment was repeated twice, and both experiments are illustrated (figure 4.3 a and b). Each cell line expressing mutant p53 showed no difference in cell growth when compared to Saos-2 cells expressing the vector alone. In fact Saos-2 parental cells showed a slightly higher growth rate (figure 4.3b) than the cell lines expressing the mutant p53 constructs but this was probably due to G418 selection procedures. Therefore none of the p53 mutants tested, including $p53^{216}$, increased the saturation density or gave a growth advantage to the parental Saos-2 cells.

Figure 4.3.

Saturation density of Saos-2 cells expressing mutant p53 constructs.



Saos-2 cells were transfected with p53 mutants 216 val → gly, 143 ala → val, 248 arg → trp or vector alone (CMV4). Equal numbers (1×10^5) were seeded into a 5cm dish and removed and counted at the times indicated. Each cell count is the mean number from duplicate plates.

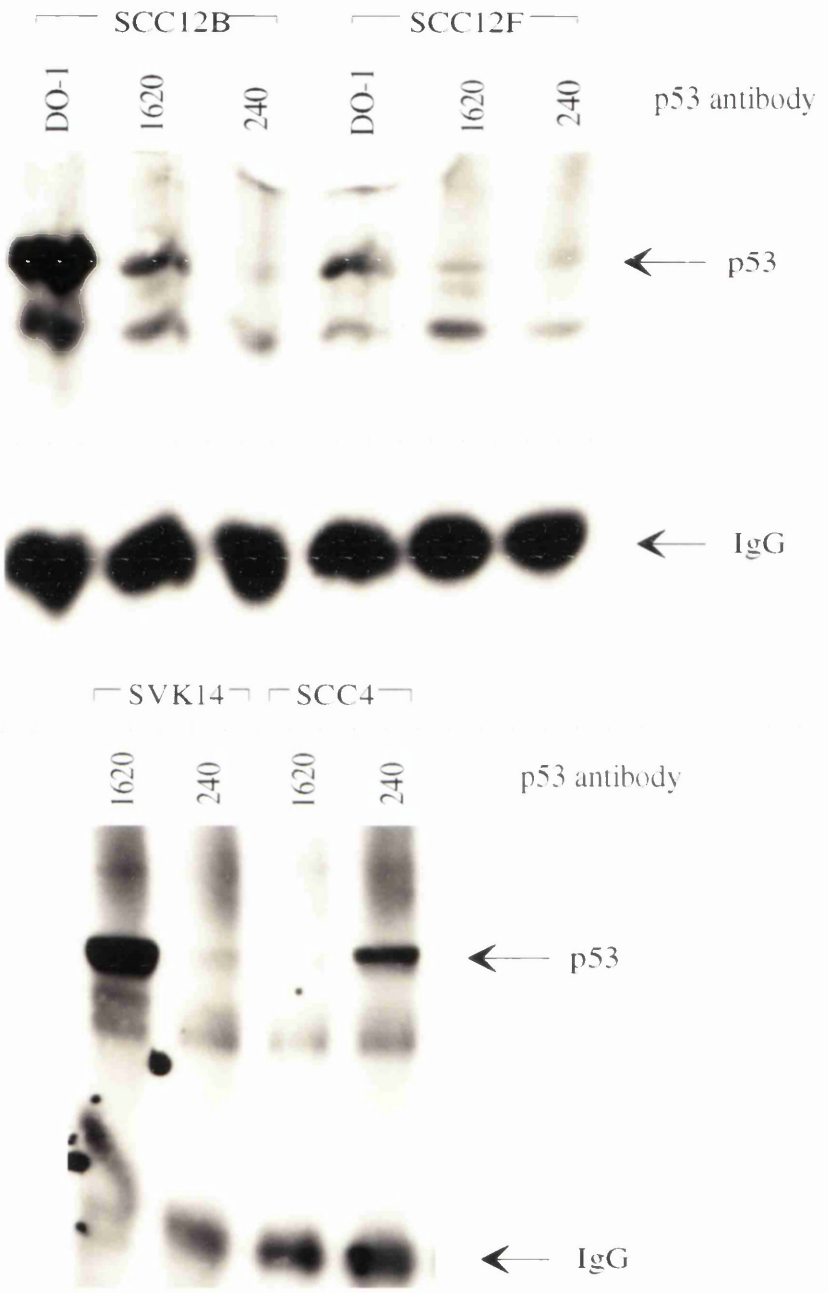
It seems therefore that the $p53^{216}$ mutant present in SCC12B and SCC12F contributes to tumour progression by losing its wild-type growth suppressive properties. It does not however seem to have gained an oncogenic function and does not give the cells in which it is expressed an increased growth advantage over neighbouring cells at least in Saos-2 cells.

4.1.3. Conformation of the $p53^{216}$ mutant.

The p53 protein in SCC12B and SCC12F was immunoprecipitated using various antibodies as shown in figure 4.4. PAb1620 (Milner *et al*, 1987) recognises an epitope mapping towards the N-terminus and reacts with p53 in a wild-type conformation only. The antibody PAb240 (Gannon *et al*, 1990; Stephen and Lane, 1992) recognises the epitope between amino acids 212-217 within the hydrophobic core of the protein. It therefore only complexes with p53 in a denatured or "mutant" conformation. DO-1 (Vojtesek *et al*, 1992) is a monoclonal antibody that reacts with all varieties of p53. SVK14 is an SV40 transformed keratinocyte cell line where the wild-type p53 is bound and stabilised by the SV40 large T antigen and is therefore readily detectable.. This cell line was used as a control as the p53 protein is precipitated by PAb1620 but not by PAb240. SCC4 has a p53 mutation at codon 151 (Burns *et al.*, 1993) which produces a stabilised p53 protein that reacts with PAb240 but not PAb1620.

SCC12B expresses a large amount of p53 protein as already shown by western analysis (figure 3.6) and confirmed by the amount of protein shown to immunoprecipitate with DO-1 (figure 4.4). All the p53 protein expressed in SCC12B appears to react with PAb1620 and not with PAb240. Figure 4.4 also confirms that SCC12F has less p53 protein than SCC12B as shown by comparing the quantity of protein bound to DO-1. The small amount of protein detected in SCC12F is also only reactive with PAb1620 and not PAb240. This therefore suggests that the $p53^{216}$ mutant protein has a wild-type conformation. This is unusual as most p53 mutants have a PAb240 reactivity and it prevents any further analysis of the relative dosages of wild-type and mutant p53 protein expressed in the two cell lines by immunoprecipitation.

Figure 4.4 **Immunoprecipitation of p53 from SCC12B and SCC12F**



100ug pre-cleared protein extract from each cell line was incubated overnight at 4^{O}C with p53 antibody as labelled. The antibody/protein complex was pelleted with protein G plus agarose beads, washed and electrophoresed by SDS-PAGE on a 12% gel. p53 protein was transferred onto immobilin P nitrocellulose and detected using p53 rabbit antibody CM-1 (1:250 dilution) and anti-rabbit Ig HRP antibody (1:5000). The filter was immersed in ECL reagent and the film exposed for 1 minute.
DO-1 recognises both mutant and wild-type p53. PAb1620 recognises only wild-type and PAb240 recognises only mutant p53.

Chapter 5

The effect of increased mutant $p53^{216}$ dosage on keratinocyte differentiation

5.1. Expression of $p53^{216}$ mutant in clone 19

5.1.1. Derivation of the target cell line, clone 19.

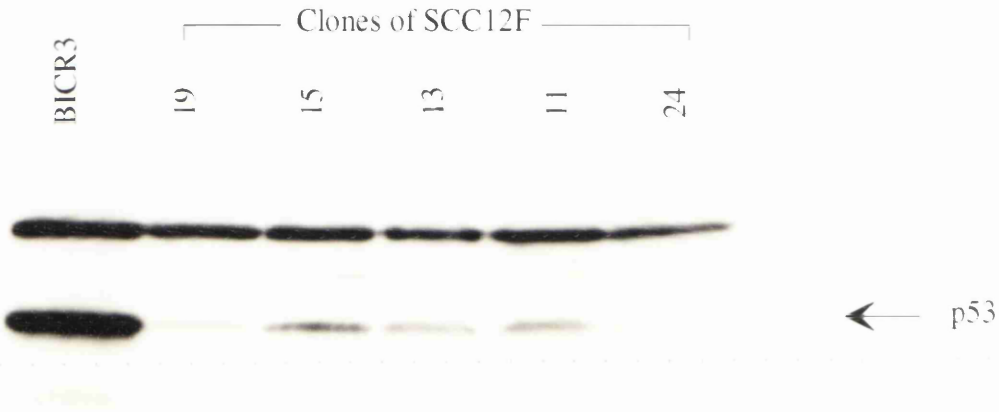
In order to analyse the effect of increasing the mutant to wild-type p53 gene dosage on malignant phenotype an appropriate target cell was needed. Whilst analysing the p53 protein levels in SCC12F and SCC12B it was noticed that expression in SCC12F appeared to be heterogeneous. Single cell clones were isolated from this cell line and examined for their p53 protein expression level as shown in figure 5.1. As this blot shows, different clones express varying levels of p53 protein. BICR3 was used as a positive control as it has been shown to have a p53 mutation at codon 282 resulting in a highly stable p53 protein (Burns *et al*, 1993).

A clone expressing low levels of p53 protein (clone 19), as shown in figure 5.1 and also in figure 3.6 was chosen as a target cell in which to alter the wild-type to mutant $p53^{216}$ gene dosage. Due to the very low level of p53 protein in this clone it presumably expresses very little stabilised mutant p53 protein and therefore has a more normal p53 phenotype than SCC12B. This was confirmed by DNA dot blot analysis in figure 3.9 where clone 19 appears to have more wild-type p53 DNA than SCC12B.

Immunoprecipitation analysis of p53 protein was carried out on clone 19 as shown in figure 5.2. No p53 protein signal was seen after the usual 30 second exposure to ECL agreeing with the above observation that clone 19 has very low levels of p53 protein. However if the film was left in contact with the blot for 20 minutes (figure 5.2) which completely overexposed signals from the control cell lines, a signal was obtained with the DO-1 antibody and PAb1620 but not with PAb240. This confirmed the observation that the p53 protein in clone 19 of SCC12F as with SCC12B has a wild-type, PAb1620 reactive conformation. It therefore cannot be revealed by this method whether the protein in clone 19 has a wild-type or mutant sequence but it is more likely that the protein is wild-type as it is not stabilised and is present at very low levels in the cell requiring a long exposure time to be visible unlike the highly stabilised mutant p53 protein in SCC12B.

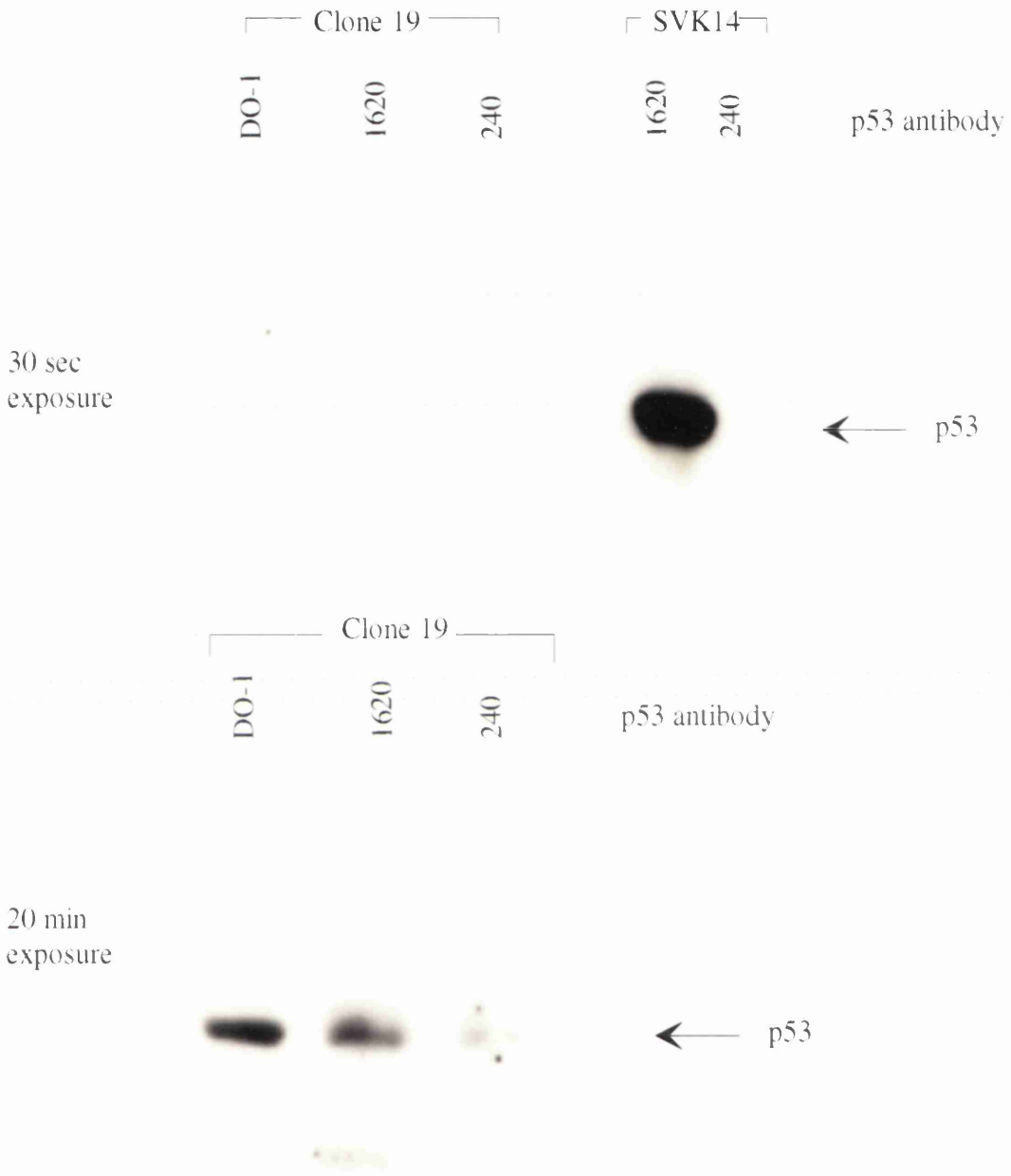
Figure 5.1

P53 protein levels in single cell clones of SCC12F



Single cell clones of SCC12F were isolated. 250ug protein extract from each clone was electrophoresed through a 12% denaturing polyacrylamide gel. as described in materials and methods. p53 protein was detected with the antibody PAb1801 at a final concentration of 1ug/ml.
BICR3 was a positive control for p53 expression.

Figure 5.2 Immunoprecipitation of p53 protein in clone 19



100ug pre-cleared protein extract from each cell line was incubated at 4^{0}C with p53 antibody as labelled. The antibody/protein complex was pelleted with protein G plus agarose beads, washed and electrophoresed by SDS-PAGE through a 12% gel. Protein was transferred onto immobilin P nitrocellulose and detected using p53 rabbit antibody CM-1(1:250 dilution) and anti-rabbit Ig HRP antibody (1:5000 dilution). The filter was immersed in ECL reagent and the film exposed for 30 seconds (top) or 20 minutes (bottom).
DO-1 antibody binds to both wild-type and mutant forms of p53. PAb1620 binds only to wild-type p53 and PAb240 binds only to mutant p53..

Although clone 19 expresses low levels of p53 protein, it still appears to contain three copies of the p53 gene as detected with FISH analysis (see figure3.11, section 3.2.5.) and three copies of chromosome 17 (figure 3.10, section 3.2.4) identical to SCC12B and SCC12F. It therefore does not appear to be the number of p53 gene copies that differs between each cell line, but the dosages of mutant to wild-type p53 genes.

Clone 19 therefore is a more relevant target cell in which to manipulate the dosages of wild-type and mutant p53 than a null p53 cell as it retains a related genetic background to SCC12B and the effects of increased mutant $p53^{216}$ protein on malignant phenotype in this cancer will be easier to interpret .

5.1.2. Introduction of mutant $p53^{216}$ into clone 19.

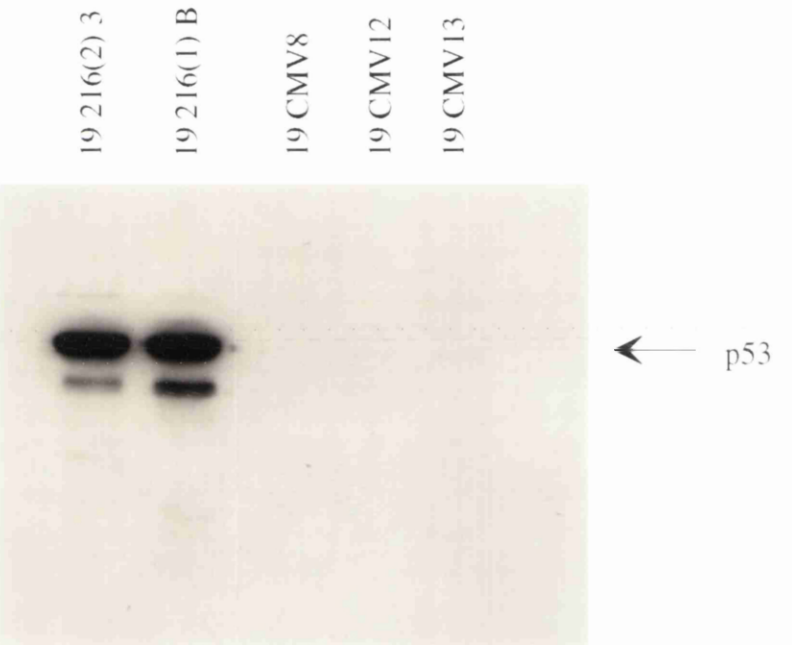
$p53^{216}$ val→gly mutant was synthesised as described in section 2.2.4.15 and 4.1.1. In order to ensure no other spontaneous mutations arise in culture giving spurious results, two $p53^{216}$ mutants were used in the following experiments, namely 216(1) and 216(2).

Wild-type and mutant DNA was transfected into clone 19 as described in section 2.2.1.4. Resistant colonies were picked, expanded and stained for p53 expression. No resistant colonies overexpressing p53 were obtained from cells transfected with wild-type p53. Large numbers of colonies were obtained by transfection of clone 19 with 216(1) and 216(2), 14 resistant colonies arose from $1x10^6$ transfected cells and 12 colonies from $7.5x10^5$ cells respectively. Fewer resistant colonies were obtained from the vector alone, 6 colonies from $1.5x10^6$ transfected cells.

High levels of p53 protein expression was obtained from the expression vector as shown by western analysis in figure 5.3. Two transfectants 19 216(2) 3 and 19 216(1) B are shown as examples. Transfectant 19 216(2) 3 shows a similar p53 expression level

Figure 5.3

Levels of p53 protein in transfectants of clone 19 expressing CMV-p53^{216}

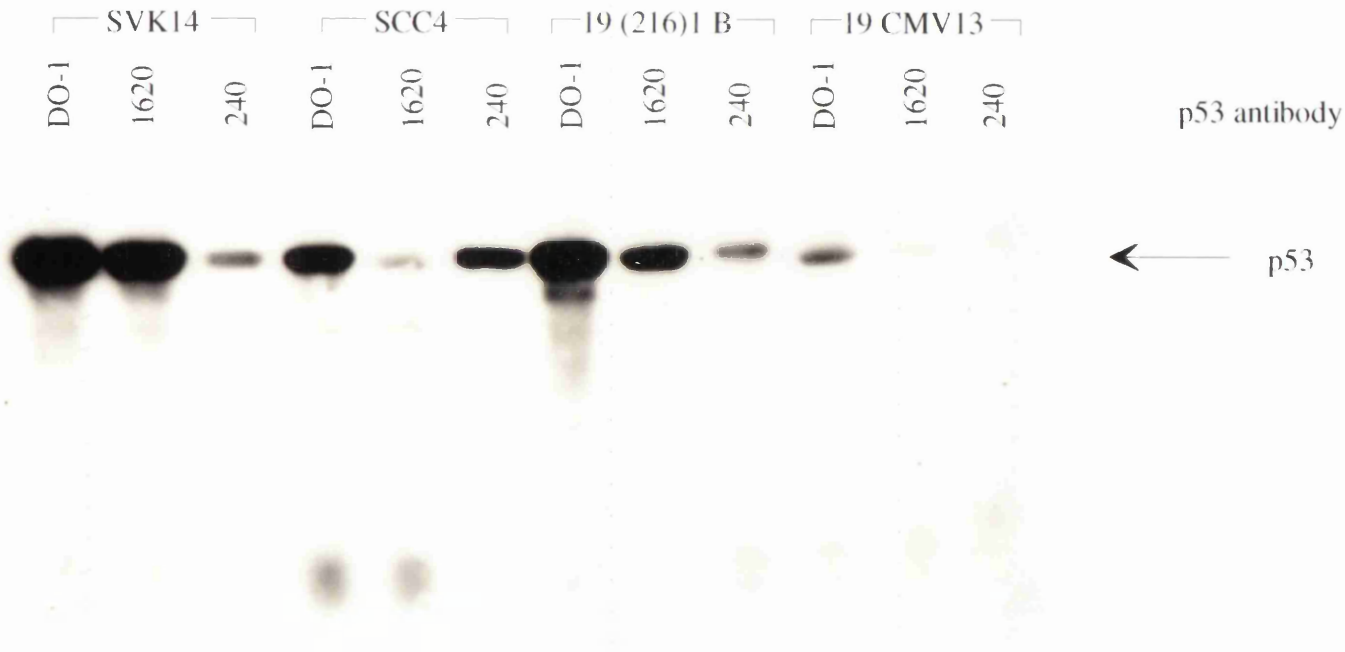


50ug protein extract from each cell line was electrophoresed through a 12% denaturing polyacrylamide gel, as described in materials and methods. P53 protein was detected with the antibody PAb1801 at a final concentration of 1ug/ml.

19 (216)1 B and 19 (216)2 3 were produced from clone 19 transfected with mutant p53 216(1) or 216(2) respectively.

19 CMV8, CMV12 and CMV13 are clone 19 transfected with CMV vector alone.

Figure 5.4 Immunoprecipitation of p53 protein in cell lines transfected with $p53^{216}$ mutant.



100ug pre-cleared protein extract from each cell line was incubated at 4°C with p53 antibody as labelled. The antibody/protein complex was pelleted with protein G plus agarose beads, washed and electrophoresed by SDS-PAGE through a 12% gel. Protein was transferred onto immobilin P nitrocellulose and detected using p53 antibody CM-1 (1:250 dilution) and anti-rabbit Ig HRP antibody (1:5000 dilution). The filter was immersed in ECl and the film exposed for 30 seconds.

DO-1 antibody binds to both wild-type and mutant forms of p53, PAb1620 binds only to wild-type p53 and PAb240 binds only to mutant p53.

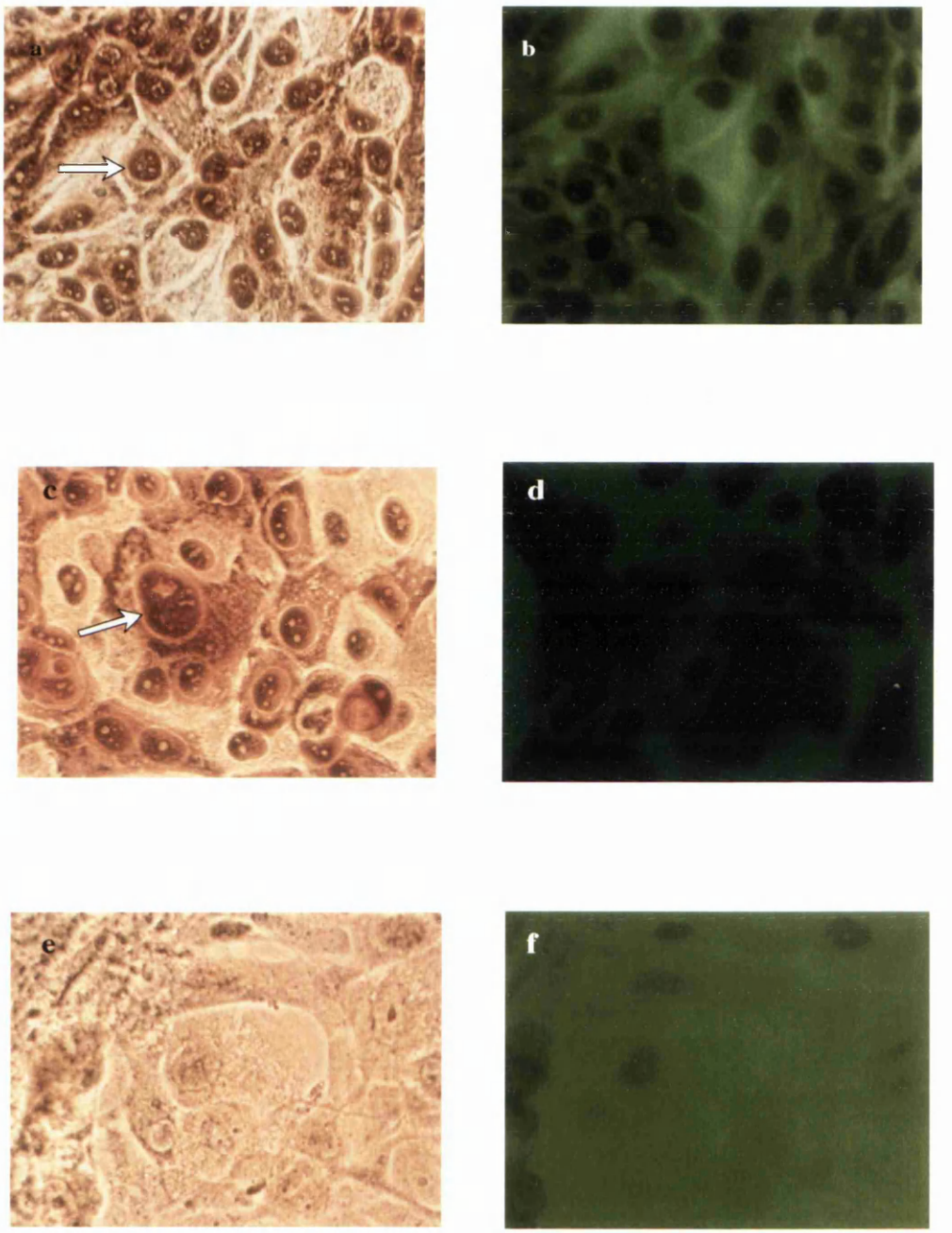
(data not shown). No stabilised p53 protein was shown as expected in three clones transfected with the vector only (19 CMV8, 19 CMV12 and 19 CMV13).

The conformation of the mutant $p53^{216}$ was analysed in the transfectants by immunoprecipitation (figure 5.4) to compare it with that analysed in SCC12B and SCC12F (figure 4.4, section 4.1.3). Large amounts of p53 protein in the two transfectants 19 216(1) B (figure 5.4) and 19 216(2) 3 (data not shown) react with antibody DO-1 that recognises both wild-type and mutant conformations. The majority of this mutant p53 protein is reactive with PAb1620 as is the p53 protein in SCC12B and SCC12F. However there appears to also be some reactivity with PAb240 (which reacts with only p53 in a mutant conformation) in the transfectants which was not apparent in SCC12B, SCC12F or clone 19 (figures 4.4 and 5.2). This reactivity in clone 19 transfectants may be due to the high levels of mutant $p53^{216}$ expression resulting in some becoming denatured during the experiment. The same controls were used as before, SVK14 containing SV40 large T antigen bound wild-type p53 and SCC4 containing mutant $p53^{151}$ (Burns *et al.*, 1993). There also appears to be some cross-reactivity with PAb240 in the control SVK14. This reactivity of normally PAb1620 reactive p53 with PAb240 may be due to slight degradation of the protein in cases where it is at extremely high levels in the cell or perhaps slightly different conditions which vary between experiments may lead to a low level of protein denaturation.

To analyse the cellular expression of the $p53^{216}$ mutant in transfected cell lines, cells were seeded onto chamber slides and stained for p53 with PAb1801 as described in section 2.2.2.1. and shown in figure 5.5. The position of the staining was enhanced by using a green filter (figure 5.5 right) and can be seen to be nuclear in 19 216 (2) 3 (a and b) and 19 216 (1) B (c and d). Only slight p53 staining was visible in 19 CMV13 expressing the vector alone as expected.

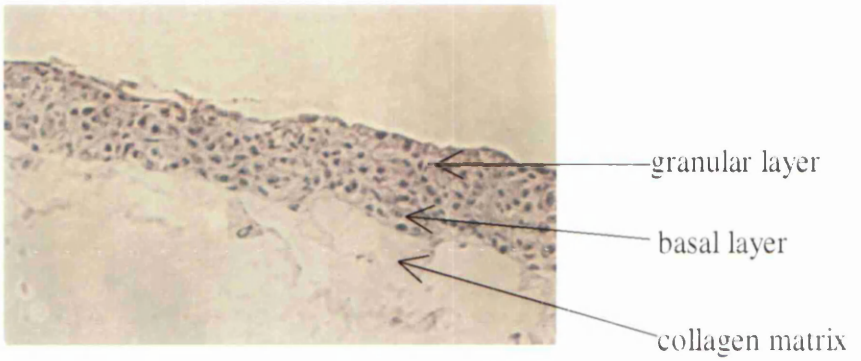
When visualised under the microscope the majority of the transfected cells (figure 55 a-d) stained for p53 protein. However where the cells differentiated to produce foci in culture the staining appeared to be more suprabasal. As the basal cells are the only proliferating cells in the epidermis, it would be expected that an increase in mutant p53 would only have an effect if expressed in these cells and not later when the cells have

Figure 5.5 Clone 19 transfected with p53 ^{216} mutant constructs.



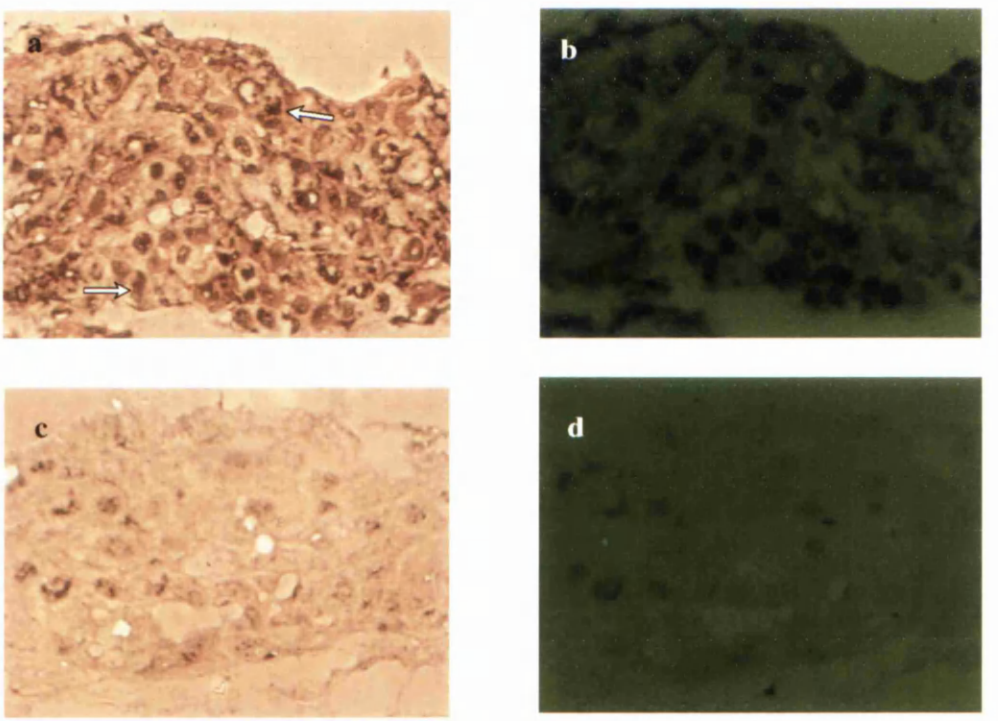
Clone 19 was transfected with p53^{216} mutant constructs and stained for p53 protein using PAb1801 as described in materials and methods. Phase constrast (left) and green filter (right) photographs are shown. Clone 19 216(2) 3 is shown in a and b, clone 19 216(1) B in c and d, and vector alone 19 CMV13 in e and f.
Arrows show expression of p53 protein. Bar =10um

Figure 5.6 Raft culture of clone 19 216(1) B stained with haematoxylin and eosin.



Clone 19 216(1)B was grown on a raft culture and stained with haematoxylin and eosin to show stratification, as described in materials and methods. Cellular layers are as shown.
Bar = 10um ▪

Figure 5.7 Raft culture of clone 19 216(1)B stained for p53 protein.



Clone 19 216(1)B was grown on a raft culture and stained for p53 protein with PAb1801 (a and b) as described in materials and methods. P53 protein is expressed in all layers of the raft culture as indicated by the arrows.
Clone 19 CMV13 showed no p53 protein expression as expected (c and d).
Phase contrast (left) and green filter (right) photographs are shown.
Bar = 10um ▪

already been committed to terminal differentiation. In order to check the spatial expression from the CMV vector, cells were grown on raft cultures (Kopan *et al.*, 1987) either completely submerged in medium or only fed from the bottom of the raft. Cryostat sections of each raft culture were stained with haematoxylin and eosin to enhance cellular morphology (figure 5.6) and show stratification. The raft sections were also stained with involucrin and staining was restricted to the suprabasal layers as expected. The basal cells were present and remained unstained. As shown in figure 5.7 when stained with the p53 antibody PAb1801 the expression of p53 protein from the CMV promoter appears to be in all layers of the raft culture and not confined to the upper layers as first thought. The CMV promoter was therefore suitable to drive the expression of mutant $p53^{216}$ protein to all layers of the stratified keratinocyte cultures.

5.2. The effect of $p53^{216}$ mutant dosage on the progression of SCC.

5.2.1. Morphology of cell lines.

Phase contrast photographs of each cell line used in experiments described in this thesis are shown in figure 5.8. HEK cells showed the normal polygonal shaped cells and were grown in serum containing medium in the presence of 3T3 feeders as described in section 2.1.1. Similar culture conditions were used for all other cell lines, i.e. DMEM supplemented with 10% serum and hydrocortisone. Clone 19, a low expressing p53 clone of SCC12F, grew very slowly in these culture conditions. Optimal conditions in which to grow this cell line were not defined and culture conditions were kept identical to other cell lines for control purposes. Like HEK cells, clone 19 preferred to be kept at a high cell density , losing cloning efficiency if the cells were extensively split. Clone 19 also maintained the characteristic cobblestone morphology of normal keratinocytes as did SCC12F (figure 5.8 B and D). The morphology of SCC12F and SCC12B have been previously described elsewhere (Dawson *et al.*, 1990; Jaffe *et al.*, 1992). SCC12B appear much flatter than SCC12F (figure 5.8C) or clone 19 and appeared to have an increased growth rate. SCC12B is able to grow in the presence of reduced serum (Jaffe *et al.*, 1992 and data not shown). 19 CMV 13 and 19 CMV8 (G and H), clones of clone 19 expressing the CMV vector alone showed no obvious differences from clone 19. Clones transfected with mutant $p53^{216}$ (19 216(1) B and 19 216(2) 3 figure 5.8 E and F) however yielded cell lines that showed increased growth rates and reduced serum dependence . Both of these cell lines also appeared to have more compact cell growth and seemed to be less contact inhibited than other cells.

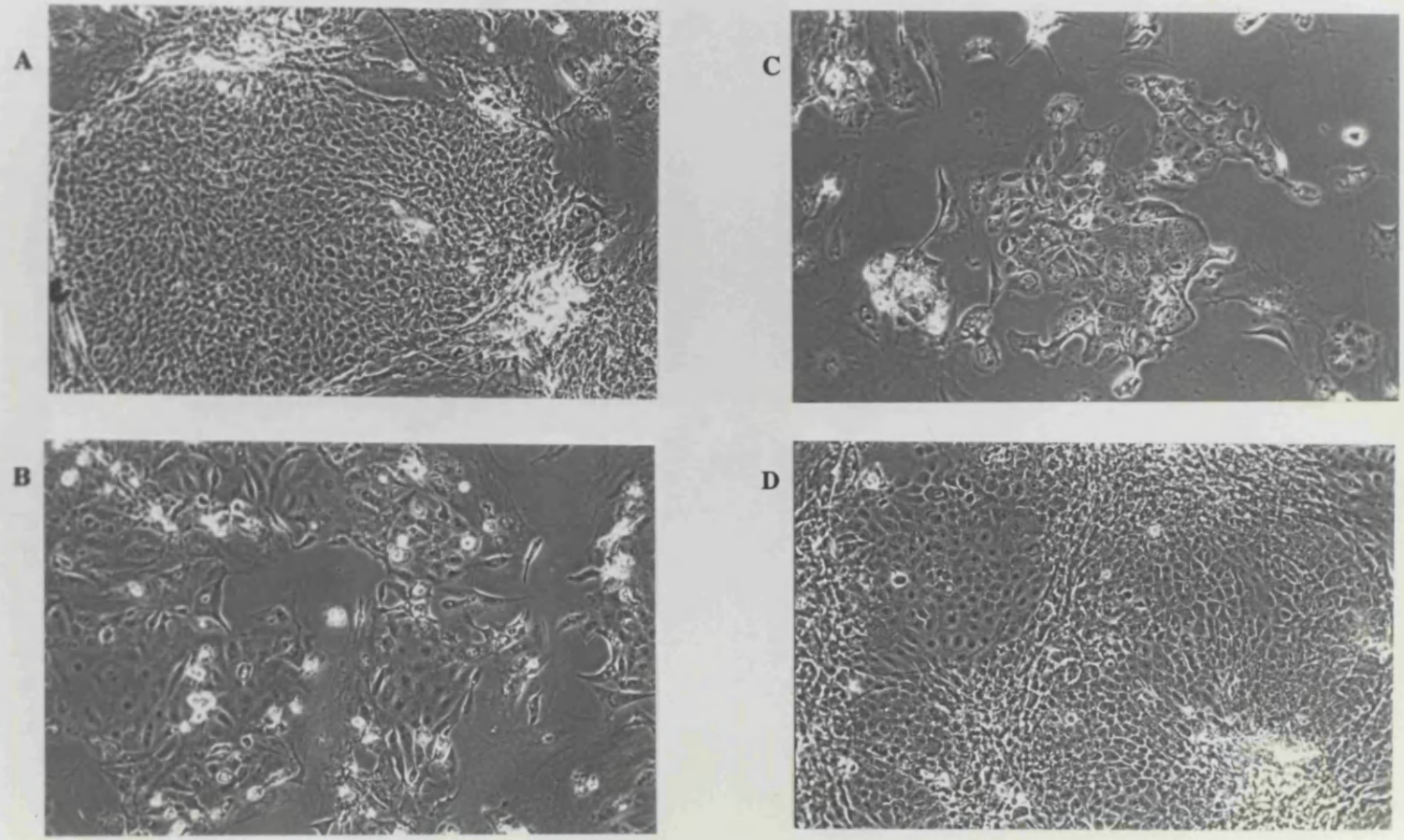
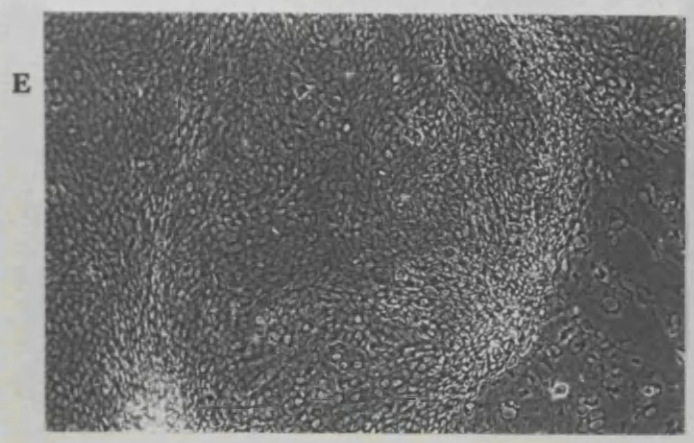
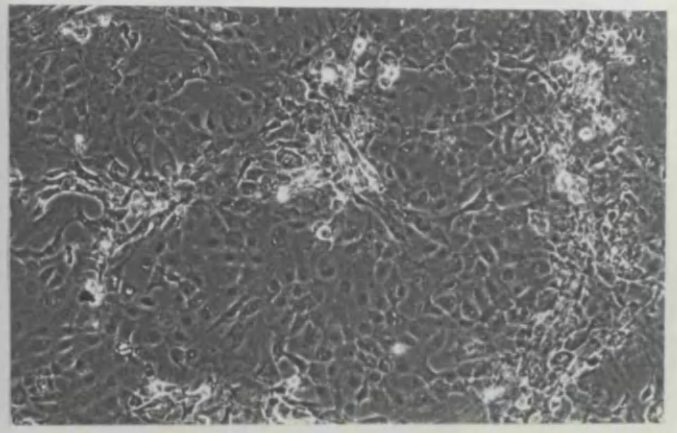


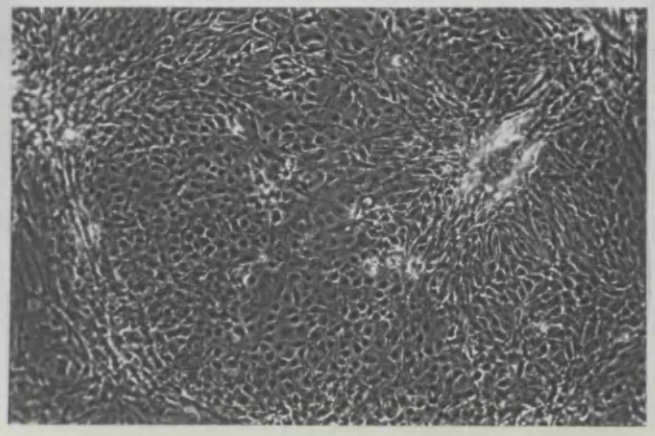
Figure 5.8 Phase contrast photographs showing the morphology of each cell line grown on a feeder layer.

HEK (A), Clone 19 (B), SCC12B (C), SCC12F (D).

Bar = 500uM







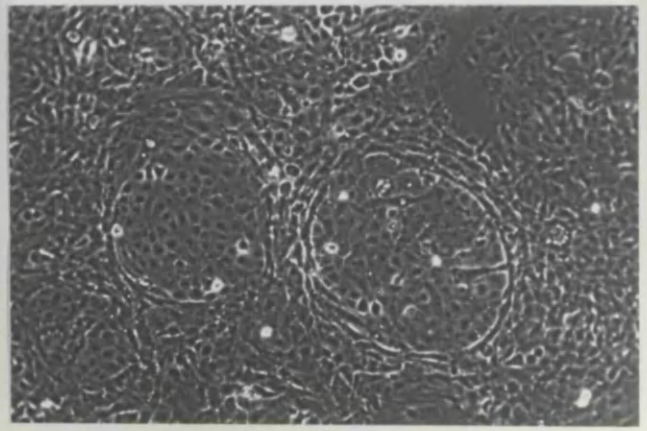


Figure 5.8b Phase contrast photographs showing the morphology of each cell line grown on a feeder layer.

19 216 1 B (E), 19 216 2 3 (F), 19 CMV13 (G), 19 CMV8 (H).

Bar = 500uM

5.2.2. Expression of involucrin.

Involucrin expression was analysed on all the cell lines shown in figure 5.8 after suspension culture for 0 and 3 days (as described in section 2.2.1.5 and section 2.2.2.2). Cell density was controlled for by growing cells at both high density ($2x10^6$cells/5cm dish) and low density ($3x10^5$ cells /5cm dish) before being placed in suspension culture.

Figure 5.9 shows the staining pattern of normal HEK cells expressing involucrin after 3 days in suspension. All other cell lines showed a similar staining pattern (data not shown) in the proportion of cells that stained (figure 5.10 and 5.11). It has previously been reported that involucrin expression correlates with cell size (Watt and Green, 1981), the larger cells being more differentiated and therefore expressing more involucrin protein (as discussed in section 1.1.3). The large dark red cells highlighted by the arrows were scored positive and any other cells were scored negative for involucrin expression.

After culturing at high density 37% of normal HEK's expressed involucrin and this increased to 64% after 3 days in suspension culture (figure 5.10a and b). A similar trend was followed by all other cell lines. A slightly lower 20-30% of these cells expressed involucrin at day 0, and this increased to 50-60% after day 3. 19 CMV13 appeared to have a much higher involucrin expression after day 3 but this was based on only one experiment (hence the absence of a standard deviation value).

No affect on involucrin expression was therefore apparent when the mutant $p53^{216}$ levels were increased and these cells were grown at high density.

An affect became clearer when the cell lines were grown at lower density before being placed in suspension (figure 5.11a and b). 5-28% of cells in all cell lines expressed involucrin at day 0. Involucrin expression then increased in most cell lines after 3 days in suspension (figure 5.11b). The largest increase was in clone 19 where expression increased from 14% to 57%. Control cell lines 19 CMV8 and 19 CMV13 showed a similar increase. However, cell lines 19 216(1) B and 19 216(2) 3 showed only a very slight increase (≈3%) in involucrin expression after 3 days in suspension culture

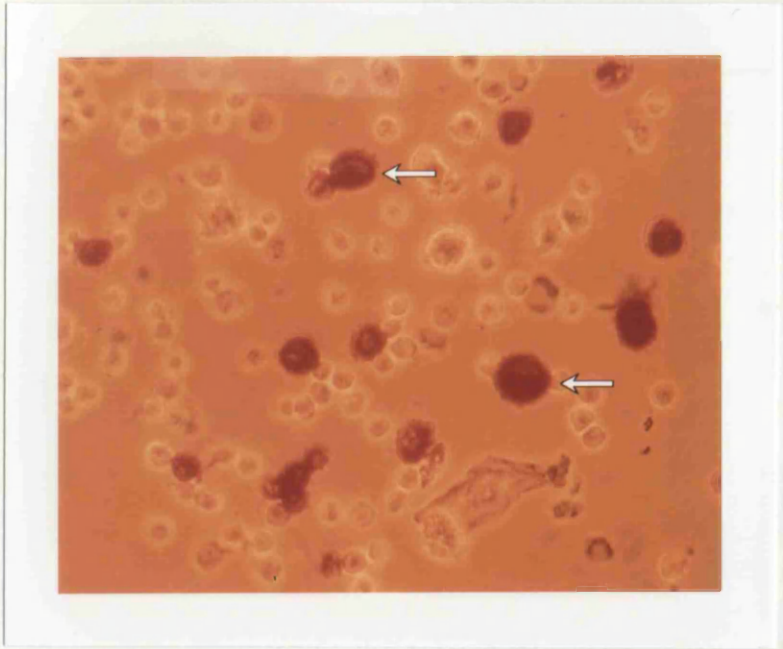


Figure 5.9

Phase contrast photograph shows involucrin staining of HEK cells 3 days after suspension culture. All other cell lines showed a similar staining pattern and the proportion of cells stained are shown in figure 5.10 and 5.11. The dark red cells (examples are highlighted by the arrows) were scored as positive.

Bar = 10uM

Figure 5.10

Involucrin expression in cells induced to differentiation by suspension culture.

High density

Cells were seeded at $2x10^6$/5cm dish and cultured for 4 days. Cells were then trypsinised and either stained for involucrin immediately (day 0) or placed in suspension culture at 10^5 cells/ml for 3 days. These cells were washed from methylcellulose and then stained for involucrin.
Cells were scored positive or negative and expressed as a percentage of the total cells counted.
Experiments were repeated 2-4 times and the average value for each cell line was calculated and plotted as shown ± standard deviation

Figure 5.10

Involucrin expression in cells induced to differentiate by suspension culture.

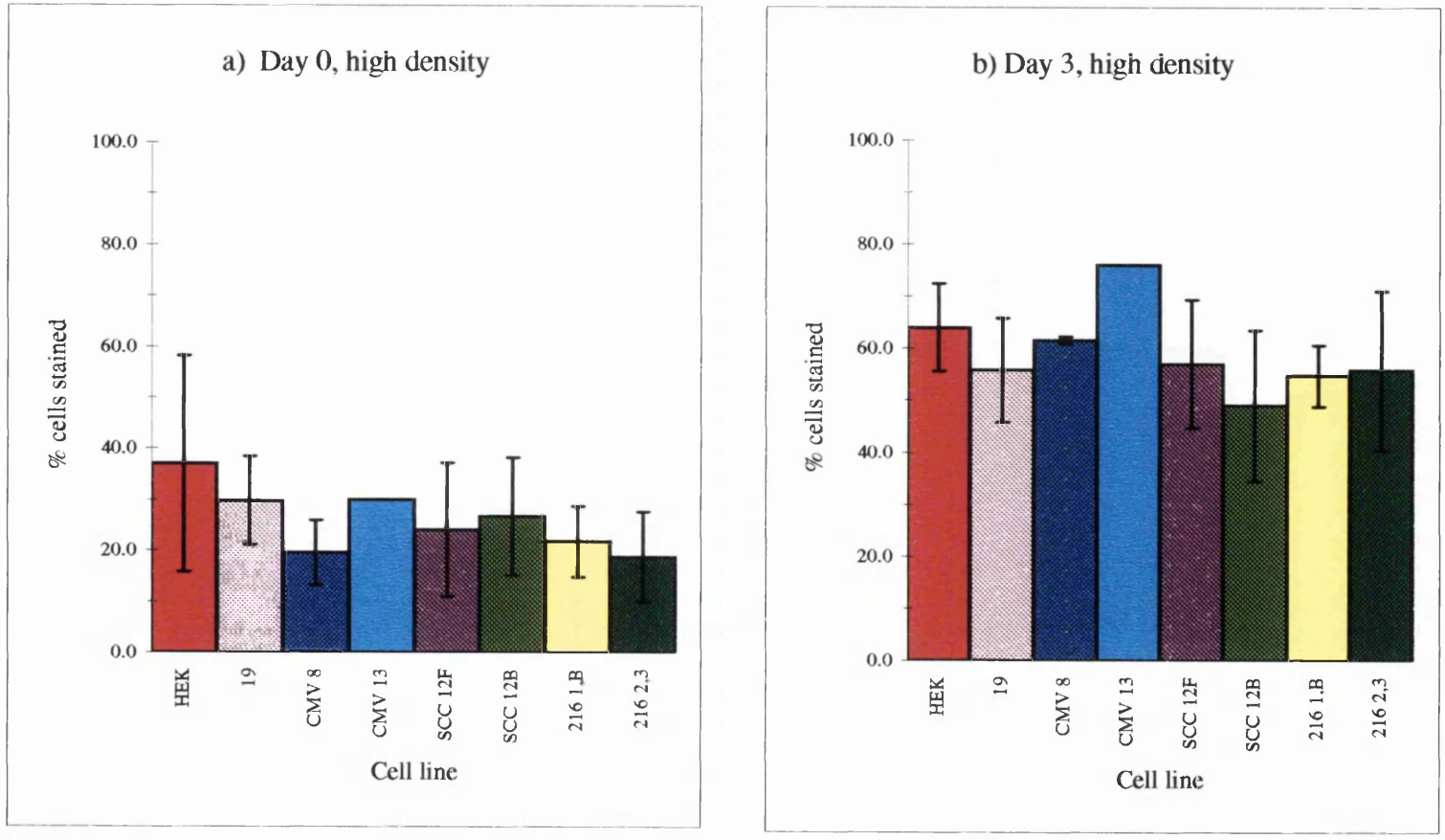


Figure 5.11

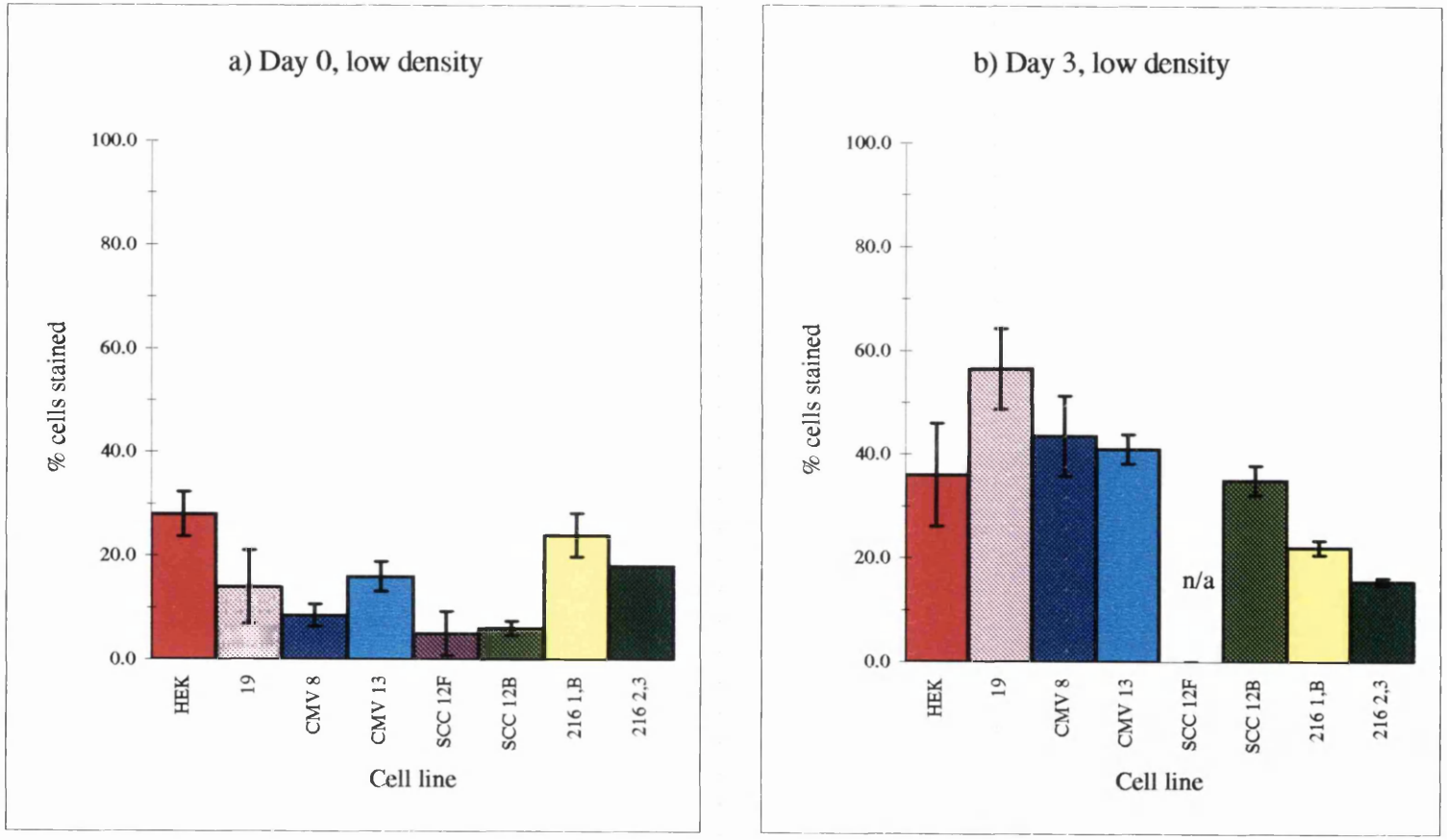
Involucrin expression in cells induced to differentiation by suspension culture.

Low density

Cells were seeded at $3x10^5$/5cm dish and cultured for 4 days. Cells were then trypsinised and either stained for involucrin immediately (day 0) or placed in suspension culture at 10^5 cells/ml for 3 days. These cells were washed from methylcellulose and then stained for involucrin.
Cells were scored positive or negative and expressed as a percentage of the total cells counted.
Experiments were repeated 2-4 times and the average value for each cell line calculated and plotted as shown ±.standard deviation.
n/a means not counted due to contamination.

Figure 5.11

Involucrin expression in cells induced to differentiate by suspension culture.



Therefore, increasing the levels of mutant $p53^{216}$ protein in clone 19 appears to inhibit the ability of these cells to express involucrin in response to suspension induced terminal differentiation, if these cells are cultured at low density.

5.2.3. Resistance of cell lines to suspension induced cell death.

The colony forming efficiency of each cell line was measured without being placed in suspension culture (day 0) and after 24 hours in suspension culture (24 hours) as described in section 2.2.1.7. Cell density was controlled for by plating cells at both high density ($2x10^6$cells/5cm dish) and low density ($3x10^5$ cells /5cm dish) before being placed in suspension culture. The resistance of cells to suspension induced cell death was plotted for each cell line (figure 5.12 and 5.13) and this was expressed as the number of colonies formed after 24 hours in suspension as a percentage of the number of colonies obtained at day 0. Cloning efficiencies without suspension culture are given in the corresponding figure legends and are expressd as a percentage of the total cells plated.

The survival of clone 19, SCC12F and SCC12B were compared to 19 (216)1 B as shown in figure 5.12. At both low and high density clone 19 and SCC12F had a much lower colony forming efficiency than SCC12B and 19 (216)1 B. SCC12B still retained a 40% colony survival after 24 hours in suspension culture, a similar figure as obtained by Parkinson *et al*, 1983. The cloning efficiencies of SCC12F and SCC12B were much lower than clone 19 and 19 (216)1 B as shown by the percentage number of colonies at day 0 as detailed in figure 5.12 legend. This was probably due to the fact that the SCC12B and SCC12F cells used were of a much lower pass than the other two cell lines and this could also explain the larger standard deviation for values obtained from SCC12B.

SCC12F, clone 19, and two clones of 19 expressing the vector alone (19 CMV13 and 19 CMV8) had similar survival patterns and their survival was lower than 19 (216)1 B and 19 (216)2 3 (figure 5.13). At high density, colony survival ranged from with between 41% (19 CMV8) to 18% (SCC12F). At lower density similar results were

Figure 5.12

Colony survival after suspension induced cell death.

Experiment 1

Cells were seeded at either $2x10^6$/5cm dish (high density) or $3x10^5$/5cm dish (low density) and incubated for 4 days. Cells were then either plated at 500 cells /T25 flask or placed in suspension culture for 24 hours. These cells were then washed from the methylcellulose and plated at 10^3/T25 flask. Cells were incubated at 37°C until colonies were easily visible and then stained with 1% rhodamine and counted.
Colony numbers were recorded per 500 cells plated.
Experiments were repeated at least 3 times and the average number of colonies per time point for each cell line recorded
Percentage colony survival is plotted for each cell line i.e. the number of colonies counted after 24 hours in methylcellulose as a percentage of the number of colonies counted without suspension culture (day 0), ± standard deviation.

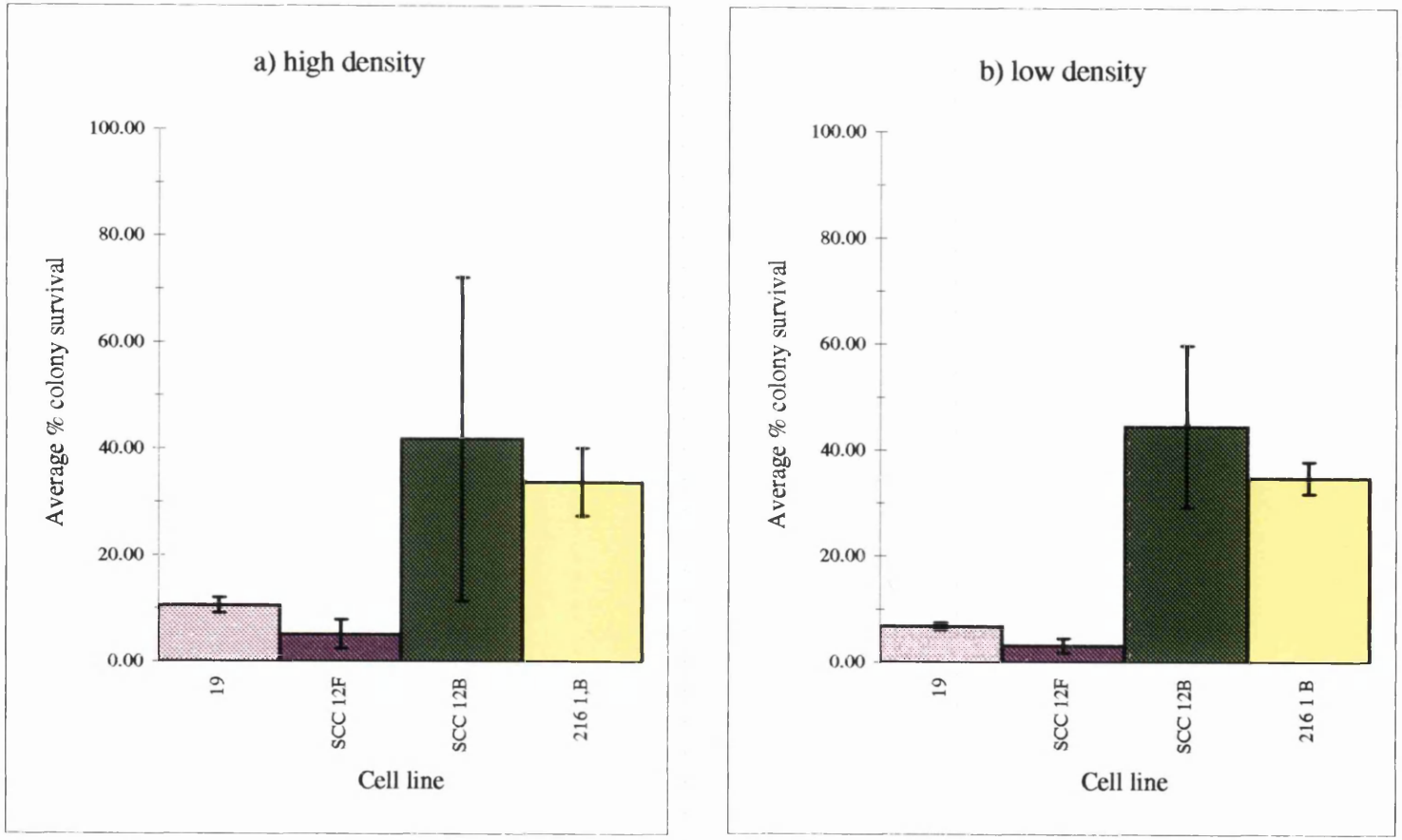
Colony survival at day 0 recorded as a percentage of total cells plated for each cell line are:

High density
Clone 19 19.9%, SCC12F 9.1%, SCC12B 5%, 19 (216)1 B 29.6%

Low density
Clone 19 33.2%, SCC12F 8.5%, SCC12B 10.8%, 19(216)1 B 26.4%

Figure 5.12

Colony survival after suspension induced cell death.



obtained with a survival range form 48% (19 CMV8) to 24% (clone 19). As expected normal HEK cells had very low resistance to suspension induced cell death with very few colonies being formed after 24 hours in suspension.

The cell lines expressing large amounts of mutant p53 (19 (216)1 B and 19 (216)2 3) were shown to have a higher resistance to suspension induced cell death (figure 5.12 and 5.13). Also colonies arose from these cell lines more quickly than other cell lines and appeared larger and healthier. Colonies were often stained upto five days before any other cell line to prevent the colonies merging. At high density 49% and 61% colonies survived after 24 hours respectively. At low density this survival increased to 73% and 70% respectively.

These results therefore suggest that 19 (216)1 B and 19 (216)2 3, which express high levels of mutant $p53^{216}$, have a higher colony forming ability and therefore a greater resistance to suspension induced cell death than cell lines that have lower levels of mutant p53. This resistance is enhanced by growing the cells at lower density where survival is approximately double that of the other cell lines.

Figure 5.13

Colony survival after suspension induced cell death.

Cells were seeded at either $2x10^6$/5cm dish (high density) or $3x10^5$/5cm dish (low density) and incubated for 4 days. Cells were then either plated at 500 cells /T25 flask or placed in suspension culture for 24 hours. These cells were then washed from the methylcellulose and plated at 10^3/T25 flask. Cells were incubated at 37°C until colonies were easily visible and then stained with 1% rhodamine and counted. Colony numbers were recorded per 500 cells plated.
Experiments were repeated at least 3 times and the average number of colonies per time point for each cell line recorded
Percentage colony survival is plotted for each cell line i.e. the number of colonies counted after 24 hours in methylcellulose as a percentage of the number of colonies counted without suspension culture (day 0), ± standard deviation.

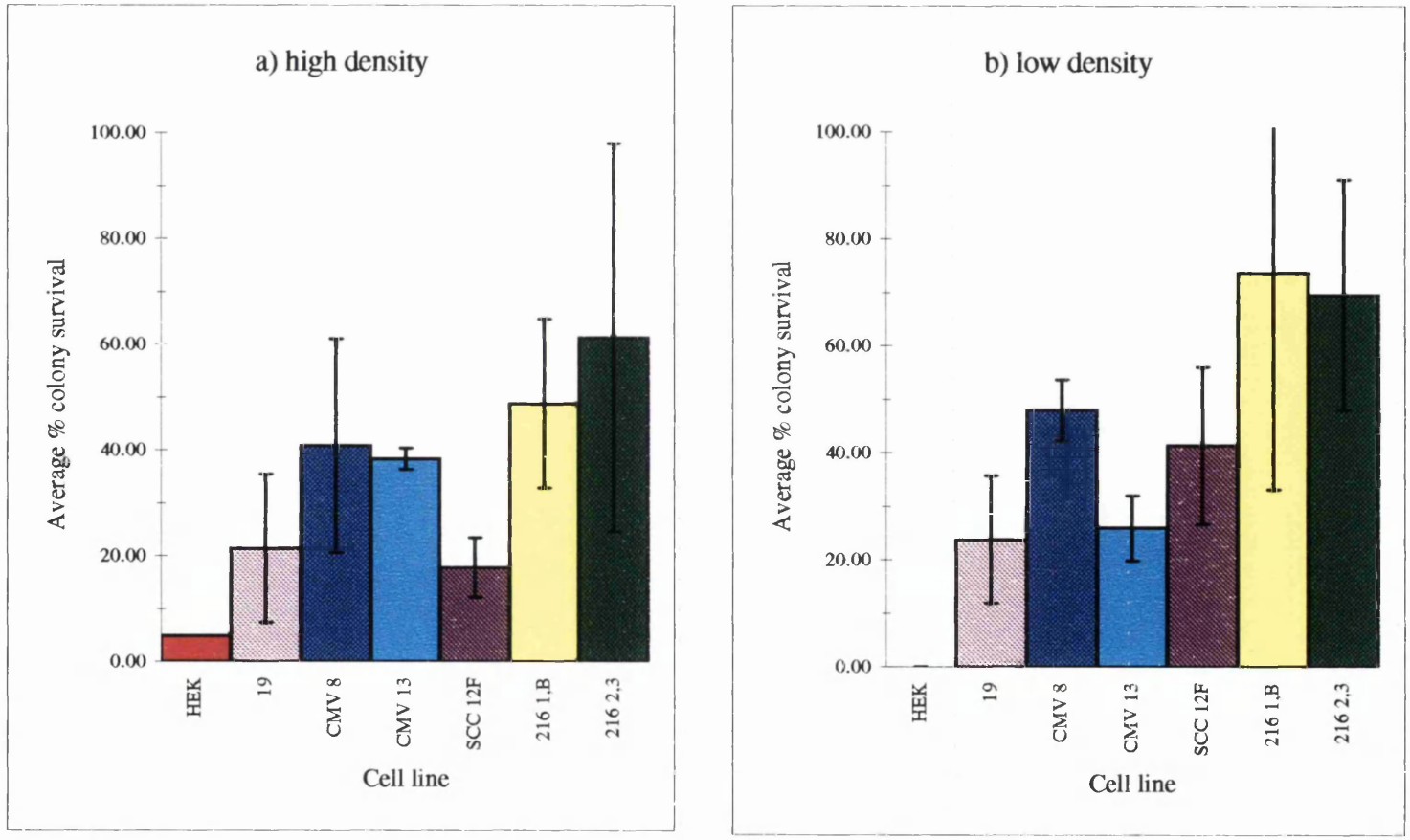
Colony survival at day 0 recorded as a percentage of total cells plated for each cell line are:

High density
HEK 8.2%, Clone 19 41.6%, 19 CMV8 38.1%, 19 CMV13 51.5%, SCC12F 37%, 19 (216)1 B 47%, 19 (216)2, 3 50.7%.

Low density
HEK 2.3%, Clone 19 47.1%, 19 CMV8 45.4%, 19 CMV13 73.9%, SCC12F 36.8%, 19 (216)1 B 46.2%, 19 (216)2, 3 58.3%.

Figure 5.13

Colony survival after suspension induced cell death.



5.2.4. Formation of cornified envelopes.

Cornified envelopes are first visible in cells of the granular layer (Sun and Green, 1976). Such cornified envelopes are formed as the cells become permeable to trypan blue (Green, 1977) by the crosslinking of several proteins by the calcium dependent cellular enzyme transglutaminase (Rice and Green, 1979). Cornified envelopes can easily be isolated by their insolublility to detergents and reducing agents (Sun and Green, 1976). HEK cells can be induced to terminally differentiate by culturing them in methylcellulose. Such cells have been shown to form insoluble cornified envelopes almost immediately and 50% of these cells form cornified envelopes by day 5 (Green, 1977).

The ability of SCC12F, SCC12B, clone 19 and clone 19 transfected with high levels of mutant $p53^{216}$ to form envelopes was investigated. Cornified envelopes were counted after cells had been cultured in methylcellulose for 5 days as described in section 2.2.1.6. Cell density was controlled for by plating cells at both high density ($2x10^6$cells/5cm dish) and low density ($3x10^5$ cells /5cm dish) before being placed in suspension culture.

Envelopes were obtained by their resistance to treatment with 5% SDS/1% β mercaptoethanol and boiling at 100°C and an example of their appearance is shown in figure 5.14, one of many being highlighted by an arrow. They are often very large rounded and transparent structures and can be seen by focussing through several planes using the phase contrast of a light microscope. Electron micrograph studies of the envelope have previously been reported (Green, 1977).

SCC12B and 19 (216)1 B develop very few cornified envelopes after 5 days in suspension culture (figure 5.15) when grown at both high and low density. SCC12F develops slightly more envelopes but still very few relative to clone 19.

This is again shown in figure 5.16 where SCC12B, 19 (216)1 B, 19 (216)2 3 and 19 (216)2 1 which all express large amounts of mutant $p53^{216}$ are deficient in forming cornified envelopes after 5 days in suspension culture. These cell lines appear to form slightly higher numbers of envelopes when grown at high density than at low density

(figure 5.16) but in comparison to controls these numbers are still very low. In this experiment too, clone 19, SCC12F and 19 CMV8 and 19 CMV13 which express the vector alone, all develop higher numbers of cornified envelopes than the cell lines expressing more mutant p53 but lower numbers than normal cells. Their ability to form envelopes was also higher when grown at high density than when grown at a lower density. Normal HEK cells, as expected, develop large numbers of envelopes after suspension induced terminal differentiation (figure 5.16).

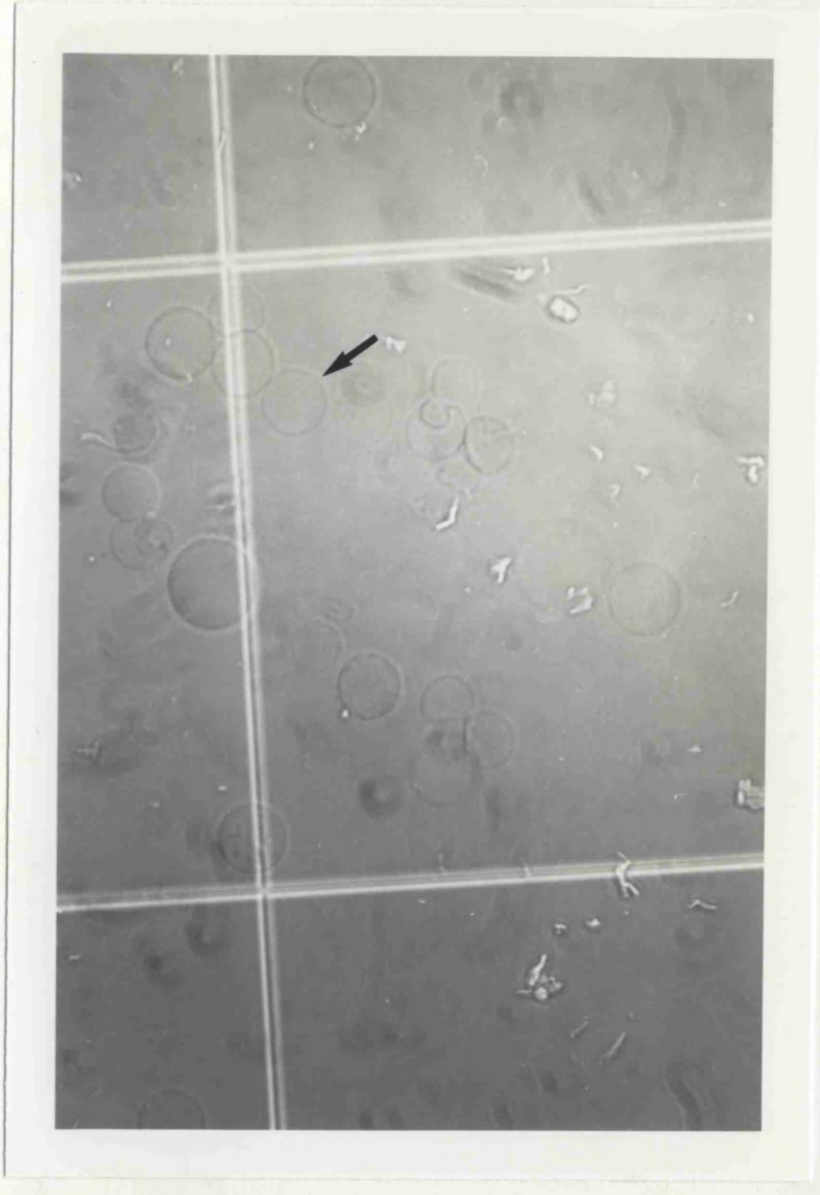


Figure 5.14

Phase contrast photograph showing the structure of cornified envelopes obtained from HEK cells 5 days after suspension culture. One of many is highlighted by the arrow. Numbers of cornified envelopes formed in each cell line are shown in figure 5.15 and 5.16.

Bar = 10uM

Figure 5.15

Cornified envelope formation after suspension culture.

Experiment 1

Cells were seeded at either $2x10^6$/5cm dish (high density) or $3x10^5$/5cm dish (low density) and incubated for 4 days at 37°C. Cells were then suspended in methylcellulose at 10^5/ml for 5 days . Cells were then washed and resuspended at $3x10^5$/ml in 5% SDS/ 1% β mercaptoethanol and solubilised by boiling for 5 minutes. Cornified envelopes were counted using a haemocytometer under a light microscope. Numbers are plotted per 10^4/ml and are the average values obtained from at least 4 experiments. Standard deviations ± are also plotted

Percentage of cells which had formed cornified envelopes at day 0 before suspension culture are:

High density

Clone 19 0.2%, SCC12F 0.33%, SCC12B 0.33%, 19 (216)1 B 0.67%.

Low density

Clone 19 0.9%, SCC12F 0.67%, SCC12B 0.1%, 19 (216)1 B 0.2%.

Figure 5.15

Cornified envelope formation after suspension culture

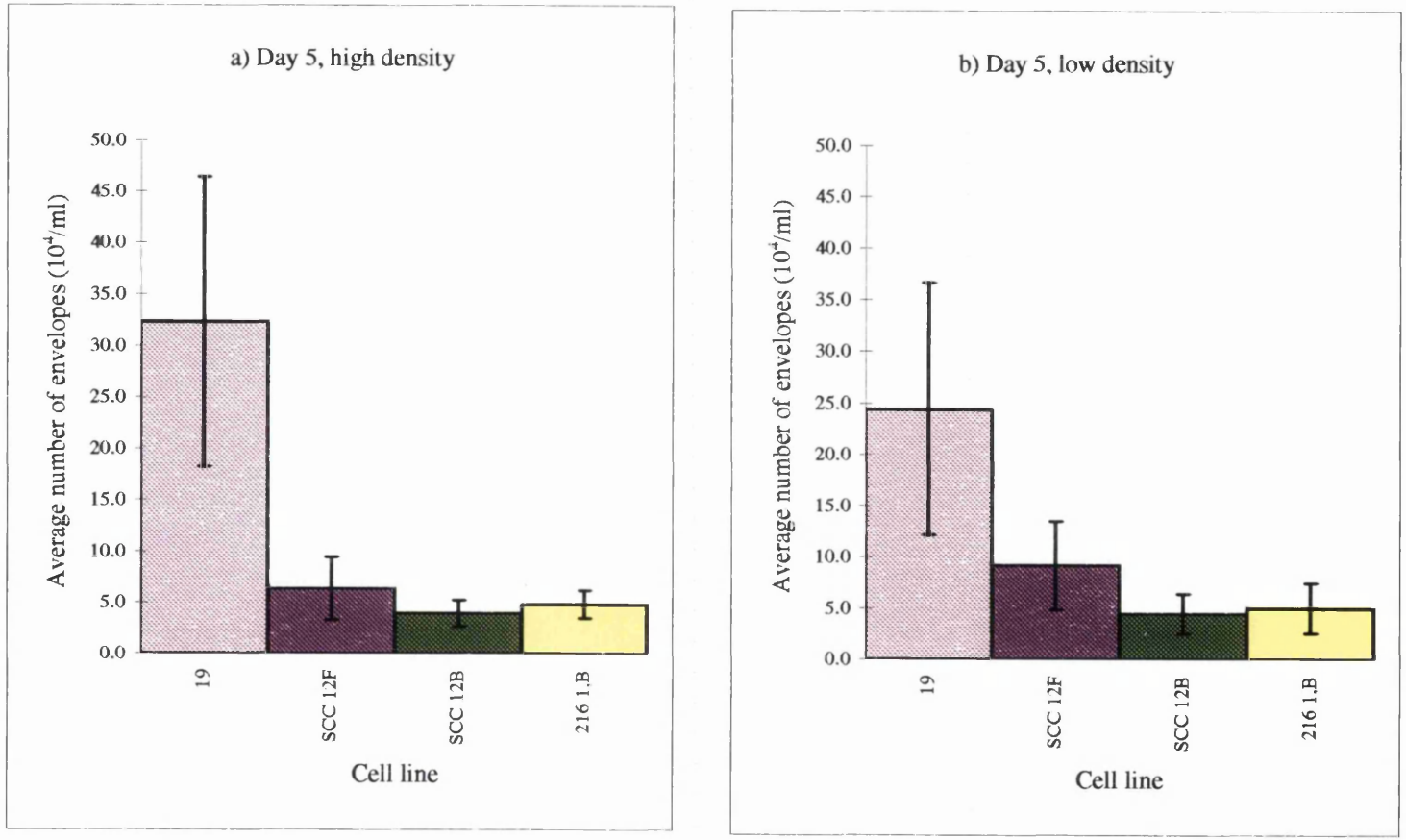


Figure 5.16

Cornified envelope formation induced by suspension culture.

Experiment 2

Cells were seeded at either $2x10^6$/5cm dish (high density) or $3x10^5$/5cm dish (low density) and incubated for 4 days at 37°C. Cells were then suspended in methylcellulose at 10^5/ml for 5 days . Cells were then washed and resuspended at $3x10^5$/ml in 5% SDS/ 1% β mercaptoethanol and solubilised by boiling for 5 minutes. Cornified envelopes were counted using a haemocytometer under a light microscope. Numbers are plotted per 10^4/ml and are the average values obtained from at least 4 experiments. Standard deviations ± are also plotted

Percentage of cells which had formed cornified envelopes at day 0 before suspension culture are:

High density

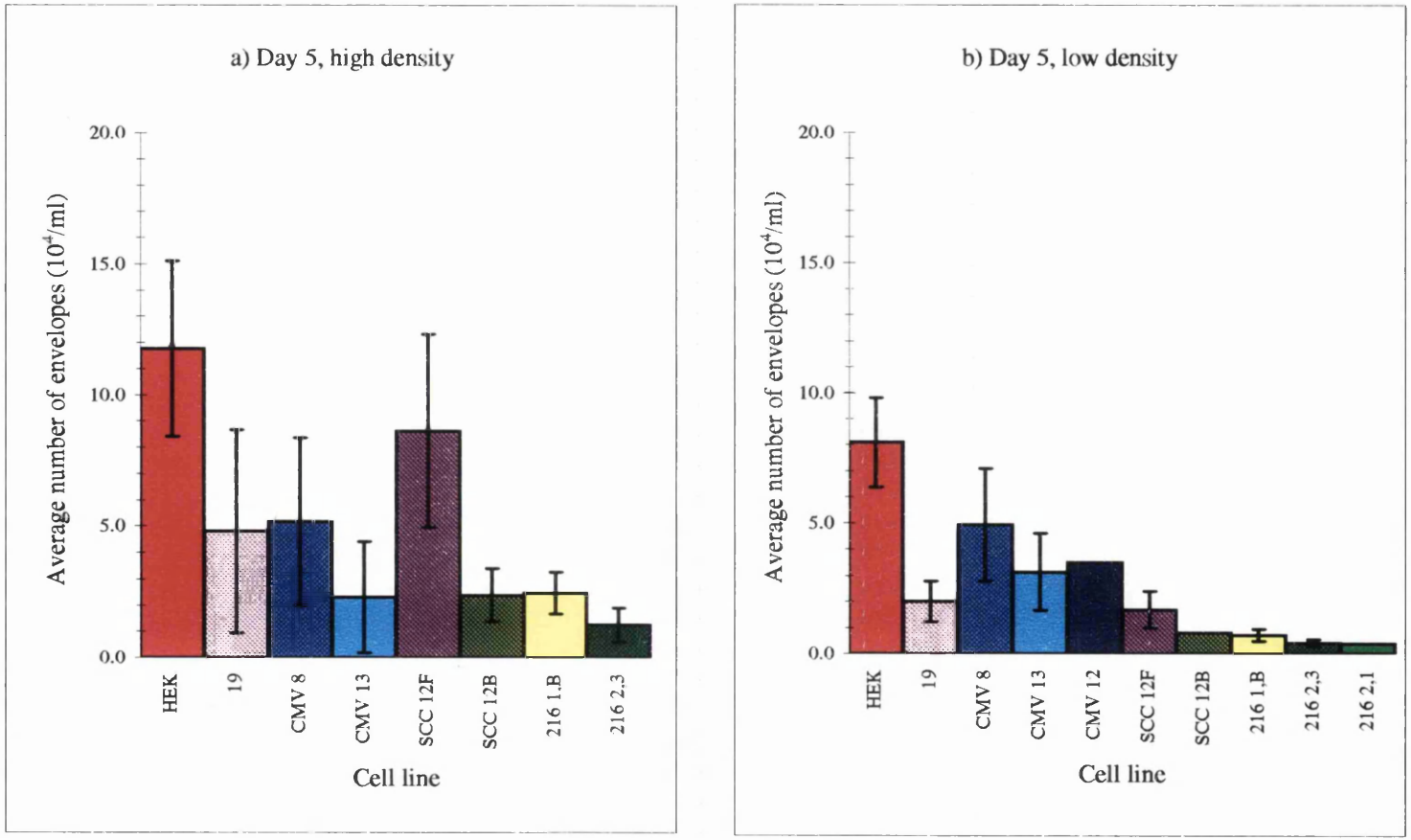
HEK 2.3%, Clone 19 1.3%, 19 CMV8 0.67%, 19 CMV13 1.3%, SCC12F 1%, SCC12B 1%, 19 (216)1 B 1.3%, 19 (216)2 3 1%.

Low density

HEK 1.3%, Clone 19 0.67%, 19 CMV8 0.33%, 19 CMV13 0.33%, 19 CMV12 0.33%, SCC12F 0, SCC12B 0.33%, 19 (216)1 B 0.33%, 19 (216)2 3 0.33%, 19 (216)2 1 0.33%.

Figure 5.16

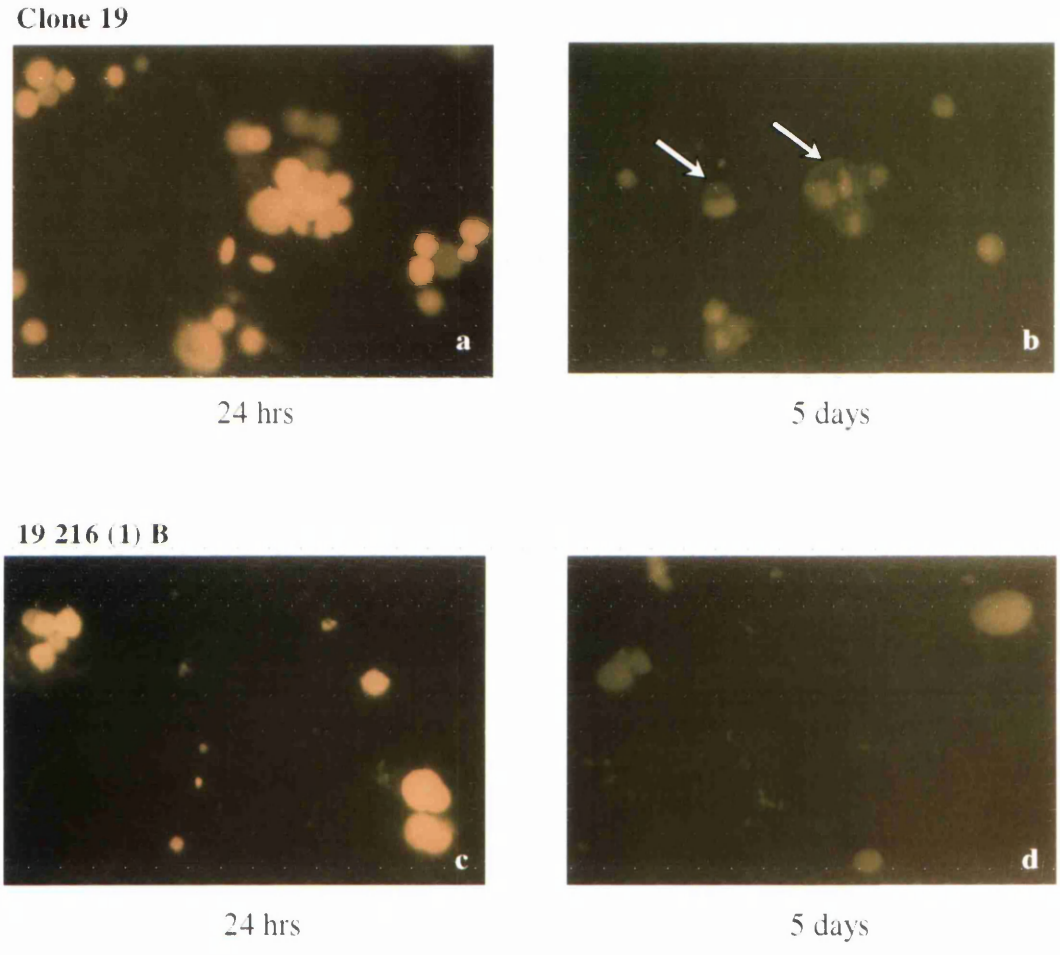
Cornified envelope formation induced by suspension culture



5.2.5. Morphology of cells after suspension culture.

Cells after 0, 1, 3 and 5 days in suspension were stained with acridine orange which binds to DNA, highlighting the position and size of the nucleus. Photographs showing the nuclear morphology after 24 hours and 5 days in suspension of clone 19 and 19 216 (1) B are shown in figure 5.17. SCC12F and clone 19 cells transfected with the CMV vector alone showed a similar pattern as for clone 19 (figure 5.17 a and b). Other cell lines expressing high levels of mutant p53 (19 216(2) 3 and SCC12B) showed a similar staining as for 19 216(1) B (figure 5.17 c and d). As part of the terminal differentiation pathway keratinocytes have been shown to degrade their nuclear DNA in a manner characteristic of apoptosis (McCall and Cohen, 1991; Meredith *et al.*, 1993) and sometimes termed anoikis (Frisch and Francis, 1994). Nuclear digestion does not occur until a few days after the cells have become permeable and formed a cornified envelope (Green, 1977) in the upper granular layer. Squames are often released from stratified colonies into the medium before their nuclei have been digested. It has previously been reported that 35% of HEK cells cultured in methylcellulose for 5 days have lost their nucleus (Green, 1977). The chromatin becomes condensed and marginated as can be seen in clone 19 figure 5.17b. During these morphological changes the DNA is fragmented into 200bp units by a calcium dependent endonuclease (McCall and Cohen, 1991) which can be visualised as a DNA ladder on an agarose gel (Meredith *et al.*, 1993; Frisch and Francis, 1994). Cell lines expressing high levels of mutant p53 (19 216(1) B) did not show any nuclear shrinking after 5 days in suspension (figure 5.17d). No nuclear shrinking was apparent in other clones expressing mutant p53 either (data not shown). From these preliminary results it appears therefore that mutant p53 has inhibited the ability of cells to destroy their nucleus, another feature of the terminal differentiation pathway.

Figure 5.17 Acrydine orange staining of cells after suspension culture



Clone 19 (a and b) and 19 216 (1) B (c and d) were placed in suspension culture for 24 hours (a and c) or 5 days (b and d). Cells were then stained with acrydine orange 10ug/ml as described in materials and methods. Photographs show examples of the typical morphology of these cells at the time points indicated.
Arrows shows nuclear condensation.

Bar = 10uM

5.2.6. Tumorigenicity of cell lines in nude mice.

Tumorigenicity data from two separate experiments are shown in figure 5.18 and 5.19. Cells were injected at two different doses, $5x10^6$ or $1.6x10^6$ / mice in either flank of four week old nude mice The lower dose rarely gave rise to tumours, but tumours were formed from some cell lines when the larger cell dose was administered. Cysts were commonly formed immediately after injection but these slowly regressed.

SCC12F and clone 19 did not give rise to persistent tumours in either experiment. Mice injected with higher doses of both cell lines developed slowly regressing tumours and by the end of both experiments these had completely regressed in SCC12F and only one small cyst remained from the injection of clone 19.

Control cell lines, 19 CMV8 and 19 CMV13 expressing the CMV vector alone, produced a similar result to clone 19. No persistent tumours were produced by these cell lines (figure 5.19).

Contradictory to published results (Parkinson *et al.*, 1984; Jaffe *et al.*, 1992) in our hands SCC12B did not form persistent tumours (figure 5.18). A relatively large tumour developed 2 weeks after the injection of $5x10^6$ cells/mouse but this slowly regressed throughout the period of the experiment.

Similarly cell lines 19 216(1) B and 19 216(2) 3, both expressing high levels of mutant $p53^{216}$ protein showed a low tumorigenic potential. 19 216(2) 3 was totally non-tumorigenic at both cell doses and in both experiments. 19 216(1) B however was tumorigenic but only when injected at 5 $x10^6$ cells/mouse. This cell line produced large cysts at the beginning of each experiment at both doses but throughout the duration of the experiment these slowly regressed. Only one tumour remained in one of each of the the three mice (figure 5.18 and 5.19) reaching our criteria for tumorigenicity i.e. a tumour remaining on the mouse for 3 months and reaching $1cm^3$ in size. The tumour from 19 216 (1) B reached $1cm^3$ in both experiments with a latent period of 63-66 days. A third clone, (19 216 (1) 2) overexpressing mutant $p53^{216}$ was also tested once for tumorigenicity and was found to be non-tumorigenic

Figure 5.18

Tumorigenicity of cell lines injected into nude mice.

Experiment 1

	Time after injection					
Cell line	2 weeks	4.5 weeks	8 weeks	9.5 weeks	11.5 weeks	19 weeks
SCC12F $5x10^6$ cells	*4x4mm* (3/3)	*3x3mm* (3/3)	*2x2mm* (3/3)	*1x1mm* (3/3)	2x2*mm* (3x3)	(0/3)
$1.6x10^6$ cells	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)
Clone 19 $5x10^6$ cells	*4x4mm* (3/3)	*2x2mm* (3/3)	*1x1mm* (3/3)	*3x3mm* (1/3)	*3x3mm* (1/3)	*2x2mm* (1/3)
$1.6x10^6$ cells	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)
SCC12B $5x10^6$ cells	5x5*mm* (3/3)	4x4*mm* (3/3)	3x3*mm* (3/3)	3x3*mm* (3/3)	4x4*mm* (3/3)	2x2*mm* (1/3)
$1.6x10^6$ cells	(0/3)	2x2*mm* (1/3)	1x1*mm* (1/3)	(0/3)	(0/3)	(0/3)
19 216(1) B $5x10^6$ cells	5x5*mm* (3/3)	5x6*mm* (2/3)	9x10*mm* (3/3)	10x10*mm* * (2/3)	11x13*mm* (2/3)	12x14*mm* (1/3)
$1.6x10^6$ cells	2x2*mm* (2/3)	3x3*mm* (1/3)	1x1*mm* (3/3)	(0/3)	2x2*mm* (2/3)	(0/3)
19 216(2) 3 $5x10^6$ cells	5x5*mm* (3/3)	4x4*mm* (1/3)	(0/3)	(0/3)	(0/3)	(0/3)
$1.6x10^6$ cells	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)

$5x10^6$ and $1.6x10^6$ cells were injected subcutaneously into the right or left flank respectively of four week old nude mice.
Size of tumour is shown in italics and measured in mm
A total of three mice was used per cell line. Numbers of mice with tumours are shown in parentheses.

* Latent period of tumour where tumour reached $1cm^3$ was 66 days after injection.

Figure 5.19

Tumorigenicity of cell lines injected into nude mice.

Experiment 2

	Time after injection		
Cell line	1.5 weeks	3weeks	10.5weeks
Clone19 $5x10^6$ cells	*2x2mm* (3/3)	*2x2mm* (3/3)	*2x2mm* (1/3)
$1.6x10^6$ cells	*2x2mm* (3/3)	*1x1mm* (2/3)	(0/3)
19 CMV8 $5x10^6$ cells	*2x2mm* (3/3)	*2x2mm* (3/3)	(0/3)
$1.6x10^6$ cells	(0/3)	(0/3)	(0/3)
19 CMV13 $5x10^6$ cells	4x4*mm* (3/3)	2x2*mm* (3/3)	(0/3)
$1.6x10^6$ cells	(0/3)	(0/3)	(0/3)
19 216(1) B $5x10^6$ cells	6x6*mm* (3/3)	7x7*mm* (2/3)	10x10*mm* *(1/3)
$1.6x10^6$ cells	4x4*mm* (2/3)	6x6*mm* (2/3)	(0/3)
19 216(2) 3 $5x10^6$ cells	2x2*mm* (3/3)	4x4*mm* (2/3)	(0/3)
$1.6x10^6$ cells	2x2mm (3/3)	2x2mm (2/3)	(0/3)

$5x10^6$ and $1.6x10^6$ cells were injected subcutaneously into the right or left flank respectively of four week old nude mice.
Size of tumour is shown in italics and measured in mm
A total of three mice was used per cell line. Numbers of mice with tumours are shown in parentheses.

* Latent period of tumour where tumour reached $1cm^3$ was 63 days after injection.

Chapter 6

Discussion

6.1 Genetic differences between SCC12B and SCC12F.

SCC12B and SCC12F represent two distinct cell populations within a single tumour (SCC12) at different stages of malignant progression (Rheinwald and Beckett, 1981). As outlined in section 1.1.2. SCC12B is more tumorigenic and more resistant to terminal differentiation signals than the sister cell line SCC12F. Somatic cell hybrids derived by fusing SCC12B with SCC12F showed a much lower tumorigenic phenotype than the parental SCC12B cell line (Jaffe *et al*, 1992). This suggests that the malignant phenotype is recessive and SCC12F was able to donate a suppressor function to the hybrid. It was therefore reasonable to assume that SCC12F possessed a gene or genes, possibly a tumour suppressor gene, that had become inactivated in SCC12B preventing it either directly or indirectly from responding normally to terminal differentiation signals and aiding its progression to malignancy.

Several known genes or loci known to be important in SCC-HN development were compared between SCC12F and SCC12B in the hope of finding a genetic difference that could be responsible for their different phenotypes.

Comparison of cyclin D1 protein levels in SCC12B and SCC12F showed no noticeable difference between the two cell lines (section 3.1.3) and very little overexpression of the protein was observed when compared with other SCC cell lines. Also transfection of cyclin D1 into SCC12F did not increase its tumorigenic potential (M.Nikolic, personal communication). These observations suggest that although cyclin D1 amplification is frequently observed in a variety of tumours including SCC-HN (Jiang *et al.*, 1992, Nikolic *et al.*, submitted) it does not play an important role in the progression of this particular SCC-HN cancer.

Analysis of microsatellite sequences on chromosomes 9 and 3p did not reveal any differences in LOH between SCC12B and SCC12F either. These two chromosomes were chosen for analysis over any other as there is currently more data linking loci on these chromosomes with SCC development (section 1.4.1.1). Chromosome 3p has been shown to have frequent loss of heterozygosity in SCC-HN (Edington *et al.*, 1995; and unpublished data). Previous analysis of chromosome 9 in SCC-HN has shown there to

be an 84% chance of LOH at 9p21 in SCC-HN (Loughran *et al.*, 1994). The majority of microsatellites used only showed amplification of one allele band in both cell lines. This was marked as non-informative, but as no normal DNA from the patient was available for comparison a loss could have occurred at that locus in both cell lines, possibly highlighting the position of a gene whose inactivation plays an important and early role in the development of this SCC. The data however does not answer the initial question by revealing any candidates on chromosomes 9 or 3p that could be responsible for the different phenotypes of these two cell lines.

On analysing the p53 tumor suppressor protein levels in SCC12B and SCC12F a difference in expression levels was observed. SCC12B appeared to express much higher levels of p53 protein than SCC12F (figure 3.6). Single clones of SCC12F could also be isolated that had varying levels of p53 protein supporting the hypothesis that SCC12F progression towards the more malignant phenotype seen in SCC12B is accompanied by the expression of high levels of p53 protein. Wild-type p53 has a relatively short half-life but this becomes increased when the protein is stabilised by a mutation. SCC12B was indeed found to express a novel p53 mutation at codon 216, a T→G transversion, substituting a valine for a glycine (figure 3.7). Interestingly, the mutation appears to be a heterozygous one with the wild-type allele being retained. This is rare as the wild-type affect of p53 is dominant as shown by the induction of growth arrest by expressing wild-type p53 into cell lines carrying endogenous mutant p53 (Mercer *et al.*, 1990; Baker *et al.*, 1990; Casey *et al.*, 1991) and usually both alleles are mutated as the cancer progresses for example in Li-Fraumeni patients (Srivistava *et al.*, 1992b).

In line with the low level of p53 protein expression, little mutant protein was detected in SCC12F which appears to express mostly the wild-type allele (figure 3.7). This suggests that accumulation of this p53 mutation occurs later in the progression of this SCC. Although this particular mutation is novel and lies outside a conserved domain where most mutations are observed (figure 1.3), other mutations at this codon have been reported (Caron de Fromentel and Soussi, 1992) and therefore this part of the protein is a relevant site for mutagenesis.

Codon 216 is positioned in the hydrophobic core of the p53 protein and is not involved directly in DNA binding (Clore *et al.*, 1994). This region coincides with the epitope recognised by PAb240 (residues 212-217) (Stephen and Lane, 1992). This epitope however is cryptic and the protein has to be denatured or this region of the protein unfolded in order for the antibody to bind. This mutant p53 is unusual in that it is not recognised by PAb240 in immunoprecipitation experiments (figure 4.4). At first glance this may not be surprising as the mutation at codon 216 may disrupt the epitope and prevent the antibody from binding. Another mutation in a Burkitts lymphoma cell line (Farrell *et al.*,1991) was identified in the PAb240 epitope region at codon 213 substituting an arginine for a glutamine. This mutation was also a heterozygous one and the protein had also lost reactivity with PAb240 presumably due to the disruption of the epitope. However the $p53^{216}$ mutant protein is still reactive with the antibody PAb1620 which recognises the wild-type conformation suggesting that the mutation has not unfolded the protein and the PAb240 epitope is not revealed. Glycine and valine amino-acids are very similar in that they both have aliphathic side chains and are non-polar although glycine is less hydrophobic than valine. Glycine has a small side chain of a single hydrogen whilst valine is slightly more bulky with two methyl groups but this substitution would have few constraining effects on the protein. Immunoprecipitation studies suggest that a substitution of a glycine for a valine in this region does not alter the protein conformation and the mutant is a class I type as discussed in section 1.4.3.2. However, if this were a silent mutation then the protein would be expected to behave as a wild-type protein. This is clearly not the case as the protein is stabilised like most mutants and when transfected into Saos-2 cells (Frebourg *et al.*, 1992) the mutant protein has lost its ability to suppress cellular growth (section 4.1.2). This mutation, then, whilst it has no effect on protein conformation, it does inactivate the growth suppressor function of p53.

Preliminary studies suggest that this mutant $p53^{216}$ protein has not gained any non wild-type functions. Saos-2 cells expressing the mutant p53 do not show an increase in saturation density (section 4.1.3) (Chen *et al.*, 1990) and therefore the mutant does not appear to promote an oncogenic phenotype by giving the cells a selective growth

advantage. However additional transcriptional and DNA binding properties of the mutant cannot be ruled out and require further analysis (Bargonetti *et al.*, 1992; Scharer and Iggo, 1992; Dittmer *et al.*, 1993)

As both SCC12B and SCC12F have retained expression of the wild-type p53 it is possible that the difference in their phenotypes may be due to the dosage effects of the mutant p53 protein. Both cell lines were found to contain three copies of chromosome 17 and three copies of the p53 gene confirming the karyotype carried out by Jaffe *et al*, 1992, showing that both cell lines were triploid. Although double hybridisations were not carried out it can be concluded from these results that all three p53 genes lie on a chromosome 17 and the position of the signal (figure 3.11) indicates each gene lies on the short arm as expected. Dot blot analysis of the mutant and wild-type gene dosages in the two cell lines confirmed that SCC12B expressed much more, perhaps double the amount of mutant p53 DNA and RNA than SCC12F (section 3.2.3). Conversely, SCC12F and a clone of SCC12F (clone 19) were shown to express more wild-type p53 than SCC12B. In conjunction with the known p53 copy number in both cell lines I suggest that SCC12B carries two mutant and one wild-type p53 genes and SCC12F and clones of SCC12F carry two wild-type and one mutant p53 genes. A third chromosome 17 was acquired by both cell lines probably by chromosomal duplication or uneven segregation during mitosis and suggests that development of aneuploidy in this tumour is an early event. The acquirement of a second p53 mutation by SCC12F during progression towards SCC12B is unlikely to occur by a second independent point mutation as the probability that the same mutation would be acquired is very small. However gaining a second identical mutation could occur by non-disjunction at mitosis leading to a loss of one of the wild-type chromosomes, followed by duplication of the mutant chromosome, or alternatively by mitotic recombination between a mutant chromosome and a wild-type one.

The presence of both mutant and wild-type p53 in a neoplastic cell suggests that the mutant has inactivated the wild-type protein in a dominant-negative manner. Usually a dominant-negative protein would bind and also sequester the wild-type protein (Milner and Medcalf, 1991). In this case the $p53^{216}$ mutant retains reactivity with the PAb1620

and therefore it is difficult to ascertain how much of the immunoprecipitated protein is wild-type. Heterozygous mutations are a characteristic of the Li-Fraumeni syndrome but the normal cells rarely accumulate p53 protein when the mutation is in this heterozygous state (Bhatia *et al.*, 1993; Srivistava *et al.*, 1992b). When the wild-type p53 is lost during tumour progression the mutant protein then accumulates. Therefore stabilisation of p53 protein in the presence of a heterozygous mutation as in SCC12B signifies that the mutation is dominant. The higher levels of mutant p53 in SCC12B as compared to SCC12F suggests that the dose of the mutant in relation to the wild-type protein is critical in the phenotype displayed. This thesis shows that increasing the levels of mutant p53 in a clone of SCC12F (clone 19) did yield a phenotype similar to SCC12B (discussed further in the next section). The $p53^{216}$ mutant therefore shows a dose-dependant dominance over the endogenous wild-type p53, SCC12F and SCC12B being two examples of the phenotype gained at different mutant doses. It is postulated that as the mutant dose increases then the heterotetramers formed with the wild-type protein contain more mutant p53 molecules and therefore may reduce the wild-type proteins affinity to DNA and transcriptional activity. Sun *et al.* 1993 showed that to completely suppress cell growth by the wild-type p53 a ratio of 3 mutant to 1 wild-type molecule in the tetramer was needed. This therefore explains why SCC12B requires such a large expression of mutant p53 to express a neoplastic phenotype and why the mutant is inactive in displaying such a phenotype at smaller doses as in SCC12F. It has yet to be clarified if the mutant has itself additional activities once it has sequestered any wild-type p53 function.

6.2 Increased mutant $p53^{216}$ blocks the ability of SCC12F to terminally differentiate.

In order to analyse the effect that mutant p53 dosage has on the cells ability to terminally differentiate $p53^{216}$ was overexpressed in a clone of SCC12F, clone 19. Only small amounts of p53 protein were detected in this clone (figure 5.1) and hence it presumably expressed very little mutant p53 protein. This clone therefore represented a

more normal phenotype than SCC12B yet still retained a related genetic background on which to manipulate the mutant p53 gene dosage. The use of normal keratinocytes as a target would have been hindered by their short lifespan in culture and the use of null or normal keratinocytes would not have taken into account any initiating event that had occurred prior to the acquisition of this mutant p53 which perhaps acts in concert with the mutant to yield the phenotype displayed by SCC12B. The use of clone 19 therefore is a very appropriate and relevant target cell for investigating the influence of genetic events on the progression of this SCC-HN.

The growth and morphology of the cell lines used in this thesis displayed subtle but important differences. Clone 19, a low expressing p53 clone of SCC12F, grew very slowly in the culture conditions used i.e. 10% serum. They also preferred to be kept at quite a high cell density, losing cloning efficiency if the cells were split harshly. The culture conditions for this cell line were not optimised but kept identical to the other cell lines for a standard throughout all the experiments. Clone 19 also maintained the characteristic cobble stone morphology of normal keratinocytes as did SCC12F (figure 5.8). SCC12B however appeared much flatter and exhibited a slightly faster growth rate. As previously reported they were able to grow in the presence of reduced serum (Jaffe *et al.*, 1992). Similarly transfection of clone 19 with mutant $p53^{216}$ yielded cell lines that showed increased growth rates and reduced serum dependence

These cell lines (19 216(1)B and 19 216 (2) 3) also showed more compact cell growth and seemed to be less contact inhibited (figure 5.8). These observations therefore indicate a correlation between p53 mutant protein levels and morphological changes.

Analysis of the ability of the mutant p53 transfectants (19 216(1)B and 19 216 (2) 3) to respond to suspension induced terminal differentiation revealed two independent signals which could influence the decision of the cell to differentiate. One signal appeared to be caused by mutant p53 which prevented or blocked the transfectants from differentiating, the second signal came from cellular crowding which stimulated the cells to differentiate and sometimes masked the inhibitory effect of mutant p53.

If the cells were grown at high density before being placed in suspension no obvious effect of increased mutant p53 levels on involucrin synthesis was observed over the controls (figure 5.10). However if the cells were grown at a much lower density, reducing the extent of cellular contacts before being placed in suspension, a marked effect was observed in the ability of the cells to express involucrin (figure 5.11). Cultured at low density, 19 216(1)B and 19 216 (2) 3 appeared to be blocked in their ability to express involucrin. The level of expression after three days in suspension was half that of the same cells grown at much higher density. At low density the expression of involucrin does not increase from day 0 to day 3 in these two cell lines compared to controls (figure 5.11). These results indicate that mutant p53 may prevent the ability of cells to express involucrin but this effect can be overridden by the effects of increased cellular crowding which stimulate involucrin expression. This stimulation of differentiation by crowding is also shown by comparing the extent of involucrin expression at day 0 in the low and high density experiments. Cells grown at low density (figure 5.11a) tend to express much less involucrin than those grown at higher density (figure 5.10a) suggesting that a stimulus induced by crowding is switching on differentiation in these cell lines even before thay are placed in suspension culture. It has been shown by others that the area of contact with the substratum and hence cell shape regulates proliferation and terminal differentiation of HEKs in culture (Watt *et al.*, 1988). When spreading is restricted as in the case of cells cultured at high density, and hence the cells become slightly rounded, involucrin expression was stimulated and DNA synthesis inhibited (Watt *et al.*, 1988). This effect of high density cultures is mimicked by suspension culture i.e. reducing contact with the substratum and forcing cells to alter their shape. The signalling pathway by which cells undergo terminal differentiation in response to cell density is not clear but is proposed to be mediated by receptors expressed on the keratinocyte cell surface. The two major classes of adhesive receptors expressed by keratinocytes are integrins (Hynes 1992) which mediate adhesion to the extracellular matrix (discussed in more detail in the next section), and cadherins which mediate cell-cell adhesion (Geiger and Ayalon, 1992). Integrin binding to the extracellular matrix has been implicated in cell signalling maintaining cell proliferation

(Adams and Watt, 1989). On commitment to terminal differentiation the ability of integrin receptors to bind ligand decreases (Adams and Watt, 1990) due to modulation of the receptors on the cell surface (Hotchin and Watt, 1992) and eventually expression of the integrin receptor is completely lost. The major cadherins expressed on keratinocytes are P cadherin (confined to the basal cells) and E cadherin and cell-cell adhesion mediated by these receptors in calcium dependent (Takeichi, 1991). Recent experiments have shown that in low calcium medium keratinocytes co-express integrins and terminal differentiation markers e.g. involucrin. On increasing the levels of calcium cells stratify and integrins are lost from the cell surface (Hodivala and Watt, 1994). Furthermore antibodies to P and E cadherin inhibited calcium induced stratification and loss of integrins (Hodivala and Watt, 1994). Therefore not only does decreased contact with the substratum and alteration of cell shape induce terminal differentiation of keratinocytes but also cell to cell adhesion mediated by cadherin interactions stimulates loss of integrin expression which is known to occur during terminal differentiation (Adams and Watt, 1989 and 1990).

Plating cells at high density may therefore have already committed or sensitised cells to any further differentiation stimulus and hence higher levels of involucrin are expressed after suspension culture in the high density experiment. It is clear however from the low density experiments that mutant $p53^{216}$ has an inhibitory effect on the production of involucrin but this inhibition can be overridden by a pathway independent of p53.

Increasing mutant p53 also had an inhibitory effect on the production of another marker of terminal differentiation, cornified envelope formation. All cell lines expressing high levels of $p53^{216}$ mutant i.e. 19 216(1)B, 19 216 (2) 3, 19 216 (2) 1 and SCC12B are unable to form cornified envelopes after 5 days in suspension unlike all the controls (figure 5.15 and 5.16). This inability to form envelopes is observed when the cells are grown at both high density and low density. However at high density the numbers of envelopes counted were higher in all cell lines compared to low density experiments suggesting again that cellular crowding increases the proportion of terminally

differentiating cells as discussed above. The differentiation signal exerted by cellular crowding does not appear to have such a strong effect on cornified envelope formation as it does on involucrin synthesis. The inhibitory effect of mutant $p53^{216}$ on envelope formation can still be observed even when the cells are grown at high density before being placed in suspension. The difference in stimulation of both markers by cellular crowding is surprising as involucrin is the precursor protein of the envelope and therefore it would be expected that both would be expressed to the same degree in response to such a signal. However it is known that involucrin mRNA is expressed early in the basal compartment (Watt and Green, 1981) unlike the cornified envelope which is formed much later in the granular layer (Sun and Green, 1976). As cells leave the basal layer, moving into the granular layer they become permeable (Green, 1977) and an insoluble cornified envelope forms beneath the plasma membrane (Sun and Green, 1976). This envelope forms from the cross-linking of several proteins by the cellular enzyme transglutaminase (Rice and Green, 1977). One such precursor of the envelope is the soluble protein involucrin (Rice and Green, 1979; Watt and Green, 1981) which as previously discussed, is synthesised early in differentiation. Other membrane proteins however are also thought to be incorporated into the envelope (Simon and Green, 1984). Cells prevented from making contact with other cells by incubating them in semi-solid medium can also undergo differentiation developing detergent insoluble envelopes (Green, 1977) it therefore seems that cell to cell contact is not required for this aspect of terminal differentiation. Others have also shown that protein synthesis is also not required and inhibitors of protein synthesis actually stimulate envelope formation (Rice and Green, 1978) therefore the majority of cells at any time must have sufficient transglutaminase and precursor proteins to form envelopes. Transglutaminase activity has been identified in HEK cells (Goldsmith and Martin, 1975) and protein crosslinking can be induced by treatment with agents which affect cell membrane permeability and increase cellular calcium levels (Rice and Green, 1979). It is unclear whether the source of calcium increase that activates the transglutaminase is extracellular or intracellular but it is likely to come from the degradation of organelles such as mitochondria which release calcium into the cell, as cells incubated in calcium-free medium will ultimately

form cross-linked envelopes (Rice and Green, 1978). Studies of transglutaminase in SCC12B and SCC12F have shown that SCC12B has as much as a 50 fold decrease in total transglutaminase activity than SCC12F (Rubin and Rice, 1986) therefore explaining the incompetence of SCC12B in forming envelopes. It would be of interest to establish whether transglutaminase activity has also decreased in the transfectants of clone 19 expressing high levels of mutant p53 or whether a separate event has caused a decrease in enzyme activity in SCC12B. Phorbal ester tumour promoter TPA treatment of cultured keratinocytes has been shown to accelerate the appearance of keratins (Steinert and Yuspa, 1978), induce synthesis of epidermal transglutaminase (Yuspa, 1980) and increase the formation of cornified envelopes (Reiners and Slaga, 1983). The method of stimulation of terminal differentiation by TPA is unknown and is independent of the hyperplasia caused by such agents but it may be due to an increase in protein synthesis. The results discussed above show that involucrin synthesis is stimulated by cell to cell contact but formation of envelopes is not affected to the same extent. This is likely to be due to the early synthesis of involucrin in the proliferative basal cells, whilst envelopes are not formed until intracellular calcium levels rise much later when cells are metabolically inactive and already committed to terminal differentiation.

Analysis of the cell morphology and DNA content during suspension showed slight nuclear shrinking and margination after five days in suspension in clone 19 but not in cells expressing high levels of mutant p53 (figure 5.17). This is in agreement with other data in this thesis showing terminal differentiation to be inhibited in cell lines with high levels of mutant p53. Degradation of the nucleus in an apoptotic manner also termed anoikis is part of the normal terminal differentiation pathway in keratinocytes (McCall and Cohen, 1991; Meredith *et al.*, 1993; Frisch and Francis, 1994). As cornified envelopes are formed the cells become permeable and calcium levels increase, the nucleus is digested some days later. 50% of HEK cells cultured in methylcellulose have lost their nucleus by day 8 (Green, 1977). A component of serum, plasminogen, has been shown to be essential for nuclear degradation and HEK cells are believed to contain an activator of this enzyme (Green, 1977) Nuclear degradation in keratinocytes is

caused by a calcium dependent endonuclease which cleaves DNA into 200bp fragments which when run on an agarose gel displays a ladder pattern, a hallmark of apoptosis (McCall and Cohen, 1991). Preliminary results here show that increased levels of mutant p53 appears to prevent nuclear margination and elimination but more detailed analysis of DNA laddering and endonuclease activity needs to be carried out to clarify the affect of mutant p53 on apoptosis.

Results in this thesis suggest that increased levels of mutant $p53^{216}$ enables the cells to overcome suspension induced cell death (section 5.2.3.). The cloning efficiencies of the transfected cell lines 19 216(1)B and 19 216 (2) 3 were much higher after suspension in methylcellulose for 24 hours than the controls that express low levels of mutant p53 (figure 5.12 and figure 5.13). The colonies that were obtained from these cell lines appeared much bigger and stronger and were often fixed for counting before confluence three to four days before any other cell line. The control cell lines expressing the vector alone, 19 CMV 8 and 19 CMV 13 also showed a high survival rate relative to the original clone 19. It is unlikely that the CMV promoter or the neomycin gene conveyed any resistance to suspension induced cell death. It is more likely that during the G418 selection, there is an unconscious bias towards cloning larger colonies that often represent fitter cells. These control cell lines still showed a lower survival rate than cell lines expressing high levels of mutant p53. Cellular density also had a slight effect on the extent of survival as it did for conified envelope formation and involucrin synthesis described above. In cell lines 19 216(1)B and 19 216 (2) 3 survival dropped from 73% and 71% at low density, to 49% and 61% at higher density respectively. Once again cells have already started to differentiate in response to cellular crowding giving a lower survival rate after suspension culture compared to lower density cultures but the survival phenotype given by increased mutant p53 appears to be dominant to this signal and the cells expressing this mutant p53 are resistant to suspension induced cell death.

These results indicate that an increase in mutant $p53^{216}$ in clone 19 prevents the cells from responding to terminal differentiation signals when placed in suspension culture. These experiments therefore show that the different dosages of mutant p53 in

SCC12F and SCC12B are responsible for the difference between the two cell lines in their ability to differentiate. Increasing the level of mutant p53 dosage in a clone of SCC12F, clone 19, produces a phenotype more like that of SCC12B with respect to terminal differentiation. and this is therefore an event that happens in the progression of this SCC.

The tumorigenicity of each cell line in nude mice was not as convincing as the terminal differentiation data (figure 5.18 and figure 5.19). In our hands SCC12B was not as tumorigenic as reported by others (Parkinson *et al*, 1984; Jaffe *et al*, 1992) this maybe due to the use of different mice or the more stringent criteria used in this thesis which required the tumour to remain for 3 months and reach a size of $1cm^3$. Relatively large tumours were visible after injection of the higher dose of SCC12B after only 2 weeks but these regressed slowly in all mice. The lower injection dose (1.6×10^6 cells /site) appeared to be less affective at inducing tumours. In accordance with published results SCC12F, clone 19 and clone 19 expressing vector alone all induced large cysts which regressed in all but 1 animal where it remained at a very small size (figure 5.18 and 5.19). The tumorigenic potential of cell lines expressing large amounts of mutant $p53^{216}$ appeared to be clonal. 19 216(2) 3 did not induce tumours at any dose in either experiment nor did a second clone 19 216(2) 1 . 19 216(1) B developed large tumours at the higher injection dose in all mice after only 8 weeks (figure 5.18). However these regressed and two tumours $1cm^3$ were noted with a latent period of 66 days. The mice were kept alive and one of these tumours regressed further leaving only one remaining 19 weeks post-injection. This latent period is much longer than that published for SCC12B (Jaffe *et al*, 1992). This data therefore shows that in our mice increasing the levels of mutant p53 does not increase the tumorigenic potential of a cell line. Only one clone (19 216(1) B) was slightly tumorigenic but this could not really be explained by the increase in mutant $p53^{216}$ levels alone since 19 216(2) 3 was not tumorigenic. No correlation between degree of resistance to suspension induced terminal differentiation and tumorigenicity was apparent when comparing 19 216(1) B with 19 216(2) 3. Interestingly, this data has shown that resistant to terminal

differentiation and tumorigenicity are separate events and cell lines can be resistant to terminal differentiation but not necessarily tumorigenic. It therefore appears that tumorigenicity depends on individual clones and increasing the levels of mutant p53 alone is not sufficient to increase the tumorigenicity of the cell line. It may be that a slightly more tumorigenic phenotype was displayed by clone 19 216(1) B due to the presence of another unknown genetic event that acts in concert with the increase in mutant p53 giving a more tumorigenic phenotype.

Loss of the ability to respond to terminal differentiation signals, increased proliferation and loss of cloning efficiency are usually early events in the development of SCC-HN (Edington *et al.*, 1995). SCC12F displays a more normal phenotype than SCC12B (Parkinson *et al.*, 1983; Jaffe *et al.*, 1992 and results in this thesis) and may have acquired only a few genetic events leading it a short way along the line to carcinogenesis. The results in this thesis show that a p53 mutation is acquired relatively early in the progression of this cancer as at least one mutant allele is present in both SCC12F and SCC12B. The rate limiting step in the progression of SCC12F to SCC12B therefore appears to be the accumulation of mutant p53 normally by the complete loss of the wild-type allele, but in this case by the loss of one of two wild-type alleles. This event dramatically changes the phenotype of SCC12F by further reducing its ability to respond to terminal differentiation and increasing its cloning efficiency after suspension culture. However a p53 mutation alone is not sufficient to affect the tumorigenic potential of the cell line and therefore another later event is required perhaps gained through genetic instability due to the loss of p53.

Interestingly, investigators have shown that expressing the latent membrane protein (LMP-1) from the Epstein-Barr virus (EBV) in SCC12F also inhibits its ability to terminally differentiate (Dawson *et al.*, 1990). The transfectants were unable to form cornified envelopes in response to treatment with the calcium ionophore A23187, nor were they able to express involucrin. Expression of LMP-1 in another human keratinocytes cell line (Rhek-1) was also shown to block differentiation and predisposes these cells to a more neoplastic phenotype (Zheng *et al.*, 1994). LMP-1 expression in

rodent cell transformation assays resembled those produced by activated *ras*, therefore SCC12F was also transfected by a H-*ras* oncogene (Dawson *et al.*, 1990). This produced a similar effect as LMP-1 with respect to differentiation. These experiments also showed the the LMP-1 trasnsfected SCC12F cells, unlike the highly malignant *ras* transfectants, produced tumours in nude mice only rarely and after a long latency period. H-ras has previously been shown not to be activated in SCC12F or SCC12B (Clark *et al.*, 1993) and therefore in these experiments it may bypass the need for a p53 mutation in order to produce a tumorigenic phenotype. LMP-1 is a transmembrane protein with unknown function. Another EBV protein, EBNA5, has been shown to interact with p53 and Rb-1 (Szekely *et al.*, 1993) and it is therefore unlikely that the results obtained by transfection of SCC12F described above resemble those in this thesis by the ability of LMP-1 to inactivate p53 directly. Although LMP-1 is not a tyrosine kinase it still may act in a signalling pathway similar to integrin signalling promoting cellular proliferation and inhibiting p53 mediated differentiation as described in the next section.

6.3 A possible role for p53 in terminal differentiation of human epidermal keratinocytes.

The results in this thesis show that an increase in mutant p53 or conversely a decrease in active wild-type p53 prevent keratinocytes from responding to suspension induced terminal differentiation signals. The involvement of p53 in the control of terminal differentiation of keratinocytes is perhaps not surprising. Numerous investigators have shown that p53 is involved in the differentiation of many cell types (details in section 1.4.3.8.). Interestingly, the differentiation pathways in such cell types involves DNA strand breaks followed by repair, for example B cell differentiation involves several DNA rearrangement events and therefore this step in differentiation may require the activities of p53 to halt cell cycle and repair DNA after rearrangements have occurred. During the differentiation of keratinocytes DNA fragmentation occurs in the upper granular layers as the cells degrade their nucleus by apoptosis (McCall and Cohen, 1991). It is very unlikely that this DNA damage triggers p53 activity as the only

proliferating cells in the epithelium are in the basal layer and cells of the granular layer would be unable to synthesis p53 protein in response to such DNA fragmentation. It is more likely that the actions of p53 enables the cell to withdraw from the cell cycle and stop DNA replication as they migrate from the basal membrane. Recent experiments have shown that anchorage independent cells grown in suspension arrest in G1 (Guadagno *et al*, 1993) which is a known action of p53. This thesis shows that mutation in p53 prevents terminal differentiation and would suggest therefore that upon withdrawal from the cell cycle wild-type p53 would stimulate the onset of terminal differentiation via an unknown pathway. Events so far shown to trigger p53 action are those that induce DNA damage, specifically strand breaks e.g. irradiation, actinomycin D and restriction enzymes. A trigger that activates p53 in keratinocyte terminal differentiation has not yet been identified but recent experiments show that when epithelial cells are prevented from contacting the basement membrane they undergo terminal differentiation (Watt *et al*, 1983) and an apoptotic-like phenomenon which has been termed anoikis (Meredith *et al*, 1993; Frisch and Francis, 1994) . The basal surface of epithelial cells adheres to the basal lamina mostly via members of the integrin family receptors (Adams and Watt, 1991). Integrins are heterodimeric transmembrane proteins consisting of α and β subunits (Hynes, 1992). Diversity in the integrin family is achieved by dimerisation between different subunits and each member binds to a different extracellular matrix protein such as fibronectin, collagens, laminin, nidogen and vitronectin and they also associate with cytoskeletal proteins such as actin. Integrins have been shown to translate external cues into signals that affect cytoskeletal organisation, cell shape and motility as well as cell-cell adhesion, cell migration and stratification. Recent evidence suggests that integrins mediate anchorage independent growth and are able to trigger terminal differentiation. More specifically the fibronectin integrin receptor $\alpha_5\beta_1$has been shown to directly affect the cells ability to undergo terminal differentiation (Adams and Watt, 1989). Terminal differentiation can be inhibited in cells grown in suspension by the immediate addition of fibronectin (Adams and Watt, 1989). A delay in the exposure to fibronectin has no effect as the cells have already been committed to terminal differentiation, the ability of the receptor to bind

fibronectin is decreased (Adams and Watt, 1990) and the receptor is gradually lost from the cell surface. Binding of fibronectin to its receptor during adhesion to the basement membrane signals the clustering and retention of receptors into focal contacts and polymerisation of actin filaments providing a positive stimulus for cell adhesion and spreading. This ligand binding also sends a second signal independent of clustering that inhibits terminal differentiation. Integrin signalling is thought to be mediated via changes in cellular pH, calcium fluxes and phosphorylation events. Treatment of cells with an anti-integrin antibody or fibronectin increases the tyrosine phosphorylation of proteins in the cell (Juliano and Haskill, 1993). One such protein has been identified as FAK (focal adhesion kinase) which is also a substrate for c-*src* proteins. The phosphorylation of this protein along with others is thought to promote cell growth and inhibit terminal differentiation and anoikis. Detachment of epithelial and endothelial cells but not fibroblasts from extracellular matrix contacts has been shown to elicit anoikis (Meredith *et al*, 1993; Frisch and Francis, 1994). *In vivo* such a response would prevent detached cells from becoming inappropriately re-attached and helps maintain the stratification and polarity of the epithelia. Meredith *et al*, 1993 has shown that the addition of sodium orthovanidate which is an inhibitor of tyrosine phosphatases, to cells in suspension blocks programmed cell death therefore suggesting that apoptosis is triggered by signals from integrins via tyrosine phosphorylation. Apoptosis in these cells can be inhibited by the addition of anti-integrin antibodies (Meredith *et al*, 1993), scatter factor or transfection with bcl-2 (Frisch and Francis, 1994)). Interestingly, bcl-2 is expressed in the basal layer of the skin but not in other layers suggesting that this may be an *in vivo* mechanism for preventing apoptosis (Hockenbury *et al*, 1991). P53 has been shown to promote apoptosis (discussed in section 1.4.3.7.) and results in this thesis also shows its inactivation prevents terminal differentiation. It is therefore possible that p53 may respond to integrin signalling when cells lose contact with the extracellular matrix, arresting cells in G1 and triggering terminal maturation which includes apoptosis as the cells migrate towards the outer epithelial layers. Activation of p53 via an integrin pathway has not yet been proved but it is clear that p53 is able to promote apoptosis and this thesis shows it to play an important role in keratinocyte differentiation. Whether p53

acts directly by controlling the expression of specific differentiation proteins e.g. involucrin or transglutaminase or whether it acts indirectly by establishing an irreversible commitment to terminal differentiation via withdrawal from the cell cycle is not clear and further experiments are required to define further the involvement of p53 in keratinocyte terminal maturation

6.4. Wild-type p53 prevents the expression of an oncogenic phenotype.

Mice null for p53 are viable and exhibit normal development (Donehower *et al.*, 1992) making it hard to establish a role for wild-type p53 in normal differentiation pathways unless some form of redundancy has taken place in these mice which is difficult to prove. Wild-type p53 has been shown to induce growth inhibition (Kastan *et al.*, 1991a; Kuerbitz *et al.*, 1992) and apoptosis (Yonish-Rouach *et al.*, 1991) in response to DNA damage preventing the accumulation of mutations and gene amplifications that are associated with malignancy. For this reason there appears to be an immense pressure for the loss of wild-type p53 and indeed the majority of tumours have acquired a mutation in a p53 allele with the concurrent loss of the remaining allele. An alternative model for the involvement of mutant $p53^{216}$ in the progression of the SCC-HN cancer dicussed in this thesis may be that its accumulation allows the expression of an oncogenic phenotype i.e. either an activated oncogene or an inactivated tumour suppressor gene which in turn blocks terminal differentiaion. The presence of wild-type p53 would normally prevent the expression of such a phenotype by inducing growth arrest or terminal differentiation. The expression of adenovirus E1A (known to bind Rb-1) in untransformed cells leads to the nuclear stabilization of wild-type p53 and loss of viability of these cells by apoptosis (Lowe and Ruley, 1993b). Levels of p53 protein reverted to normal in cells that had lost E1A expression. Similarly deregulated c-*myc* expression induces apoptosis in low serum conditions (Evan *et al.*, 1992). E1B which is known to bind to p53 was shown to protect cells form this toxic effect of E1A suggesting the role for E1B in viral infection is to counter cellular responses to E1A oncogene transformation (Lowe and Ruley, 1993b). The stabilization of p53 in these cells appears to guard cells against unscheduled

proliferation induced by E1A and p53 induced apoptosis may play a natural defense against tumour progression *in vivo*. The use of embryonic fibroblasts from p53 null mice highlighted the role of p53 in the suppression of oncogenic transformation. The absence of wild-type p53 enhances cell growth and survival in the presence of E1A (Lowe *et al.*, 1994). Cells heterozygous for p53 when transfected with E1A formed almost as many colonies as did null fibroblasts and displayed an intermediate resistance to apoptosis. This dose-dependent suppression of oncogene transformation by p53 suggests that mutations leading to the partial loss of p53 (for example SCC12F) allows the outgrowth of cells that have completely lost wild-type p53 activity (such as SCC12B) and therefore can no longer suppress the oncogenic phenotype of the cell. In such a way the accumulation of a p53 mutation allows the progession of a tumour which was initiated by oncogenic events. Studies of tumour formation in null and heterozygous mice showed that loss of wild-type p53 is not an initiating event and does not give rise to an increased number of papillomas. It does however appear to enhance the rate of malignant progression (Kemp *et al.*, 1993). The progresssion rate was also greater in heterozygous mice as compared to normals and was associated with the loss of the remaining wild-type allele (Kemp *et al.*, 1993; Harvey *et al.*, 1993b). Interestingly, this study also shows that the carcinomas from p53 null and heterozygous mice were markedly undifferentiated in comparison with wild-type carcinomas (Kemp *et al.*, 1993). Similarly papillomas from the null mice also showed a similar undifferentiated phenotype compared to wild-type and showed areas of conversion to early stage carcinomas. Therefore not only does the inactivation of p53 increase the rate from benign to malignant conversion, it also influences the degree of differentiation and malignancy of a tumour. These results suggest that loss of wild-type p53 whilst not initiating allows the expression of oncogenic events that occur in multistep carcinogenesis which promotes malignant progression and dedifferentiation.

This thesis therefore shows that the accumulation of mutant $p53^{216}$ is responsible for the progression of SCC12F to SCC12B. The increased levels of this mutant p53 may promote a more malignant phenotype *in vivo* by either one of the above mechanisms.

Either by the direct inhibition of terminal differentiation induced by signalling from integrin receptors or indirectly by allowing the expression of an oncogene which inturn blocks terminal differentiation and promotes proliferation.

The acquisition of mutant p53, whilst not an initiating event in carcinogenesis as previously discussed, appears to be an early event in the development of SCC-HN. In SCC12, a p53 mutation has occurred at an early stage in SCC12F before a more malignant phenotype has developed. There is intense pressure for this cancer to lose any active wild-type p53 in order for it to progress (SCC12B). A study of the epithelia at distant sites from the primary tumour in SCC-HN (Nees *et al.*, 1993) showed that although these biopsies were histologically inconspicuous they contained high levels of p53 protein. This increased level of protein was not due to a response to DNA damage but was associated with a p53 mutation and an increase in cellular proliferation as shown by an increase in histone H3 mRNA. Whatsmore different tumour distant sites from the same biopsy expressed different p53 mutations suggesting the development of multiple primary tumours frequently seen in head and neck cancer patients is a multifocal polyclonal process. Cells that showed high levels of p53 also showed a dedifferentiated phenotype by the presence of high levels of cytokeratin 8 and 18 which are consistently detected in squamous cell carcinomas. Similar conclusions were drawn from the discovery of early p53 mutations in cases of Bowens disease (Campbell *et al.*, 1993) a pre-invasive lesion which shows a significant rate of progression to invasive SCC. Therefore taken together results from this thesis and and others (Nees *et al.*, 1993; Campbell *et al.*, 1993) suggest that the role of p53 mutation in SCC of the skin and head and neck are different from that seen in colonic cancer where a mutation rarely precedes invasion (Baker *et al.*, 1989). On the contrary results here show that *in vivo* SCC12F acquired a p53 mutation early in its development which gave it a slight growth advantage and a delay in the response to terminal differentiation caused by rounding and cellular contact in the basal cells as compared to normal cells. This increased proliferation allowed other genetic events to occur in this cancer which were suppressed by the presence of the remaining wild-type p53. There was then increased pressure for the cells

to lose wild-type p53 by the accumulation of more mutant p53 which resulted in a dramatic progression of cells to a more malignant phenotype (SCC12B).

6.5. Future prospects.

Experiments are currently underway to show that the inhibition of terminal differentiation shown in this thesis is directly dependent on the presence of mutant $p53^{216}$ and not by any other effect of suspension culture. The mutant p53 is being expressed under the inducible metallothionein promoter in clone 19 and the experiments are being repeated in the presence and absence of mutant p53 expression. It is also important to establish whether it is the knockout of wild-type p53 activities by increased expression of the mutant or a novel function gained by the mutant protein that blocks suspension induced terminal differentiation. Experiments are planned whereby the activities of endogenous wild-type p53 protein in clone 19 can be inhibited either by HPV infection, transfection with antisense RNA or transfection with the C terminal fragment of p53 that acts by oligomerising with the wild-type protein and prevents its action. It will be interesting to see if the removal of wild-type activities from clone 19 are sufficient to inhibit terminal differentiation and if so this would provide evidence to suggest that the mutant $p53^{216}$ was acting in a dose-dependent dominant negative manner. If knocking out wild-type p53 is not sufficient to obtain the results shown in this thesis then it would suggest that the mutant had gained a property that interfered with terminal differentiation.

Further molecular analysis of the mutant $p53^{216}$ would be interesting so as to understand how the mutation affects the protein. Analysis of its DNA binding and transcriptional activities using a CAT construct would show if it has retained any wild-type p53 characteristics. Also analysis of whether the mutant can knockout wild-type p53 transactivation of a reporter construct *in vitro* and whether such an activity is dependent on mutant dosage as seen *in vivo* in SCC12F and SCC12B would highlight

whether the mutant acts in a dominant negative manner to prevent wild-type activites and inhibit terminal differentiation.

The experiments in this thesis did not investigate the extent to which each cell line can undergo apoptosis. As p53 has been implicated in controlling this process and keratinocytes have been shown to induce an apoptotic-like process when cells become detached from the substratum or are grown in suspension (McCall and Cohen.,1991; Meredith et al., 1993; Frisch and Francis, 1994), it would be of interest to study the effect of increased mutant p53 on this process. DNA laddering is technically quite difficult to show in some cell lines (Wyllie, 1980). Other markers of apoptosis such as protein crosslinking due to the activation of intracellular transglutaminases, detergent solubility of degraded DNA, and the expression of the apoptotis specific gene TRPM-2 by northern analysis (Meredith *et al.*, 1993) could be studied.

If the inability of the cells to respond to suspension induced terminal differentiation was due purely to the inactivation of wild-type p53 it would be interesting to establish just how wild-type p53 would normally trigger the differentiation pathway. Identification of p53 consensus binding sites in promoters of epithelial differentiation proteins such as involucrin, keratins or transglutaminase, similar to that found in the muscle differentiation gene MCK promoter enhancer (Weintraub *et al.*, 1991), would suggest a direct involvement of p53 in terminal differentiation of keratinocytes. If a positive involvement was identified it would be interesting to see how this pathway has become redundant in cells without wild-type p53 such as p53 knockout mice which clearly display normal development and differentiation of the epidermis. If p53 played a more indirect role by switching the commitment to terminal differentiation on, comparisons of the proportion of cells able to arrest in G1 during suspension culture or removal from the basement membrane in cell lines with high and low doses of mutant p53 would highlight a pathway for p53 involvement.

A closer examination of the relationship between integrin signalling and p53 activation is required in order to support the model described above which proposes terminal maturation of keratinocytes is triggered by release of ligand from the integrin receptor and mediated by p53. Little is known of the pathways involved in integrin-

mediated signalling either growth promoting or those involved in anoikis. Investigation of such pathways and any implication of p53 involvement could highlight an exciting new role for wild-type p53 in the terminal maturation of keratinocytes.

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