Genetic analysis of the immortal phenotype in human squamous cell carcinoma

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Abbreviations

DNA	deoxyribonucleic acid
RNA	ribonucleic acid
mRNA	messenger RNA
tRNA	transfer RNA
dNTP	deoxyribonucleoside triphosphate
dATP	deoxyadenosine triphosphate
dTTP	deoxythymidine triphosphate
dGTP	deoxyguanosine triphosphate
DNAse	deoxyribonuclease
RNAse	ribonuclease
bp	base pairs
kb	kilobase pairs
OD	optical density (absorbance)
BSA	bovine serum albumin
EDTA	ethylenediamine tetra-acetic acid
PBS	phosphate-buffered saline
PEG	polyethylene glycol
SDS	sodium dodecyl sulphate
DMEM	Dulbecco's modified Eagle's medium
TEMED	N,N,N',N'-tetramethylethylenediamine
DMSO	dimethyl sulphoxide
HGPRT	hypoxanthine guanine phosphoribosyl transferase
HPV	human papillomavirus
SCC	squamous cell carcinoma
HEK	human epidermal keratinocyte
PCR	polymerase chain reaction
LOH	loss of heterozygosity

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Abstract

There is accumulating evidence that the escape from the phenomenon of *in vitro* senescence, or immortality, is an important step in tumour progression. Senescence is tightly linked to terminal differentiation and is genetically programmed; furthermore, the genes regulating senescence are candidate tumour suppressor genes.

The work described in this thesis investigates immortality in a human head and neck squamous cell carcinoma (SCC) system. Prior to this work there were no cell lines available where loss of heterozygosity could be studied in SCC, and few in other systems. In addition, most SCC cell lines had been derived from recurrent tumours and/or without feeder layers of irradiated mouse Swiss 3T3 fibroblasts. Therefore it was not possible to distinguish the genetic events causing the tumour and those resulting from therapy and adaptation to tissue culture.

Twenty-two primarily untreated tumours were collected along with blood samples and seven immortal cell lines (the BICR cell lines) were isolated. We have also obtained several premalignant erythroplakia cultures, so it is now possible to grow all the stages of SCC progression from normal keratinocytes through premalignant erythroplakias to carcinomas and lymph node metastases.

DNA fingerprinting confirms that blood, fibroblast and cell line DNA came from the same patient and each cell line is unique. Similarly keratin & involucrin staining and electron micrographs show they are SCCs.

Immortality appears to be a late-emerging phenotype during carcinogenesis as the erythroplakia cells eventually senesce and it is difficult to establish cell lines from early (TNM clinical stage T2) tumours relative to late (T4) tumours. Erythroplakias and T2 cell lines are non-tumorigenic in nude mice, whereas late-stage lines are tumorigenic.

Human papilloma viruses (HPV) 16 & 18 can immortalise keratinocytes. Their transforming genes E6 & E7 bind p53 and pRb respectively and thus p53 and pRb may be involved in senescence. None of the cell lines contain HPV 16 or 18, however loss of heterozygosity (LOH) analysis confirms data showing high frequencies of p53 mutation obtained by Burns *et al* (1993). Interestingly, although it had been previously shown that SCC cell lines have normal RB mRNA and no abnormalities in pRB size, location or phosphorylation status, 3/6 informative cases exhibit LOH at RB.

Chromosomes 1, 4, 6 & 9 have been shown by several other groups to induce senescence in various target cells when introduced by microcell-mediated chromosome transfer. These chromosomes were investigated in the BICR cultures, again by LOH analysis. Regions 1q, 4p and 6q do not show a significant degree of LOH at any stage, but there are high levels of loss at both 9p and separately at 9q in later stage immortal cultures. The information allows tentative assignment of a tumour suppressor gene involved in SCC progression and possibly in escape from senescence to 9p22-23.

Chapter 1

Introduction

1.1 Introduction

Senescence is the *in vitro* phenomenon of limited lifespan exhibited by normal human cells (Hayflick and Moorhead, 1961). Many cancers however contain immortal variants which in contrast can be subcultured for over 100 population doublings (Barrett, 1980, Rheinwald and Beckett, 1981, Smith *et al*, 1987). Yet senescence is poorly understood, and the role of escape from senescence in carcinogenesis is controversial (Newbold *et al*, 1982, Newbold and Overell, 1983, Weinberg, 1989, Hunter, 1991). It is important to establish this role in order to determine whether senescence will be useful as a cancer therapy, and it is difficult to see how this can be achieved without an understanding of the molecular and genetic basis of immortalisation. Recently the advent of molecular techniques for genotype analysis have enabled the study of the genetic basis of immortality.

1.2 Senescence

Normal cells undergo a limited number of population doublings before ceasing proliferation and entering a viable state of growth arrest (Hayflick and Moorhead, 1961). The number of population doublings is reproducible within narrow limits for a mass culture of a specific cell type, and is inversely proportional to the age of the donor (Dell'Orco *et al*, 1973, Harley and Goldstein, 1978). In addition, cells from shorter-lived species proliferate for a shorter time in culture, as do cells derived from individuals with premature ageing syndromes (Martin *et al*, 1970, Norwood *et al*, 1979, Rohme, 1981). Indeed the population doubling maxima under similar conditions of normal embryonic fibroblasts *in vitro* are proportional to the mean maximal lifespan of the donor species (Rohme, 1981). This process has been proposed as an *in vitro* model for cellular ageing (Hayflick, 1965) and the viable state of growth arrest is known as senescence.

The phenotypic characteristics of senescent cells are:

a) They do not undergo population doubling within four weeks or more.

b) Incorporation of ^{3}H-thymidine is low (Cristofalo and Sharf, 1973).

c) Nucleus:cytoplasm ratio is low (Hayflick, 1965).

d) Cells enlarge (Hayflick, 1965).

Hayflick and Moorhead (1961) described three growth phases in cells derived from fetal tissues. Phase 1 was the early growth phase or primary culture when the cells had been freed from the tissue and were establishing themselves in the flask. Phase 2 was characterised by exponential growth and cells required continual sub-cultivation. When cultures entered phase 3 the cells started to accumulate in the post-mitotic state and consequently there were longer time periods between population doubling. At the end of this phase the culture gradually dies. Cultures in phase 3 were termed senescent.

Senecent cells are thought to be blocked at late G1 of the cell cycle and this is supported by several lines of indirect evidence. They contain the 2n complement of DNA characteristic of G1 (Hart and Setlow, 1974). Thymidine kinase activity and thymidine triphosphate synthesis are similar upon serum-stimulation of density-arrested cells in senescent and young cells, however young cells subsequently initiate far more DNA synthesis than old cells (Cristofalo, 1973, Oloshaw *et al*, 1983). Serum also induces C-MYC and C-HA-RAS expression in senescent human diploid fibroblasts (Seshadri and Campisi, 1990). As these events are cell cycle-dependent and occur at late G1 we can deduce senescent cells may be blocked here. Nuclear fluorescence patterns of senescent WI-38 cells are also typical of late G1 (Gorman and Cristofalo, 1984), and flow microfluorimetry analysis shows 80% of late-passage keratinocytes are in G1 (Wille *et al*, 1984).

Cyclins and cyclin-dependent kinases (cdks) drive the cell cycle, possibly in response to signals from growth factors acting at G1 (reviewed in Sherr, 1993). The cell

cycle is shown in figure 1. D and E cyclins seem to act at G1/S, and A and B cyclins at S and M phases. Cyclin dependent kinases 2 and 4 are strong candidates for phosphorylating pRb-1 and thus allowing exit from G1, possibly targetted by the D and E cyclins (Akiyama *et al*, 1992, Kato *et al*, 1993, Sherr, 1993). It has been found that both cyclins A and B and their associated kinase p34cdc2 (cdk1) are down-regulated in senescent fibroblasts and are not expressed on serum stimulation (Richter *et al*, 1991, Stein *et al*, 1991). Conversely in immortal keratinocytes these same proteins are elevated (Rice *et al*, 1993). Cdk 2 and 4 are also suppressed in senescent fibroblasts, but surprisingly cyclins D1 and E are elevated (Lucibello *et al*, 1993, Dulic *et al*, 1993). Dulic *et al* have found that cyclin E is unphosphorylated and inactive in senescent fibroblasts, cdk2 is unphosphorylated and inactive, and cyclin D1-cdk2 complexes contained exclusively unphosphorylated cdk2. They suggested that the failure to activate cyclin E-cdk2 kinase activity in senescent cells accounts for the inability of the cells to phosphorylate pRb-1 in late G1, which then blocks expression of late G1 genes such as cyclin A (see figure 2).

1.2.1 Theories of senescence

Several theories for the mechanism of senescence are currently thought valid in different ways. However none satisfy all aspects of the available data .

1.2.1.1 The error catastrophe theory

It was first proposed by Orgel (1963) that the transcriptional and translational apparatus is potentially unstable. Errors in newly synthesised proteins, especially those involved in replication, transcription and translation, could feed back and cause additional errors. Error levels could then either stabilise at a steady state level or increase until a lethal error catastrophe is reached (Orgel, 1970).

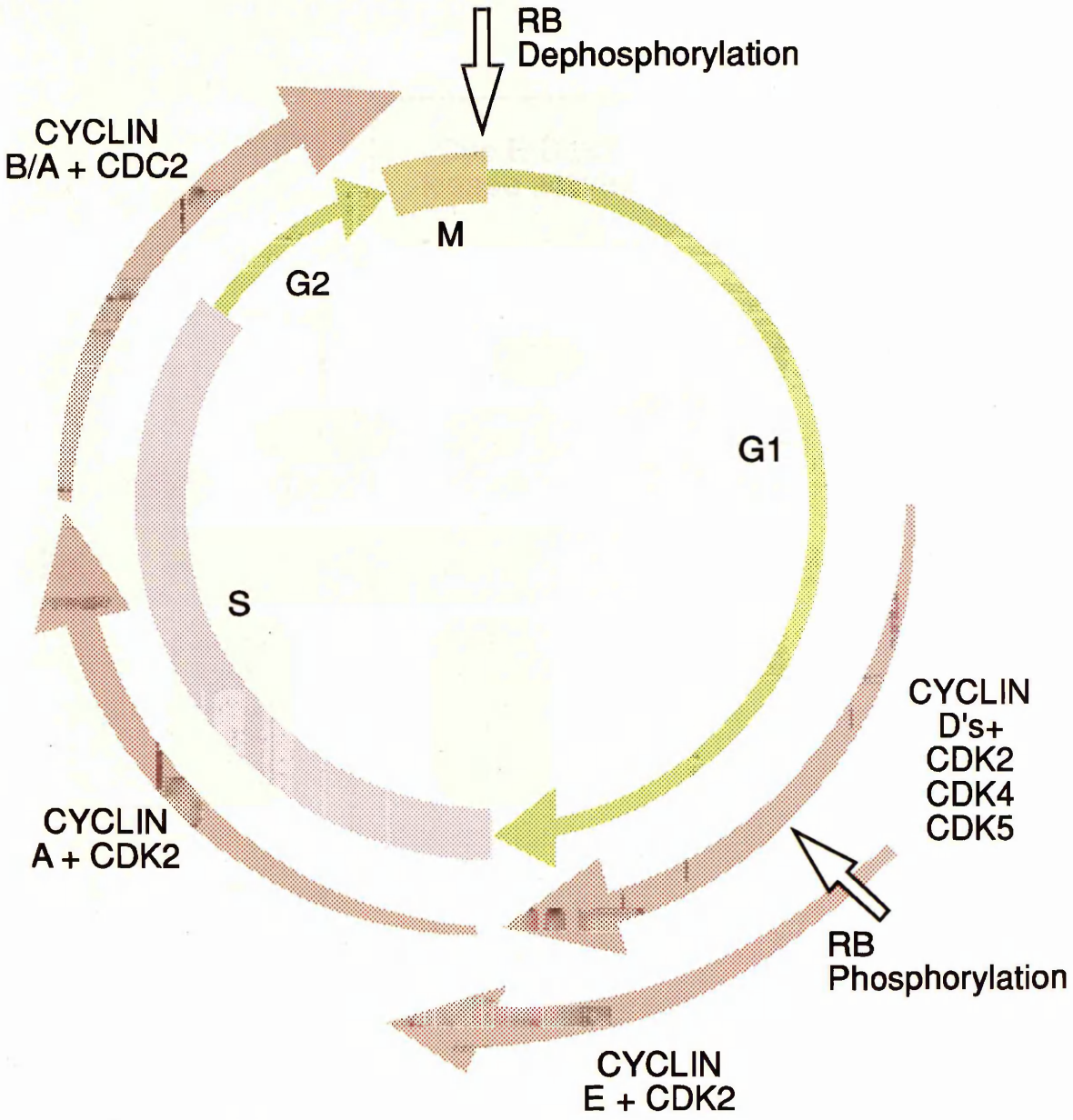


Figure 1 The interaction of cyclins and cyclin-dependent kinases during the cell cycle.
Taken from Sherr, 1993

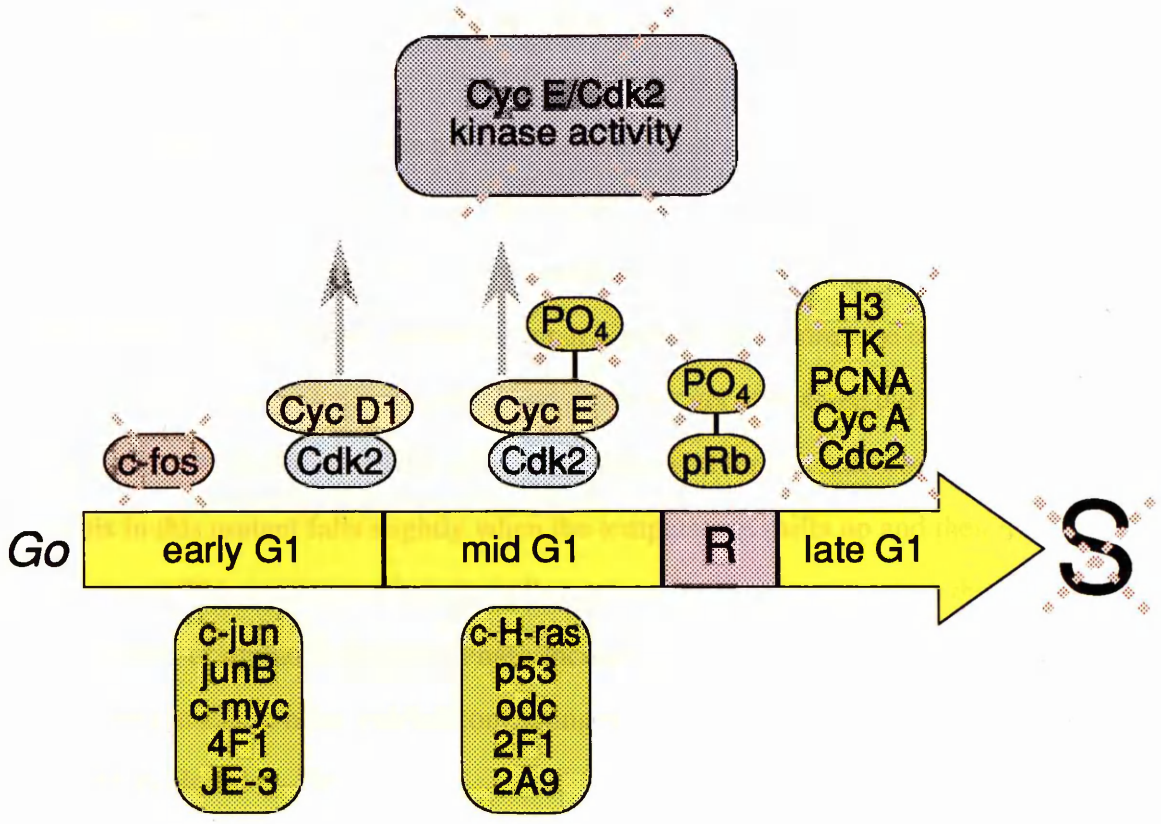


Figure 2 A summary of serum-inducible events in senescent and early-passage quiescent cells. Senescent cells are deficient in the events crossed out with dashed lines and have an overabundance of cyclin D1 & E complexes as indicated by the arrows. Placement of pRb phosphorylation at R is not proven. H3 = Histone H3, TK = thymidine kinase, PCNA = proliferating cell nuclear antigen, odc = ornithine decarboxylase.
Taken from Dulic *et al* 1993

With regard to steady state levels, it is possible that a protein synthesis apparatus containing errors can synthesise a new apparatus with fewer errors (Orgel, 1970). In addition, there are enzymes which scavenge error-containing proteins (Goldberg, 1972).

Attempts have been made to discover whether error catastrophes do occur in living systems. Direct evidence for an error catastrophe comes from the LEU-5 mutant of *Neurospora*, which makes a temperature-sensitive leucyl transfer RNA synthetase. At low temperatures the strain grows normally, but at higher temperatures the enzyme incorporates incorrect amino acids at leucine codons and death occurs after three days (Printz and Gross, 1967). Lewis and Holliday (1970) subsequently showed that the accuracy of protein synthesis in this mutant falls slightly when the temperature shifts up and then remains constant until 70 hours later, when the cells age rapidly. At the same time the thermolability of glutamic dehydrogenase increases while its specific activity falls. Experiments in *E.coli* have yielded conflicting results which have not been resolved (reviewed in Holliday, 1984).

Attempts to measure error frequencies in mammalian cells have not been successful (Holliday, 1984). However some indirect evidence for error accumulation is provided by Holliday and Tarrant (1972), who found that as cultures of MRC-5 human fibroblasts age: (i) the proportion of thermolabile glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase increases, (ii) the ratio of thermolabile glucose-6-phosphate dehydrogenase to thermolabile 6-phosphogluconate dehydrogenase remains constant, (iii) treatments which quiesce cells do not increase the amount of thermolabile protein, (iv) cells grown in the presence of 5-fluorouracil (a base analogue which induces errors in protein synthesis) age prematurely, accumulating thermolabile protein after only a few subcultures, and (v) cultures of cells derived from individuals with Werner's syndrome, a premature aging disorder, already contain thermolabile protein when they are placed in tissue culture. These experiments agree with the error accumulation hypothesis but it has not been proven that the increases in thermolabile protein are due to errors in protein synthesis (Orgel, 1973). Fulder on the other hand has shown directly that glucose-6-

phosphate dehydrogenase mutants are more common in old than young cells (Fulder, 1972). Morley too has shown that numbers of human T lymphocytes resistant to 6-thioguanine increase with the age of the donor, and that these resistent cells are mutant (Morley *et al*, 1982, 1983).

Another piece of indirect evidence comes from Curtis' observation that gross chromosomal abnormalities increase in frequency with age in cells taken from whole animals (Curtis, 1967). They also increase with age in lymphocytes and cultured fibroblasts (Saksela and Moorhead, 1963, Jacobs *et al*, 1964, Thompson and Holliday, 1975).

Finally, DNA polymerase α from senescent cells is several times less accurate than enzyme from young cells (Murray and Holliday, 1981).

Evidence against Orgel's theory comes from the fact that senescent cells can remain viable in culture for at least a year (McKeehan *et al*, 1977, Matsamura *et al*, 1979). SV40 large T antigen expression can bypass part of the senescence mechanism in human diploid fibroblasts and stimulate replication, so the DNA synthesis machinery is intact (Wright *et al*, 1989). Finally, Seshadri and Campisi (1990) have shown that serum induces C-HA-RAS, C-MYC and ornithine decarboxylase mRNAs normally, showing that at least the signal transduction pathways for these genes are intact.

The error catastrophe theory provides a straightforward link between cell and organismal ageing in that one can understand why organisms can age due to their cells accumulating defects (reviewed in Kirkwood, 1991).

The theory also provides a reason for evolutionary development of ageing. Accurate macromolecular synthesis depends on many processes which require energy, so organisms must balance their energy use and error levels. Resource diversion into reproduction ensure overall survival of the species but leads to aging and eventually death (Holliday, 1984, Kirkwood, 1991).

However there is no simple way of explaining transformation. Viruses and chemical carcinogens cannot increase the accuracy of transcriptional and translational machinery. In

addition one must explain continued accuracy in the germline, although many oocytes which begin to mature eventually degenerate (Orgel, 1973).

Premature ageing syndromes provide an interesting aspect to the error accumulation theory. Several diseases of defective DNA repair show features of premature aging, examples being Cockayne syndrome and ataxia telangiectasia (Martin, 1978). There is also correlation between rates of DNA-excision repair and lifespan in mammals (Hart and Setlow, 1974). It is not yet clear whether Werner's syndrome or progeria, which are considered models of premature ageing, are more sensitive to DNA damaging agents than normal cells. For Werner's syndrome chromosome instability has been reported from both cytogenetic studies and mutation frequency studies (Hoehn *et al*, 1975, Salk *et al*, 1981), but unscheduled DNA synthesis is reportedly normal (Fujiwara *et al*, 1977, Saito and Moses, 1991). In progeria there are conflicting results for responses to ultraviolet radiation, gamma rays, X-rays and agents such as bleomycin (Epstein *et al*, 1973, Regan and Setlow, 1974, Weichselbaum *et al*, 1980, Saito and Moses, 1991).

On the other hand in both Werner's syndrome and progeria oxidised proteins increase at a much higher rate than is normal (Stadtman, 1992). One important factor in longevity appears to be basal metabolic rate, which is higher in species with shorter lifespans (reviewed in Ames *et al*, 1993). Metabolic rate influences quantities of endogenous oxidants and mutagens produced as by-products, and the level of oxidative DNA damage is roughly related to metabolic rate in several mammalian species (Adelman *et al*, 1988, Shigenaga *et al*, 1989, Loft *et al*, 1992). Chronic infection and inflammation also contribute to cancer (Beasely, 1987, Marsh and Mossman, 1991, Yu, 1991, Korkina *et al*, 1992), possibly because leukocytes and other phagocytic cells combat bacteria and virus-infected cells by destroying them with an oxidant mixture (Ischiropoulos *et al*, 1992, Stamler *et al*, 1992). Finally, caloric restriction significantly increases lifespan and decreases cancer incidence in rodents, possibly by enhancing maintenance functions and resulting in less oxidative damage (Roe, 1987, Weraarchakul *et al*, 1989). These data and

hypotheses tie in with the disposable soma theory in that metabolism has costs and diet restriction means resources go to maintenance of the body until food resources are available for reproduction (Holliday, 1989).

1.2.1.2 Terminal differentiation

Bell *et al* (1978) were the first to argue that cessation of proliferation in vitro could represent a state of terminal differentiation. Their theory was that a culture of human diploid fibroblasts (HDF) consists of cells which are cycling, cells which are prepared to cycle, and cells which have further differentiated.

Fibroblasts in culture are stimulated to divide but have a strong tendency to leave the cycle and differentiate. Haemopoietic cells (Schofield, 1987), fibroblasts (Smith and Whitney, 1980), and keratinocytes are also a non-synchronous population in that different clones have different division potential (Barrandon and Green, 1987). Cells in the organism have this tendency but less so than in culture where all cells may be ultimately forced to differentiate. In development all cell types would undergo divisions and then differentiate further. Later in life cells can re-enter the cycle, for example in wound healing, and then leave the cycle to complete their differentiation. Adult populations would have a higher proportion of further differentiated cells than young populations. Bell *et al* propose that phase 3 cultures are difficult to grow because culture techniques favour proliferating and not differentiated cells. For example, aminoguanidine can enhance the survival of terminal non-dividing WI-38 cells by four months or more (Duffy and Kremzner, 1977). The fact that post-mitotic populations have been kept in culture for over a year supports this (McKeehan *et al*, 1977).

It is common in many different cell types that transformation and reduction in expression of the differentiated phenotype occur in parallel (Rheinwald and Beckett, 1980, Song *et al*, 1992, Sparks *et al*, 1993). Defective terminal differentiation can even be used to select malignantly transformed keratinocytes (Rheinwald and Beckett, 1980, Yuspa and Morgan, 1981). However there have been instances where limited lifespan and differentiation have been uncoupled to an extent. A spontaneously immortalised keratinocyte cell line (HaCaT) was established by Boukamp *et al* (1988) which fully expresses the normal keratinization program, and dexamethasone treatment of rhabdomyosarcoma cells causes virtually complete block of differentiation, but only causes 20-30% growth inhibition (de Giovanni *et al*, 1993). In these cases though differentiation may well be slowed. In keratinocytes growing in serum-containing medium where a low rate of differentiation is favoured, lifespan is extended (Rheinwald and Green, 1977, E.K.Parkinson, unpublished data).

Bayreuther and colleagues (1991) have demonstrated in man and other species that fibroblasts in the stem cell system develop and differentiate along an 11-stage sequence within five compartments. Three stem cells develop in the stem cell compartment along the lineage S1-S2-S3. In the fibroblast progenitor compartment three mitotic fibroblasts develop along the sequence MF1-MF2-MF3; in the maturing compartment three post-mitotic fibroblasts develop, and the sequence ends with either apoptosis in the degenerating compartment or transformation in the transforming compartment. These phenotypes were observed in young primary explant populations and were highly reproducible. Donor age-related differences in the frequencies of the cell types, numbers of post-mitotic fibroblasts increasing with age, were also highly reproducible (Bayreuther *et al*, 1992), so it was felt that equivalents existed *in vivo*. It was possible to select for mitotic and post-mitotic types, which also exhibited differences in labelled protein patterns (Francz *et al*, 1989).

The idea of terminal post-mitotic differentiation fits in with Kirkwood and Holliday's early proposal of cellular commitment (1975). Their theory proposed that prior to the establishment of primary fibroblast cultures all cells are immortal and uncommitted.

During cell division they give rise to committed cells with probability P. Committed cells continue to divide but eventually die out, and the number of divisions between commitment and death is the incubation period M. It follows that the proportion of uncommitted cells eventually dies out as well, and that population size influences longevity. Various "bottleneck" experiments where transient reductions in culture size were introduced showed that this was indeed the case (Holliday *et al*, 1977, 1981). However the theory has proved untenable (Harley and Goldstein, 1990). Measurements of percentages of dividing cells at various stages of replicative lifespan are not those predicted, and the fact that no immortal diploid cells have been reported is also at odds with the theory.

1.2.1.3 The telomere hypothesis for cellular aging

Telomeres are the simple G-C-rich sequence repeats at the ends of eukaryotic chromosomes. They stabilise chromosome ends, protecting them against illegitimate recombination and possibly directing their attachment to the nuclear membrane (Orr-Weaver *et al*, 1981, Agard and Sedat, 1983, Haber and Thornburn, 1984). In addition they may prevent loss of coding DNA from the chromosome ends resulting from chromosomal replication (Greider, 1990). Because DNA polymerase synthesises DNA only in the 5' to 3' direction and requires an RNA primer, removal of the primer at the 5' ends of newly synthesised strands means that they shorten by at least the length of the primer at each division (Walmsley, 1987).

The enzyme telomerase (telomere terminal transferase) was first discovered in *Tetrahymena* (Greider and Blackburn, 1985) and has since been detected in two other ciliates, *Euplotes* and *Oxytricha*, and in human and mouse transformed cell lines (Zahler and Prescott, 1988, Morin, 1989, Shippen-Lentz and Blackburn, 1989, Prowse *et al*, 1993). Telomerase synthesises telomeric repeats using an RNA template which is an essential component of the enzyme (Shippen-Lentz and Blackburn, 1990, Yu *et al*, 1990). Thus telomere sequence loss during replication is balanced by *de novo* addition. Recent evidence

suggests telomere length may be regulated partly by the processivity of telomerase (Prowse *et al*, 1993), and the large number of yeast genes affecting telomere length may mean regulation is a complex process involving telomerase, telomere-binding proteins and other components such as rap-1 (Lundblad and Szostak, 1989, Conrad *et al*, 1990, Lustig *et al*, 1990).

Several observations implicate a role for telomere shortening in cellular senescence. Telomeres shorten during ageing of cultured human fibroblasts (Harley *et al*, 1990) and other cell types in vivo including skin and colon epithelia and peripheral blood leukocytes (Hastie *et al*, 1990, Lindsey *et al*, 1991). In somatic cells telomeres may shorten due to the absence of telomerase activity: telomerase was not detectable in primary human embryonic kidney cells (Counter *et al*, 1992).

There is increased frequency of dicentric chromosomes due to telomeric associations in senesceing fibroblasts (Saksela and Moorhead, 1963, Benn, 1976, Sherwood *et al*, 1989). These abnormalities are typical of terminal deletions (McClintock, 1941).

The presence of telomerase in immortal cells but not in transformed cells which are not immortal correlates with the stabilised telomere length over time in such cells (Morin, 1989, de Lange *et al*, 1990, Counter *et al*, 1992).

Finally, sperm telomeres are longer than those of somatic cells and do not decrease with donor age (Allsopp *et al*, 1992). Presumably telomerase is active in the germline.

These data led Harley (1991) to propose that loss of telomere sequence due to incomplete DNA replication in the absence of telomerase activity provides a mitotic clock that signals exit from the cell cycle. In fact it seems that telomere length is a better predictor of replicative capacity in culture for normal cells than donor age (Allsopp *et al*, 1992). This explains variation in replicative capacity of clones of mass cultures and implies a causal role for telomere loss in ageing (Allsopp *et al*, 1992). In addition, fibroblasts from progeria donors had relatively short telomeres at a very young age, consistent with their reduced division potential (Allsopp *et al*, 1992). This tight correlation suggests that

telomere loss may initiate cell cycle exit once a critical threshold number of telomeric repeats is reached, which could represent the Hayflick limit (Allsopp *et al*, 1992).

It also appears that altering the telomere length equilibrium decreases cell viability. *Tetrahymena* transformed with mutant genes for the telomerase RNA template grow very slowly and the cells die after several weeks in culture. The length of the telomeres was altered: some were longer and some shorter depending on the introduced mutation (Greider, 1990). Yeast EST mutants eventually senesce, which correlates with progressive decrease in telomere length as cells divide (Lundblad and Szostak, 1989). It is thought that the EST-1 gene encodes a protein component of an essential yeast telomerase (Lundblad and Blackburn, 1990). Furthermore, a small proportion of EST-1- cells which survive have acquired and amplified subtelomeric fragments (Lundblad and Blackburn, 1993).

Counter *et al* (1992) showed that SV40 large T antigen and adenovirus 5 E1A and E1B extend the lifespan of human embryonic kidney cells without directly activating telomerase, which was detected only in those rare immortal clones which survived crisis. Telomeres continue to shorten during extended lifespan but it may be necessary to derepress telomerase for immortality.

It is more difficult to explain the dominance of senescence over immortality in hybrids in terms of telomeres. A trans-acting repressor of telomerase may be present in the mortal parent (Allsopp *et al*, 1992).

Mice are interesting in that they are a short-lived species but have very long telomeres (Kipling and Cooke, 1990, Starling *et al*, 1990). Mouse cells do immortalise easily in culture and telomerase has been detected in several immortal lines (Prowse *et al*, 1993). Mouse telomerase adds only one or two repeats onto telomeric primers *in vitro*, in contrast to the human enzyme which under the same conditions adds hundreds of repeats (Prowse *et al*, 1993). This is evidence that telomere length may be controlled by factors other than telomerase *in vivo*.

1.2.1.4 Senecence is a genetically programmed event

There is increasing evidence now in favour of senescence being genetically programmed. For example, quiescence and senescence are biochemically distinct. The RB-1 protein, which is phosphorylated after serum stimulation of quiescent fibroblasts, remains hypophosphorylated in senescent cells (Stein *et al*, 1990). Senescent cells continue to express fibroblast growth inhibitory protein (GIP) and the 57kd protein statin after serum stimulation whereas quiescent cells do not (Wang *et al*, 1989), and senescent fibroblasts express a protein unique to their state, pSEN (Giordano and Foster, 1989).

a). Are stimulatory genes switched off?

C-HA-RAS, C-MYC, and ornithine decarboxylase can be induced normally although ornithine decarboxylase shows low activity. Actin is inducible to a lesser extent (Seshadri and Campisi, 1990). There are conflicting reports as to whether c-fos can be induced in senescent cells (Lucibello *et al*, 1993, Campisi, 1992). One study has found it appears to be under specific transcriptional repression since it was not serum-inducible in senescent human diploid fibroblasts and contains the same serum response element regulatory sequence as actin, which is inducible (Seshadri and Campisi, 1990).

There are also conflicting reports as to whether genes such as histone H3 and thymidine kinase, which are commonly linked to S phase, can be serum-induced (Rittling *et al*, 1986, Chang and Chen, 1988, Seshadri and Campisi, 1990).

As was mentioned earlier (section 1.2) cyclins D and E are inactive as is cdk2, and cyclin A is down-regulated.

b). Inhibitory proteins

As was mentioned earlier statin, pSEN and fibroblast GIP are expressed constitutively in senescent cells.

Statin is also found in confluent cultures of young fibroblasts (Sester *et al*, 1990) and disappears on entry to S phase (Wang and Lin, 1986). It shows more than 92% homology to the elongation factor EF-1α but is distinct from it and regulated differently (Ann *et al*, 1990).

pSEN is homologous to statin (Giordano and Foster, 1989). Using differential screening of a cDNA library from senescent WI-38 fibroblasts with probes from senesent and young cells Giordano and Foster recognised a pSEN clone with 100% homology to EF-1α. Its transcript accumulates towards the end of the lifespan but the processed and functional transcript is present in high levels only in early passage cells. Thus the pSEN clone may be a senescence-specific form of EF-1α.

It has been found that enucleated cytoplasts from senescent fibroblasts inhibit DNA synthesis in young cells, and this effect is abolished by treating the senescent cells with cyclohexamide prior to enucleation (Dresher-Lincoln and Smith, 1984). Cells constructed from WI-38 cytoplasts and nuclei from their SV40-transformed counterparts have a finite lifespan, and heterodikaryons of senescent and young cells senesce, implying the presence of a diffusible inhibitory factor (Muggleton-Harris and De Simone, 1980). There is also a plasma membrane-associated inhibitory protein in senescent fibroblasts since membrane preparations have the same effect. Trypsinising the cells removes the activity (Pereira-Smith *et al*, 1985).

In addition to the proteins described above, Stein and Atkins (1986) have partially characterised a protein found in the cytoplasm of both senescent and quiescent human diploid fibroblasts. It does not inhibit cells transformed by DNA tumour viruses but does inhibit carcinogen-transformed cells. It also depends on a protein at the cell surface, its

activity being removed by trypsin. The quiescent inhibitor is regulated differently and disappears at late G1.

Recently the gene for prohibitin was cloned (Nuell *et al*, 1991). This is a mammalian antiproliferative protein: microinjection of syntheitic prohibitin mRNA blocks entry to S phase in both normal fibroblasts and HeLa cells. It is mutated in approximately 20% of sporadic breast cancers (Sato *et al*, 1992).

It is not yet clear whether all the lines of evidence described above involve a single protein or several proteins.

In contrast to normal cells, transformed cells have very often escaped senescence to become immortal. *In vitro* models of this process and what we have learned of its genetic basis are discussed in the next section.

1.3 Immortality

The role of immortality in carcinogenesis is as yet unknown. Senescence can be a mechanism of tumour suppression (O'Brien *et al*, 1986), and in several systems immortalisation is either a prerequisite for full transformation or increases the frequency of full transformation (Newbold and Overell, 1983, Steinberg and Defendi, 1983, Rhim *et al*, 1985, Strauss *et al*, 1990). However immortality is separable from tumorigenicity in that there are many cell lines which are immortal and yet are non-tumorigenic in a nude mouse assay (Namba *et al*, 1985, Boukamp *et al*, 1988, Rinehart *et al*, 1993). There is also a report of primary rat embryo fibroblasts transfected with mutant polyoma virus which were not immortalised but induced tumours in nude mice (Freund *et al*, 1992).

If we consider the theory of cooperation of oncogenes for full transformation, or tumorigenicity, of primary cells proposed by Weinberg (1985), the idea was that a nuclear and a cytoplasmic oncogene were required. The nuclear oncogene often induced immortality and the cytoplasmic oncogene led to reduced growth factor requirements, morphological changes and anchorage independence. Single oncogenes cannot cause full

transformation unless the transfected cells are removed from the influence of normal neighbours and allowed to proliferate. It is now thought that the action of a nuclear oncogene can be replaced by inactivation of tumour suppressor genes involved in control of, for example, the cell cycle (reviewed in Weinberg, 1989, and Hunter, 1991).

The relationship of immortality and tumorigenicity may also be complicated by the fact that nude mouse assays may not be suitable to all types of cells: for example squamous cell carcinoma lines do not easily induce nude mouse tumours in our experience (Edington *et al*, submitted for publication).

Numerous carcinogenic agents and tumour viruses can immortalise normal cells (Newbold *et al*, 1982, DiPaolo, 1983, Rhim *et al*, 1985, Hawley-Nelson *et al*, 1989, Parkinson, 1989, Hudson *et al*, 1990, Strauss *et al*, 1990). Rodent cells can immortalise spontaneously in culture (Todaro and Green, 1963, Yuspa *et al*, 1980, Hermann and Rice, 1983) but this has only been reported a few times for human cells (Baden *et al*, 1987, Boukamp *et al*, 1988, Rice *et al*, 1993). Correspondingly, rodent cells are much more easily transformed by carcinogenic agents and viruses (DiPaolo, 1983, Parkinson,1989). The reason for this is unknown. Most studies of immortality have used rodent cells, and since rodents seem to be quite different from humans in this respect and for reasons of brevity this discussion will be confined to human systems.

Evidence from cell hybrid analysis shows that immortality is a recessive event involving gene inactivation. Several investigators have shown that HeLa cells (Bunn and Tarrant, 1980, Pereira-Smith *et al*, 1990), SV40-transformed fibroblasts (Muggleton-Harris and DeSimone, 1980, Pereira-Smith and Smith, 1981), fibosarcoma cells (Pereira-Smith and Smith, 1983), and glioblastoma cells (Pereira-Smith and Smith, 1983) form hybrids of limited division potential when fused with normal diploid fibroblasts. Similar results are seen when SV40-transformed fibroblasts are fused with lymphocytes (Pereira-Smith *et al*, 1990), normal bronchial cells are fused with bronchial carcinoma cells (Kaighn *et al*, 1990), HPV-transformed keratinocytes are fused with normal keratinocytes (Chen *et al*, 1993), and SCC cells are fused with normal fibroblasts (Berry *et al*, in press). Taken

together these observations demonstrate that the process of senescence is dominant to immortalisation in human somatic cell hybrids, and that immortalisation is due in part to loss of gene function.

Additionally, further hybrid experiments have shown that hybrids between certain immortal lines senesce, whereas other cell line combinations do not (Pereira-Smith and Smith, 1988, Whitaker *et al*, 1992). It was thought that such senescent hybrids had genetically complemented defective genes in the constituent lines. Pereira-Smith and Smith (1988) were able to assign approximately 20 cell lines to one of four complementation groups, so it may be that immortality results from changes in a relatively small number of genes. However this is still not clear since work from our group (Berry *et al*, in press) showed that two epidermal SCC lines could not readily be placed in one complementation group, and Duncan *et al* (1993) have had similar results for SV40-immortalised cells.

1.3.1 Carcinogenesis is a multistage genetic process

The multistage nature of cancer was first proposed by Foulds (1954). It has now been possible to elucidate genetic alterations occurring at different stages in several human tumours including colon cancer (Vogelstein *et al*, 1988), malignant melanoma (Balaban *et al*, 1986), glioma (James *et al*, 1988) and small-cell lung carcinoma (Naylor *et al*, 1987). These alterations are a combination of mutations which activate cellular proto-oncogenes and inactivate tumour suppressor genes.

Proto-oncogenes may be thought of as genes whose products regulate the growth of the cell in a positive way. They become activated and thus overexpressed by point mutation, amplification or rearrangement, and have been identified by their transforming ability in cultured cells. In contrast, tumour suppressor genes in the normal cell suppress inappropriate cell growth. Their activities are frequently lost by deletion or mutation in neoplastic cells, giving the cells a selective advantage. At least in colon cancer it appears

that inactivation of tumour suppressor genes predominates over oncogene activation (Vogelstein *et al*, 1989).

There are two human systems where it has been possible to culture cells in vitro from various premalignant and malignant stages: colorectal cancer and malignant melanoma. Paraskeva *et al* (1984) were able to derive an immortal cell line from a large premalignant colorectal adenoma, and in melanoma lifespan was extended at radial growth phase primary melanoma and permanent cell lines were established from the next stage of progression, vertical growth phase primary melanoma (Mancianti and Herlyn, 1989). In both cases the immortal phenotype was acquired late in the benign-malignant transition.

Thus far it appears that immortality is an important step in progression from a benign to a malignant tumour, at least in the two systems described.

Squamous cell carcinoma (SCC) of the head and neck is another excellent system in which to study the role of immortalisation in multistage carcinogenesis. It follows clearly defined stages of progression (section 1.5.1), there are very good techniques for manipulation of keratinocytes in vitro (Rheinwald and Green, 1975), and there are many markers of terminal differentiation which are easily measurable (Green, 1977). Finally immortal variants are frequent in SCC (Rheinwald and Beckett, 1981)

Several rodent models exist which share some but not all of these features, and will not be discussed.

Although adenovirus 5 E1A, Epstein-Barr virus and polyoma virus large T antigen can immortalise human cells (Moran *et al*, 1986, Karran *et al*, 1990, Strauss *et al*, 1990), the two most popular systems for the study of immortality have been human papilloma virus (HPV) infection of primary human keratinocytes and SV40 infection of human diploid fibroblasts (HDF) and keratinocytes.

1.3.2 The human papillomavirus model

Human papillomaviruses cause epithelial hyperproliferation (reviewed in McCance, 1986). DNA from many HPV types has been identified in genital cancers (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52 and 56). Approximately 80% of cervical carcinomas contain one of these high-risk HPVs (reviewed in Howley, 1991). Non-viral factors are likely to be required in addition, since benign lesions progress to carcinomas relatively infrequently and after a long latency period (reviewed in Pfister, 1987).

Those HPV types most closely associated with cervical malignancies (HPVs 16, 18, 31 and 33) can immortalise normal human foreskin keratinocytes and human cervical epithelial cells (Kaur and McDougall, 1988, Woodworth *et al*, 1988, Schlegel *et al*, 1989). The cell lines contain integrated copies of HPV DNA and express viral proteins (Durst *et al*, 1987, Kaur and McDougall, 1988, Woodworth *et al*, 1988). The lines are also aneuploid, as are cervical carcinoma cells *in vivo*. Unlike immortalisation by SV40 (see section 1.3.3), HPV-infected cells become immortal without an obvious crisis: either the papillomaviruses encode enough transforming information to bypass or inactivate all mechanisms of senescence, or crisis has not been observed due to escaping clones growing out very rapidly, since transfected cultures are usually grown under non-differentiating conditions (in defined low-calcium medium without serum or feeder cells). Another possibility is that HPV may immortalise via a different mechanism than SV40.

Work on HPV 16 and 18 has shown that the E6 and E7 genes cooperate to immortalise human keratinocytes (Hawley-Nelson *et al*, 1989, Munger *et al*, 1989a, Hudson *et al*, 1990). The immortalising function has not been assigned to a specific gene, although HPV 16 E7 leads to hyperproliferation and limited extension of lifespan, and HPV 18 E7 has been reported to immortalise at very low frequency (Hawley-Nelson *et al*, 1989, Hudson *et al*, 1990). E6 alone has no immortalising activity. There may be some tissue-specificity though since it is reported that HPV 16 E6 and E7 induce hyperproliferation of primary human fibroblasts but will not immortalise them (Watanabe *et al*, 1989), and Shay

et al (1993) found HPV 16 E7 had no effect on human mammary epithelial cells but E6 extended their lifespan.

E6 binds the *p53* tumour suppressor gene and E7 the RB-1 tumour suppressor gene (Munger *et al*, 1989b, Werness *et al*, 1990). In fact in HPV-immortalised cells E6 stimulates degradation of p53 protein via the ubiquitin pathway (Scheffner *et al*, 1990). E6 proteins of both oncogenic and benign HPV types associate *in vitro* with p53, but only oncogenic types target p53 for degradation via N-terminal sequences conserved between the E6 genes of these HPV types (Crook *et al*, 1991). More transforming types of HPV also express E7 more efficiently so presumably sequester pRb-1 to a greater extent (Barbosa *et al*, 1991). The functions of p53 and pRb-1 and their possible role in senescence will be discussed later (sections 1.3.3.1.A and B).

1.3.2.1 HPV infection of oral squamous epithelium

HPV DNA has been detected in esophageal SCC, with HPV 16 being the most common (Perez-Ayala *et al*, 1990, Benamouzig *et al*, 1992). Frequency of detection varied from 30-40% of tumours. Other types of HPV found at low frequency are 2, 4, 6, 11, 18, 32 and 57 (Nagashfar *et al*, 1985, Adler-Storthz *et al*, 1986, de Villiers *et al*, 1989). These generally occur in benign proliferative lesions of oral mucosa such as papillomas and verruca vulgaris. HPV has also been found in head and neck SCC cell lines (Bradford *et al*, 1991b). In addition Maitland *et al* (1989) have detected HPV 16 and Yeudall and Campo (1991) have found HPV 18 at very low levels in normal oral mucosa samples.

Due to these results and in parallel with cervical cancer a role has been postulated for HPV in the etiology of oral SCC. There is as yet no evidence for viral integration in oral epithelium as there is with cervix (de Villiers *et al*, 1985, Matsukuru *et al*, 1986, Maitland *et al*, 1987). Importantly, HPV 16 has been shown to immortalise human oral keratinocytes *in vitro* (Parks *et al*, 1991), and since viral DNA has been detected in normal epithelium its role may be to cause an early event such as an increase in cell proliferation or

immortalisation (reviewed in Yeudall, 1992). However there is no direct data supporting this and HPV in SCC development remains controversial.

This system is of course extremely useful in studying mechanisms of immortalisation in the HPV-associated cancers and also points out genes involved in immortalisation which could be altered in other human cancers. However there will be several pathways to immortalisation, some of which may not involve proteins which are altered by these viruses.

1.3.3 The SV40 model

Human cells are semi-permissive for SV40 virus production with low frequency of transformation and thus relatively rarely give rise to immortal lines (Tooze, 1980, Ozer *et al*, 1981, Huschtscha and Holliday, 1983). Small *et al* (1982) found transformation efficiency in human cells increased with the use of a replication-defective virus which had a deletion within the origin of replication.

Human cells infected with SV40 express large T antigen, develop altered morphology and acquire extended lifespan *in vitro* (Ide *et al*, 1984, Neufield *et al*, 1987). The immortalising function of SV40 has been localised to the large tumour antigen and an intact T antigen may be required in human cells (Colby and Shenk, 1982, Chang *et al*, 1985). In fibroblasts lifespan is extended by approximately 20 population doublings (Wright *et al*, 1989) before crisis occurs. During crisis cell number remains constant, cell death balancing successful cell division (Stein, 1985). Rare variants survive crisis, giving rise to cell lines with unlimited growth potential (Huschtscha and Holliday, 1983, Neufield *et al*, 1987). These cells are still dependent on T antigen expression (Wright *et al*, 1989).

This two-stage escape from senescence led Wright's group to propose there are two mechanisms for senescence, mortality mechanisms 1 and 2 (M1 and M2). M1 is active but bypassed in T antigen-expressing cells with extended lifespan. It causes loss of mitogen-

responsiveness and accumulation in G1. M2 then causes failure of cells in division at crisis, and variants which survive crisis have genetically inactivated M2 (Wright *et al*, 1989).

Fusing SV40-transformed cells with normal cells produces hybrids with limited division potential, therefore SV40 immortalisation is still recessive (Muggleton-Harris and DeSimone, 1980). The viral genome integrates stably and T antigen is functional for induction of DNA synthesis in these senescent cells (Pereira-Smith and Smith, 1981). Thus T antigen alone is not sufficient for immortalisation, and both M1 and M2 inactivation are overcome by the normal cell without extinguishing T antigen expression.

T antigen causes karyotypic instability, expressing cells being aneuploid both before and after crisis, and this may contribute to genetic inactivation of M2 (Ray *et al*, 1990, Ray and Kraemer, 1993). This could explain why not all SV40-transformed cells escape crisis, because endoreduplication of chromosomes carrying M2 genes would mean it was highly unlikely that M2 would be inactivated (reviewed in Shay *et al*, 1991).

1.3.3.1 Mortality mechanism 1

As with HPV 16 & 18 E6 and E7, SV40 T antigen binds the p53 and Rb-1 suppressor gene products (Lane and Crawford, 1979, DeCaprio *et al*, 1988). E6 & E7 and also T antigen can be replaced in extending lifespan by adenovirus 5 E1A and E1B, which also bind pRb-1 and p53 (Sarnow *et al*, 1982, Moran *et al*, 1986, Whyte *et al*, 1988). Therefore these genes are good candidates for producing M1 senescence. In SV40-transformed cells p53 protein has an increased half-life, generally a sign of mutant protein (Oren *et al*, 1981). Shay *et al* (1991) have shown that in SV40-immortalised human fibroblasts where the T antigen has been de-induced and the cells senesced, proliferation can be stimulated by introduction of T antigen from another source; deletion mutants lacking the p53-binding domain do not have this activity.

A second reason p53 and pRb-1 are implicated specifically in M1 is that treatment of diploid fibroblasts with both an RB-1 and a *p53* anti-sense oligomer extends their lifespan and increases their sensitivity to serum (Hara *et al*, 1991).

A discussion of the role of p53 and pRb-1 in immortalisation and their possible functions follows.

1.3.3.1.A The p53 tumour suppressor gene.

Convincing evidence for a role of p53 in limited lifespan comes from studying LiFraumeni syndrome. This is an inherited autosomal dominant cancer syndrome, and Malkin *et al* (1990) recently identified the inherited mutation as inactivation of one allele of *p53*. It was then found that fibroblasts from these patients escape senescence in tissue culture (Bischoff *et al*, 1990). The cells acquire altered morphology and chromosome abnormalities and then enter a growth crisis from which they escape and grow for over 200 population doublings. Further, loss of cell cycle control in the LiFraumeni fibroblasts was correlated with loss of the remaining wild type allele (Yin *et al*, 1992).

Fibroblasts from homozygous *p53*-deficient mice produce cell lines without entering any kind of crisis or slowed growth phase (Harvey *et al*, 1993, Tsukada *et al*, 1993), as did lens and mammary epithelial cells. However bone marrow, hepatocyte and cardiac muscle cells did not immortalise (Tsukada *et al*, 1993), and keratinocytes go through crisis before immortalising (D.Stuart, personal communication). Fibroblasts from heterozygous mice senesced in culture even though they lost the wild type allele early on (Harvey *et al*, 1993). Homozygous fibroblasts were highly aneuploid and heterozygous fibroblasts were moderately aneuploid. These results suggest that loss of *p53* alone is insufficient to confer immortality on a cell and that the primary role of *p53* loss is to facilitate genetic instability and thus other alterations which influence immortality directly. This is consistent with a role for p53 in M1.

p53 appears to be a very complex protein with varied roles in the normal cell, many of which are compatible with senescence. First, there is evidence that p53 is involved in the cell cycle. Steady state levels of p53 are at their highest prior to DNA synthesis in late G1 (Reich and Levine, 1984). Induction of wild type protein in growth-stimulated human cells also prevents progression from G1 to S phase (Diller *et al*, 1990, Mercer *et al*, 1990, Michalovitz *et al*, 1990). Finally, fibroblasts from *p53*-deficient mice spend less time in G1 (Harvey *et al*, 1993).

This cell cycle regulation may involve phosphorylation of p53. Two carboxy-terminal sites in mouse p53 are phosphorylated *in vitro* by p34cdc2 and casein kinase II (Samad *et al*, 1986, Sturzbecher *et al*, 1990, Herrmann *et al*, 1991). Several phosphorylation sites have been identified at the amino-terminal portion of the mouse protein (Wang and Eckhart, 1992). A casein kinase I-like enzyme from 3T3 cells acts here and the human enzyme DNA-activated protein kinase is also active on the mouse protein (Milne *et al*, 1992, Wang and Eckhart, 1992). Phosphorylation by cdc2 is cell cycle-dependent and mutation of the serine-15 site reduces the ability of p53 to inhibit cell cycle progression (Bischoff *et al*, 1990, Fiscella *et al*, 1993).

p53 also seems to have some transcription factor activity. It functions as a transcription activator when coupled to a heterologous DNA-binding domain (Fields and Jang, 1990, Unger *et al*, 1992), but has DNA-binding activity itself to specific sequences (Kern *et al*, 1991) and activates transcription of reporter genes coupled to these sequences (Aoyama *et al*, 1992, Farmer *et al*, 1992).

Third, p53 may be important for the stability of the genome (Lane, 1992). Normal cells lack the ability to amplify DNA (Tlsty, 1990, Wright *et al*, 1990), but gene amplification is common in tumour cells (Tlsty *et al*, 1989). Cells retaining one wild type *p53* allele arrest in G1 as do normal cells when placed in the uridine biosynthesis inhibitor N-(phosphonoacetyl)-L-aspartate (PALA). Cells losing the second allele fail to arrest and amplify the gene for the enzyme CAD in order to survive in the presence of the drug

(Livingston *et al*, 1992). Furthermore, expression of wild type *p53* in immortal and tumour cells containing mutant *p53* restored G1 control and reduced gene amplification (Yin et al, 1992). More recently it was found that alterations in *p53* precede and facilitate divergence of aneuploid subclones in colorectal cancer (Carder *et al*, 1993).

Finally, p53 may play a part in terminal differentiation and apoptosis. Wild type p53 induces apoptosis in colon cancer and leukaemia cell lines and is important in at least one pathway to apoptosis in thymocytes (Yonish-Rouach *et al*, 1991, Shaw *et al*, 1992, Clarke *et al*, 1993). p53 was also able to induce expression of B-cell specific markers in a murine leukaemia virus-transformed pre-B-cell line (Shaulsky *et al*, 1991), and induces involucrin expression in normal keratinocytes (Woodworth *et al*, 1993), involucrin being a marker of squamous differentiation. Finally, squamous cell carcinomas produced by carcinogen treatment of *p53* heterozygous and null mice were markedly undifferentiated compared to wild type carcinomas (Kemp *et al*, 1993), and in human head and neck cancer patients p53-positive tumour-distant epithelia were less differentiated (Nees *et al*, 1993).

1.3.3.1.B The RB-1 tumour suppressor gene.

Aside from being bound by viral immortalising proteins, further evidence for a role for pRb-1 in senescence comes from experiments by Shay *et al* (1991) where they de-induced SV40 T antigen in immortalised human fibroblasts and rendered them senescent. An alternative source of T antigen restored proliferation but T antigen deletion mutants lacking the pRb-binding domain were unable to do so.

Antisense RB and *p53* oligonucleotides cooperate to extend the lifespan of human fibroblasts as mentioned above (Hara *et al*, 1991).

This area does remain controversial though because as regards the papillomaviruses HPV 16 E6 alone can bypass M1 and extend lifespan in human mammary epithelial cells (Shay *et al*, 1993). Additionally these cells may occasionally be immortalised with HPV 16 E6/E7 plasmids which do not bind pRb as can human epithelial keratinocytes (Band *et al*,

1991, Jewers *et al*, 1992). Other experiments suggested that HPV 16 E7 alone can immortalise human keratinocytes at low frequency (Halbert *et al*, 1991).

Tedesco *et al* (1993) have suggested these conflicts could be explained by the multifunctional nature of the transforming genes. These functions have not only additive but partially interchangeable effects.

The role of pRb-1 in the cell cycle is becoming clearer. pRb-1 phosphorylation levels vary through the cell cycle (Buchkovich *et al*, 1989, DeCaprio *et al*, 1989). This phosphorylation appears to regulate its activity in that hypophosphorylated pRb forms a complex with the transcription factor E2F at G1, and this complex silences transcription from E2F-regulated genes such as DNA polymerase α, C-MYC and dihydrofolate reductase (Shirodkar *et al*, 1992, Weintraub *et al*, 1992). E2F-regulated genes play an important part in cellular proliferation. As the cells enter S phase pRb becomes hyperphosphorylated and E2F complexes with p107, a pRb-related protein, and cyclin A. In the absence of pRb E2F is a positive element for the genes described (Schwartz *et al*, 1993).

SV40 T antigen preferentially binds the hypophosphorylated form of pRb-1 (Ludlow *et al*, 1989), and hence may block the G1 phase function of pRb (Goodrich *et al*, 1991). In HPV 16 and 18-immortalised keratinocytes it has been demonstrated that the pRb-E2F complexes are disrupted and complexes containing E2F and cyclin A are maintained (Pagano *et al*, 1992).

1.3.3.2 Mortality mechanism 2

Little is known about the gene products involved in M2. The main three changes which have been observed at crisis are, first, telomerase is activated (Morin, 1989, Counter *et al*, 1992). Second, the frequency of dicentric chromosomes stabilises (Counter *et al*, 1992), and third, telomere length stabilises (Counter *et al*, 1992).

There are also several chromosomes which have been postulated to carry senescence genes mainly by virtue of their ability to produce a senescence phenotype when transferred into various cell lines. These genes may be involved in bringing about crisis, and the evidence for their existence is described below.

1.3.3.2.A Evidence for senescence genes on specific chromosomes

Several groups have transferred chromosomes 1, 4 and 9 into various cell lines by microcell-mediated chromosome transfer and noted signs of senescence. These results are summarised in table 1.

Several other studies point to the involvement of chromosome 1 in senesence. Firstly, Sugawara *et al* (1990) fused human diploid fibroblasts with cells of an immortal but non-tumorigenic Syrian hamster cell line. Most hybrids senesced, but karyotype analysis of those that did not revealed that all these clones had lost both copies of the normal human chromosome 1. All the other human chromosomes were observed in at least some of the immortal hybrids. Furthermore, application of selective pressure to retain human 1 resulted in a higher percentage of senescent clones. Criteria for senescence included cellular enlargement and flattening, failure to increase cell numbers within two weeks, failure to form colonies at clonal density and lack of significant ^{3}H-thymidine incorporation.

The group then demonstrated that the gene(s) responsible for this effect was located on the long arm of chromosome 1. They used normal human fibroblasts with a translocation between chromosome X and chromosome 1 such that the HPRT gene on X and the region 1q23-qter were retained, HPRT being used to apply selection pressure for retention of the translocated chromosome. Hybrids between these cells and the hamster cells senesced at over twice the frequency.

Paraskeva *et al* (1989) reported the isolation of an immortal, non-tumorigenic, adenoma-derived epithelial cell line by virtue of continuous passage in vitro. At early passage the cells were diploid but at late passage every cell had an isochromosome 1q. This

Chromosome	Target cell line	Reference
1	10W-2 Syrian hamster cells	Sugawara *et al*, 1990
	HHUA (uterine endometrial carcinoma)	Yamada *et al*, 1990
4	HeLa (cervical carcinoma)	Ning *et al*, 1991
	J82 (bladder carcinoma)	
	T98G (glioblastoma)	
9	Hs294T (melanoma lymph node metastasis)	Porterfield *et al*, 1992)
	K562 (myelogenic leukaemia)	
	105AJ (chondrosarcoma)	Jagasia *et al*, 1993)
X	Ni-2/TG (nickel-transformed Chinese hamster cells)	Klein *et al*, 1991 Wang *et al*, 1992

Table 1. Chromosomes causing a senescence phenotype by microcell-mediated monochromosome transfer into the cell types shown.

result implies the presence of a suppressor of immortality on 1p, but is the only result that does so that we are aware of.

There is less evidence for chromosome 4 involvement in senescence and the involvement does not appear to be universal. Ning *et al* (1991) transferred a normal human chromosome 4 into immortal lines representative of the four complementation groups for indefinite division which they had identified earlier (Pereira-Smith *et al*, 1988). They found chromosome 4 was able to reverse the immortal phenotype of cell lines assigned to complementation group B such as HeLa, J82 and T98G. However it had no effect on HT1080 cells, amoungst other lines from other complementation groups, and another group (Benedict *et al*, 1984) have found that tumorigenic segregants of hybrids between normal human fibroblasts and HT1080 cells had lost chromosome 4. On the other hand it is not clear whether the growth suppression observed in group B cells was specifically senescence and nor were hybrids with cells from other complementation groups tested for their tumorigenicity in nude mice.

Chromosome 6 shows alterations when SV40-transformed fibroblasts pass through crisis to become immortal. Hubbard-Smith *et al* (1992) generated pre- and post-crisis SV40-immortalised cell lines and found all the immortal lines had alterations of chromosome 6, involving the distal portion of 6q21, as compared with their pre-immortal parent cells.

A rare human line of spontaneously immortalised keratinocytes has been observed to acquire a single isochromosome 6p along with increases in cell cycle proteins as it increased in colony-forming efficiency during the early stages of extended lifespan, implying again a role for 6q in suppressing immortality (Rice *et al*, 1993).

Chromosome 9 has been transferred by microcell-mediated chromosome transfer into several different cell lines by different groups (table 1). Using the MTAP gene at 9p21

as a natural selectable marker Porterfield *et al* (1992) showed that hybrids without an intact 9p were not affected, but those with the entire chromosome arm senesced after six population doublings.

Chinese hamster embryo (CHE) cells treated with nickel produce a high percentage of transformants with non-random deletions of Xq (Conway and Costa, 1990). The frequency of Ni-induced transformation is significantly higher in male than female cells, leading to the hypothesis that this mechanism of transformation involved a suppressor gene on X. Microcell-mediated chromosome transfer of an active hamster X into Ni-transformed CHE cells in fact produced 80% senescent colonies (Klein *et al*, 1991). Control transfer of Xp and Xq derived from Ni-transformed lines had no such effect, indicating that it was specific to the normal chromosome.

Later the same group found that passage of X in A9 cells (microcell donors) led to it being less active in the senescence assay. Treatment of the A9s with 5-azacytidine restored activity, showing that X, and particularly putative senescence genes on X, may become inactivated epigenetically by methylation.

In a second paper the group reported transferring a human X into the CHE cells led to a much lower rate of senescence (Wang *et al*, 1992), possibly because of deletion of important sequences during fusion since human X is more fragile.

Figure 3 summarises information to date about M1 and M2 based on the viral models of 2-stage escape from senescence.

Since M1 involves p53 and pRb-1, and gene inactivation appears to be necessary to overcome M2, and in the light of the data above, senescence genes are most probably tumour suppressor genes. Thus we may approach their isolation by methods normally used for tumour suppressors.

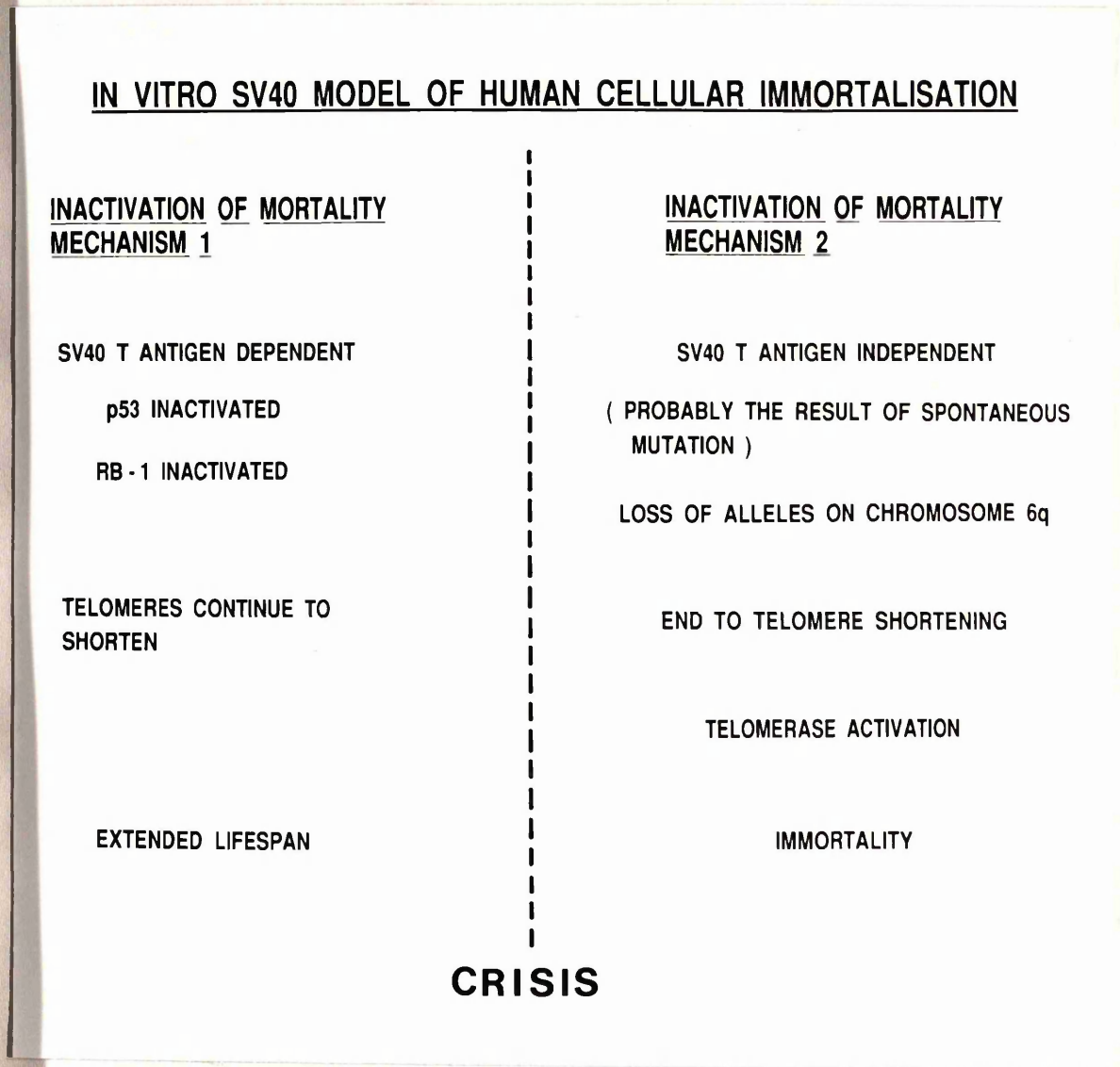


Figure 3. Summary of known data for the two-stage SV40 model of escape from senescence.

1.4 Tumour suppressor genes

Work on tumour suppressor genes has realised its importance much more recently than that on oncogenes. This may be partly due to their being more difficult to detect and functionally analyse. The first evidence for the existence of genes with the ability to suppress malignancy came from fusion of malignant and normal cells as described in the introduction to section 1.3. The study of karyotypes of various tumours showed that specific regions were consistently lost, and cellular proteins which are bound by DNA tumour viruses were identified. RB-1 was the first tumour suppressor to be cloned. The method by which the mutational mechanism of RB in tumours was detected provides a valuable clue to the presence of a tumour suppressor gene locus which is widely used today.

1.4.1 Restriction fragment length polymorphism analysis: Pinpointing the location of the retinoblastoma gene

Retinoblastoma is a childhood cancer arising in cells of the embryonal neural retina. It occurs in both sporadic and familial forms, the latter accounting for 30% of cases.

The incidences of unaffected carriers, unilateral and bilateral retinoblastomas led Knudson (1971) to propose a "two hit" model for development of the cancer. His hypothesis was that in the dominantly inherited form a mutation is passed through the germ line. The second mutation occurs in the somatic cells. In the sporadic form both mutations are somatic.

It had been noticed that approximately 5% of retinoblastoma patients had constitutional deletions of the long arm of chromosome 13. The region of smallest overlap was band 13q14 (Lele *et al*, 1963, Orye *et al*, 1974, Francke, 1976). Furthermore, upon analysis of tumour cells from patients with normal constitutional karyotypes, 5% showed visible deletions of chromosome 13, including 13q14 (Balaban-Malenbaum *et al*, 1981,

Balaban *et al*, 1982). However these alterations were obviously not enough to cause the disease as they affected such a small proportion of cases. In addition, in cases where there was a constitutional deletion not all retinal cells were neoplastic, and there were other consistent chromosomal aberrations in retinoblastomas (Squire *et al*, 1985).

The investigation was assisted by the discovery that the enzyme esterase D was linked to the disease locus (Sparkes *et al*, 1983). The enzyme has several isoforms so cosegregation of a specific allele with the disease could be followed within families, and activity levels were reduced in patients with deletions of 13q14 (Sparkes *et al*, 1980). Further substantiation of 13q14 as a possible disease locus came from cytogenetic analysis of a family who carried a constitutional deletion of 13q14. Unaffected members also had a translocation of 13q14 to chromosome 3 (Strong *et al*, 1981). Thus family members who had two copies of this region and one chromosome with a deletion did not develop retinoblastoma, but those with one copy (no translocation) and one chromosome with a deletion did. This showed that the deletional event was the predisposing event but was not acting in a genetically dominant way. It was possible that the inherited retinoblastoma mutation was a recessive allele and inactivation of the normal homologue was required for its expression in the tumour.

Bearing Knudson's proposal for two-step mutation in mind, several chromosomal mechanisms were discussed which would lead to phenotypic expression of a recessive allele (Cavenee *et al*, 1983). These are depicted in figure 4. Figure 4 initially represents a person with a recessive mutation in one copy of the RB gene. This could either be inherited or somatic. Any subsequent somatic event resulting in homozygosity for the mutant allele produces a mutant clone. Such an event could be any one of figure 4a-f. Firstly, non-disjunction at mitosis with loss of the normal chromosome would result in hemizygosity at all loci on chromosome 13. This could be followed by duplication of the mutant chromosome producing homozygosity at all loci. Recombination at mitosis between the RB locus and the centromere could produce a daughter cell with two copies of mutant RB, with heterozygosity at points above the crossover and homozygosity below. Finally, other events

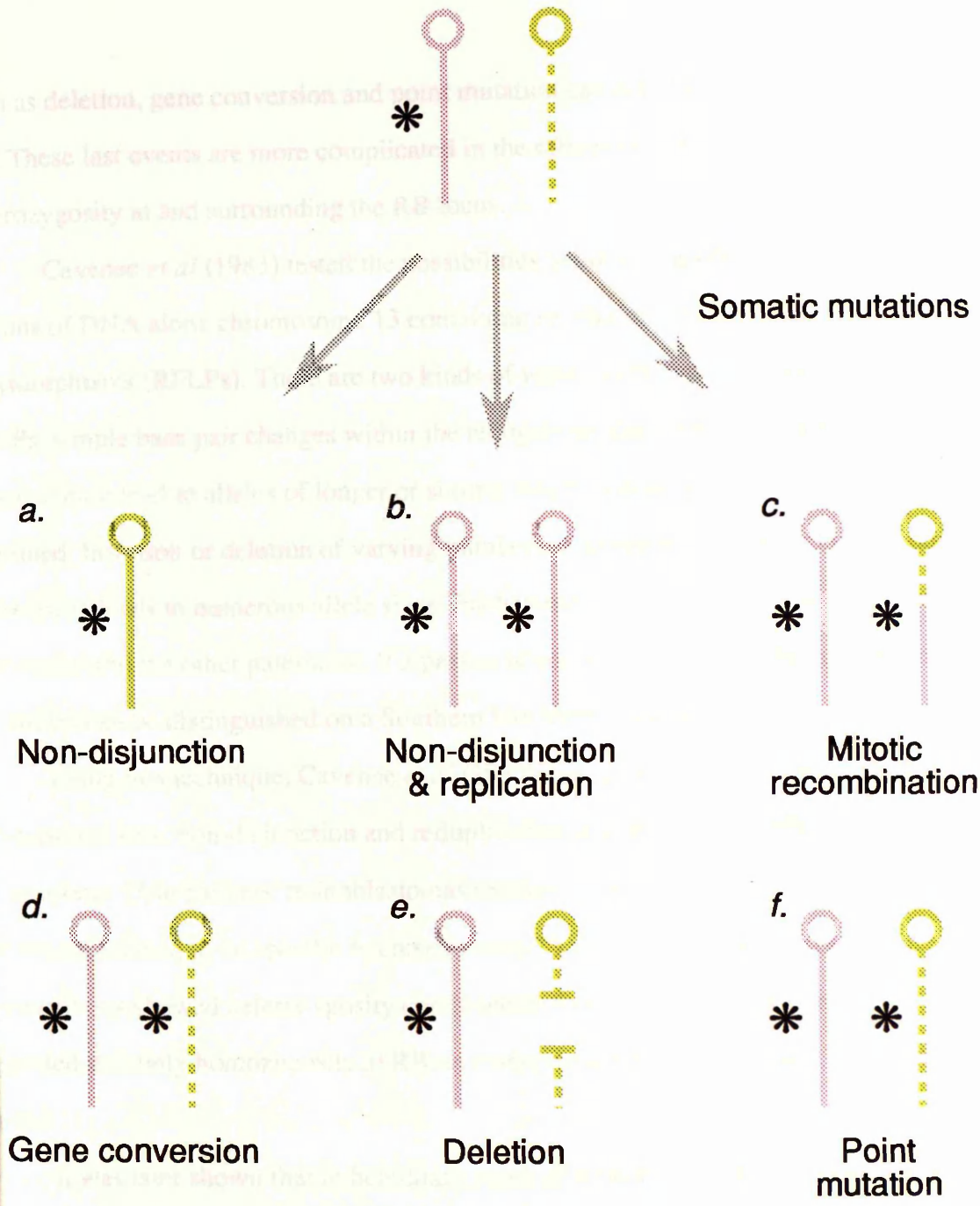


Figure 4 Mechanisms of development of loss of heterozygosity. The two chromosomes at the top represent a cell with an inactivated tumour suppressor allele ✱. The remaining allele can be inactivated by events *a* to *f* as shown. Events *a* to *e* lead to loss of heterozygosity.

such as deletion, gene conversion and point mutation can also lead to homozygous mutant RB. These last events are more complicated in the effects they would have in terms of heterozygosity at and surrounding the RB locus.

Cavenee *et al* (1983) tested the possibilities using a series of probes homologous to regions of DNA along chromosome 13 containing restriction fragment length polymorphisms (RFLPs). There are two kinds of variation between alleles which result in RFLPs: simple base pair changes within the recognition site sequence of a restriction endonuclease lead to alleles of longer or shorter length depending on whether a site is lost or gained. Insertion or deletion of varying numbers of segments of similar DNA sequences at the locus leads to numerous allele sizes. Each person has two alleles, one inherited maternally and the other paternally. If a person is polymorphic at the site in question then the alleles can be distinguished on a Southern blot hybridised with an homologous probe.

Using this technique, Cavenee *et al* (1983) found evidence for non-disjunction and chromosome loss, non-disjunction and reduplication, and mitotic recombination at chromosome 13 in patients' retinoblastomas relative to their normal constitutional DNA. The events seemed to be specific to chromosome 13 since use of markers on other chromosomes showed heterozygosity continued at those loci in the tumours. They suggested that only homozygosity at RB on chromosome 13 could cause a retinoblastoma tumour.

It was later shown that in hereditary cases of retinoblastoma the chromosome 13 which remained in the tumours was inherited from the afflicted parent, and indeed the chromosomal changes detected had uncovered a recessive mutation (Cavenee *et al*, 1985).

1.4.2 Microsatellites, another type of polymorphic marker

The use of microsatellites as markers has increased enormously in the last few years. They are a faster method of analysing loss of heterozygosity than Southern blotting. In addition a relatively small quantity of DNA is required.

Microsatellite sequences are CA-GT repeat regions of unknown function. They are highly polymorphic in length and occur approximately every 30-60 kb throughout the genome (Hamada and Kakunaga, 1982, Weber and May, 1989). They are inherited in Mendelian fashion in the same way as RFLPs (Weber and May, 1989).

Use of the polymerase chain reaction to amplify the sequences and separation of the products on a polyacrylamide gel enables the two alleles to be distinguished in heterozygous individuals (Weber and May, 1989). Since microsatellite sequences are on average 70% polymorphic, they are much more efficient than RFLP analysis. The frequency of their occurrence is an added advantage since most regions of DNA can be examined in some detail. Their main disadvantages are that it is not easy to judge allele copy numbers and their amplification is often complicated by "shadow" bands, making interpretation occasionally difficult.

1.4.3 Microcell-mediated monochromosome transfer as another method of detection of suppressor genes

This technique refines that of hybrid analysis because it enables transfer of a single chromosome into a specific target cell. It has been used recently to search for suppressive activity in conjunction with techniques previously described, providing functional evidence for tumour suppressors.

Human chromosomes are tagged with a selectable marker and form micronuclei in the presence of the mitotic inhibitor colcemid. The micronuclei are isolated by cytochalasin B enucleation and fused with the desired cell type in the traditional way with polyethylene glycol. A human chromosome library can then be constructed and passaged in mouse cells before transfer in a similar way to the appropriate target cell (McNeill and Brown, 1980, Saxon *et al*, 1985).

A previous problem had been how to obtain a selectable marker on the chromosome of interest where it did not occur naturally. This has been solved by transfecting human

cells with plasmids carrying markers such as the bacterial neomycin resistence gene or by infection with retroviral vectors. For example, human HPRT- cells have been transfected with plasmids carrying *E.Coli* xanthine-guanine phosphoribosyl transferase (XGPRT). This integrates into human DNA and transformants are selected by growth in hypoxanthine-aminopterin-thymidine medium (Srivatsan *et al*, 1984). XGPRT confers resistence to aminopterin in HPRT- cells. Transformants containing a single integrated copy are isolated and their site of integration determined by in situ hybridisation (Saxon *et al*, 1985).

It is more difficult to make microcells from human cells than from mouse, and this is why the chromosomes are passaged in mouse cells (Shows and Sakaguchi, 1980, Saxon *et al*, 1986). Rodent-human hybrids are very efficient in making microcells (Saxon *et al*, 1986). Resulting hybrids (target cell plus the chromosome of interest) may contain mouse chromosomes as well. These may be discarded (Saxon *et al*, 1986) but in experiments where they have not, no correlation was noted between the presence of the mouse chromosome and the phenotypic alterations observed after fusion (Kugoh *et al*, 1990).

1.4.4 Advantages and disadvantages of loss of heterozygosity analysis as a means of locating tumour suppressor genes

Loss of heterozygosity (LOH) analysis as described for RB-1 (section 1.4.1) has been widely used both alone and in conjunction with some of the other techniques described earlier to look for candidate tumour suppressor genes. It refines cytogenetic analysis as it detects changes in kilobase sequences of DNA rather than hundreds of kilobases.

LOH itself does not induce malignancy, but reproducible LOH is an indicator of the possible location of a gene of interest in pathogenesis. Regions of overlap of deletions in areas where LOH has been detected can be relatively small. This lack of complete loss of large regions of genetic material indicates the presence of important genes and pinpoints areas for further analysis.

However high background levels of LOH can make it difficult to decide which loci are important. Not all LOH may constitute inactivation of tumour suppressor genes, but may be simply the result of genomic instability. Indeed some are of the opinion that gene deletion is a general process in tumour progression and not a specific mechanism of carcinogenesis (Chigira *et al*, 1993). These problems can be avoided to an extent by looking at early tumours to see which alterations happen first and are presumably causative for abnormal growth.

Contamination of tumour with normal tissue tends to reduce the sensitivity of LOH assays (Lasko *et al*, 1991), resulting in underscoring of LOH frequency. This could be a reason to notice lower levels of LOH than would be thought of import when studying cell lines or purer material, and underlines the need to look at large numbers of samples. It is also important to determine background levels of LOH in the system in use for comparison.

One advantage of LOH analysis over cytogenetics is that some chromosomal mechanisms for the allele losses can be distinguished as was described in section 1.4.1. A tumour suppressor inactivated by two point mutations would not be detected.

Very recently a novel kind of tumour suppressor gene activity was identified which affects stability of specific sequences of DNA. Peltomaki *et al* (1993) mapped a locus on chromosome 2 which predisposes to hereditary non-polyposis colorectal cancer. This suppressor gene might be a replication factor and the mutation may cause general instability or alternatively instability may be associated only with microsatellite-associated genes (Aaltonen *et al*, 1993). The gene was not detectable by LOH analysis and, further, replacing it by chromosome transfer may not suppress the effects of previous damage.

Molecular evidence from LOH, linkage studies, cytogenetics and monochromosome transfer is circumstantial and does not provide sufficient functional evidence for the existence of a particular suppressor gene. This comes from cloning and subsequent transfection into cells which lack the relevant gene to test for suppressor activity. In most cases there is no specific assay apart from testing for tumorigenicity in nude mice.

Once the candidate RB gene had been cloned from chromosome band 13q14 (Friend *et al*, 1986, Lee *et al*, 1987), tumours were examined to check that the transcript was absent and the gene mutated in significant numbers (Fung *et al*, 1987, Lee *et al*, 1987, Bookstein *et al*, 1988). Huang *et al* (1988) then undertook to develop an assay system for pRb function by introducing the gene into retinoblastoma and osteosarcoma cell lines which had inactivated their endogenous Rb genes. Expression of exogenous RB suppressed growth rate in culture, anchorage-independent growth and tumorigenicity in nude mice. It also caused the cells to enlarge and flatten. Expression of Rb protein was later shown to suppress other types of tumour which tended to have inactivated RB genes such as prostate (Bookstein *et al*, 1990), and also to arrest normal cells (Fung *et al*, 1993). Finally, transgenic mice with no functional RB are non-viable (Clarke *et al*, 1992, Lee *et al*, 1992). These evidences all support the role of RB as a tumour suppressor gene with an important role both in development and the normal cell. Candidate suppressor genes must be investigated in this way to be categorised as suppressor genes.

1.4.5 Imprinting and LOH

Genomic imprinting is gamete-specific, differential expression of the two alleles of a gene. It has been demonstrated in mice, where parental-specific monoallelic expression of insulin-like growth factor 2 (IGF2), small nuclear ribonucleoprotein-associated polypeptide, the IGF 2 receptor and H19 have been detected (DeChiara *et al*, 1991, Barlow *et al*, 1991, Bartolomei *et al*, 1991, Cattanach *et al*, 1992, Leff *et al*, 1992). Functional differences between maternal and paternal alleles in humans are demonstrated by disorders associated with either all paternal or all maternal genomes (hydatidiform moles of the uterus and ovarian teratomas respectively), and also by disorders associated with uniparental disomy (UPD) of specific chromosome regions, such as paternal UPD at 11p15 in 20% of Beckwith-Wiedemann syndrome patients (Linder *et al*, 1975, Henry *et al*, 1991, Ozcelik *et al*, 1992).

There have been many reports of preferential LOH in both sporadic and inherited tumours (reviewed in Feinberg, 1993), providing indirect evidence for imprinting involvement in cancer. The bias is towards retention of the maternal allele; studies in mice have shown that while paternal UPD leads to prenatal overgrowth, maternal UPD of the same region causes growth retardation, so imprinted tumour suppressor genes may tend to be expressed by the maternal allele (Barton *et al*, 1991).

One hypothesis is that imprinting could be one of the two hits required to inactivate tumour suppressor gene expression (Scrable *et al*, 1989). LOH then results from loss of the active copy, or may not occur if the active gene mutates or is also aberrantly imprinted.

Some evidence against this idea comes from analysis of Beckwith-Wiedemann syndrome (BWS). In the 20% of patients with UPD at 11p15 there is 64% incidence of embryonal tumours such as Wilms tumour, or pediatric nephroblastoma, hepatoblastoma, and rhabdomyosarcoma, which is much higher than the incidence normally associated with the disease (Feinberg, 1993). We cannot distinguish two possible effects of the UPD, namely overexpression of the duplicated allele (the allele is a growth promoter) or loss of expression of the deleted allele where it is a tumour suppressor. Only the latter is consistent with the above hypothesis.

In non-UPD BWS patients there are eight known balanced germline translocations of 11p15, and these are of maternal origin. Fourteen of fifteen known germline 11p15 duplications are of paternal origin (Brown *et al*, 1990). This would suggest the paternal allele is active, the maternal inactive, and BWS is caused by overdosage of the paternal or activation of the maternal alleles. One candidate for the BWS gene at 11p15 is insulin-like growth factor 2. This gene is imprinted in man and is expressed from the paternal allele (Ohlsson *et al*, 1993).

WAGR syndrome is a hereditary predisposition to Wilms' tumour with other associated developmental problems. A minority of tumours from these patients have deletions at 11p13 (Francke *et al*, 1979) and the WT-1 tumour suppressor has been cloned from this region (Call *et al*, 1990, Gessler *et al*, 1990). Other Wilms' tumours have no

deletions of WT 1 but have LOH at 11p15 (Reeve *et al*, 1989). Finally some tumours show no linkage to any region of chromosome 11 (Huff *et al*, 1988). 70% of Wilms' tumours showing no LOH at either region demonstrate loss of imprinting at IGF 2 (Rainier *et al*, 1993, Ogawa *et al*, 1993). In other words both alleles are expressed. This loss of imprinting is much more common than the LOH (Wadey *et al*, 1990, Little *et al*, 1992).

Thus evidence from preferential LOH and UPD support conflicting hypotheses. A final hypothesis which would incorporate both is the idea that preferential LOH could involve a tumour suppressor gene on the same chromosome arm as an imprinted growth promoter (Feinberg, 1993, see figure 5). The preference arises because loss of the paternal allele would also delete the linked active copy of the growth promoter, which would be deleterious to tumour growth. In support of this Koi *et al* (1993) have isolated an 11p15 fragment which is distinct from IGF 2 and suppresses the growth of rhabdomyosarcoma cells. In this case LOH would still indicate the presence of a suppressor gene (see figure 5). Alternatively the function of LOH associated with duplication of the remaining allele could be simply to allow overexpression of the growth promoter. LOH has been detected in the constitutional tissue of some Wilms' tumour patients (Chao *et al*, 1993). In this case LOH would not be related to tumour suppression at all.

Further work is required before any of these theories can be proved or disproved. It is likely that some will be appropriate to some situations and others to others.

1.5 Squamous cell carcinoma

A simplified diagram of the structure of the skin is shown in figure 6. Keratinocytes proliferate in the basal layer and then migrate outwards. In the spinous layer they lose proliferative ability and begin to express markers of differentiation such as involucrin, which is a precursor of the cornified envelope. In the granular layer proteins are cross-linked to form the insoluble cornified envelope and the cells enlarge and flatten out. Finally at the cornified layer the nucleus begins to disintegrate and the cells die and become

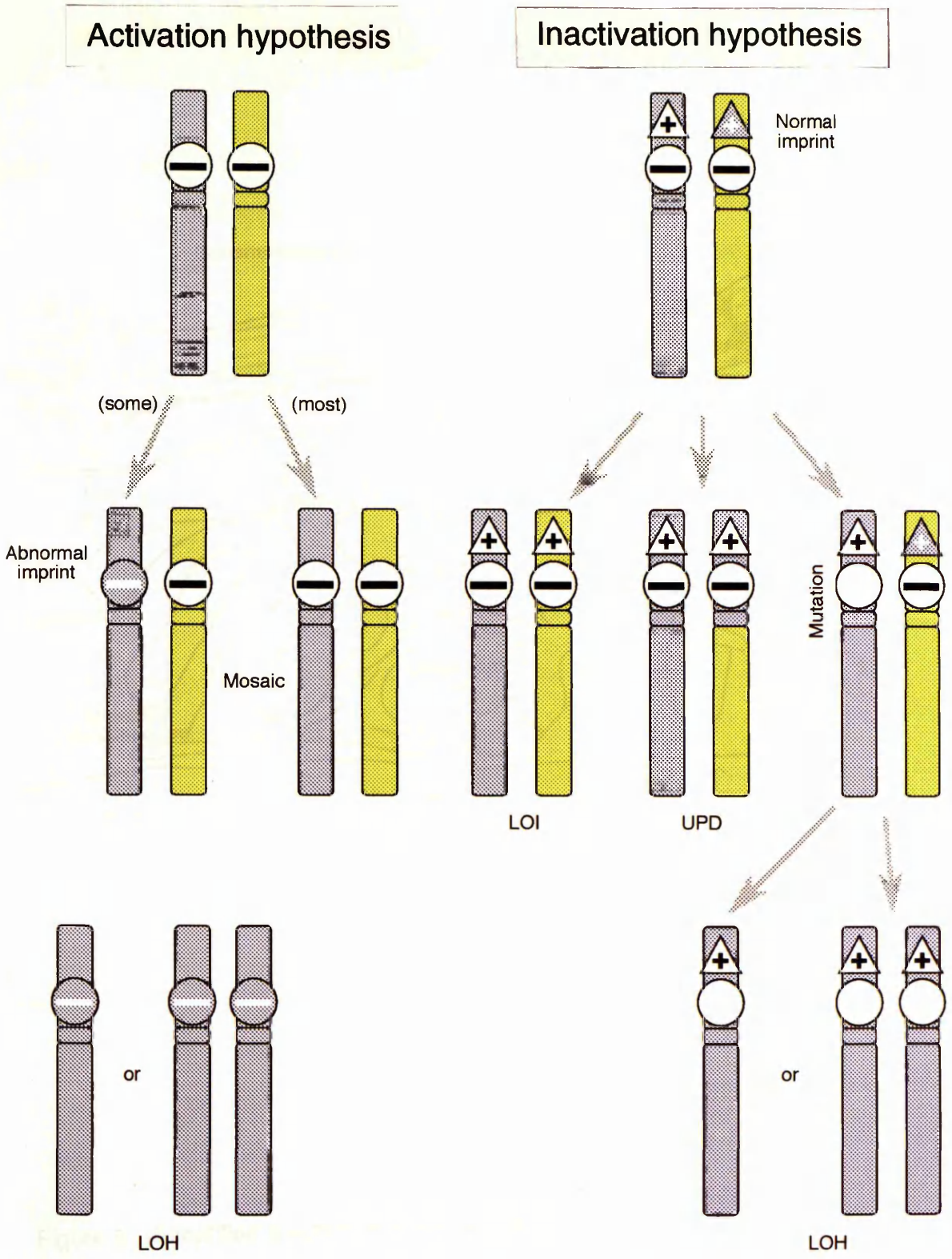


Figure 5 Contrasting hypotheses of genomic imprinting in cancer. The inactivation hypothesis involves loss of expression of a tumour suppressor gene ⊖ by abnormal imprinting. The activation hypothesis involves normal imprinting of a growth promoting gene. ⊕ Overexpression can result from loss of imprinting (LOI) of the growth promoter, uniparental disomy (UPD), or loss of the chromosome carrying the imprint. Loss of heterozygosity of the tumour suppressor would be deleterious to growth if it involved the chromosome carrying the active growth promoter.
Taken from Feinberg, 1993.

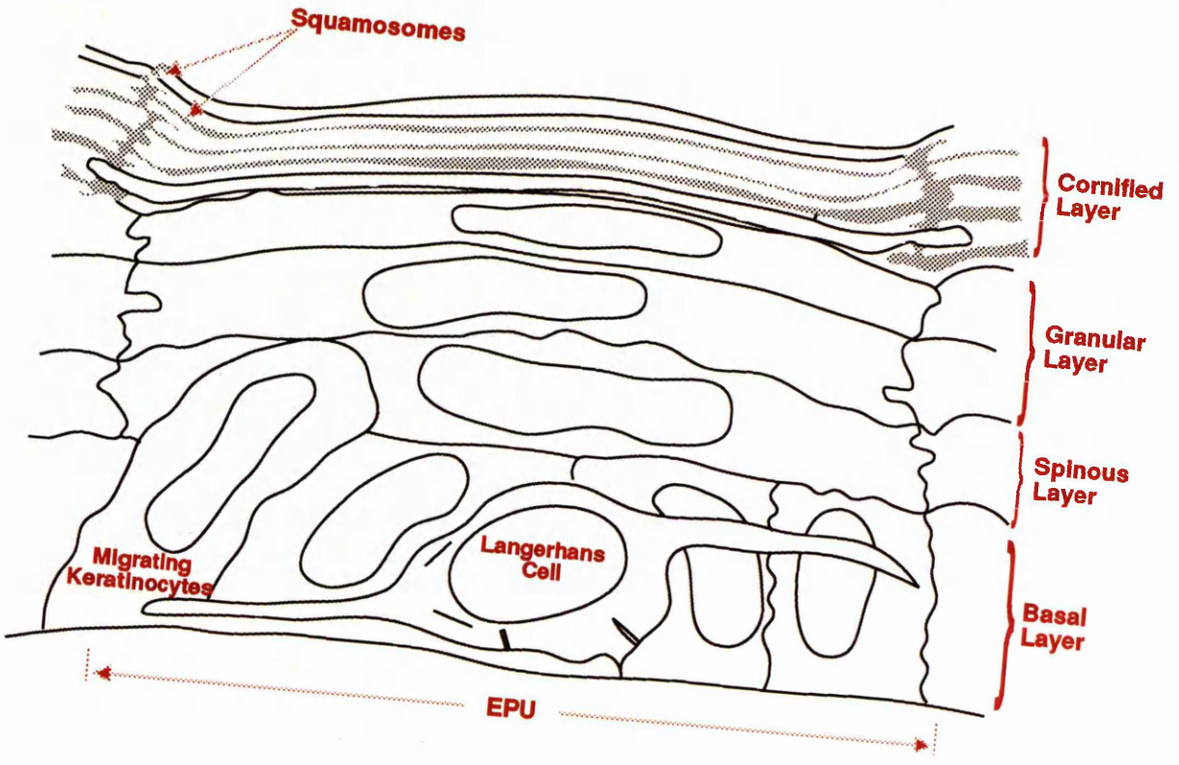


Figure 6 Simplified diagram of the structure of the epidermis.

squames and are eventually sloughed off. Thus *in vivo* keratinocyte differentiation takes place in well-defined compartments. This also occurs *in vitro* when keratinocytes are grown under fairly specialised conditions such that they can form colonies which stratify and allow cells at the centre to differentiate and express the markers described above (Rheinwald and Green, 1975a, 1975b, Sun and Green, 1976, Green, 1977)

1.5.1 Progression of oral SCC

Oral squamous cell carcinoma (SCC) is a major cause of death in developing countries. It comprises 40-50% of malignancies in India and Southeast Asia (Sanghvi, 1981). In the West it comprises 5% of total malignancies (Vokes *et al*, 1993) but its mortality rate and incidence is increasing (Franceschi *et al*, 1992, Macfarlane et al, 1992).

A diagram of oral SCC progression and the physiological changes occurring at each stage is shown in figure 7. Premalignant lesions which can progress to SCC are papillomas, leukoplakias and erythroplakias, erythroplakias having the highest probability of progression. Erythroplakias are therefore the only premalignant lesion which are routinely removed. Approximately 50% of the time however SCC develops without a prior lesion. Spindle cell carcinomas tend to be recurrent or metastatic tumours (Pindborg, 1985).

In India oral SCC is associated with chewing tobacco (Jussawalla and Deshpande, 1971) and is often preceded by premalignant leukoplakias (Daftary *et al*, 1990). Western SCC is associated with cigarette smoking, the use of snuff and alcohol (Wynder and Stellman, 1977) and is more commonly preceded by erythroplakias, but often occurs without a premalignant lesion (Binnie, 1976).

Esophageal cancer frequently requires radical surgery so reduces the quality of the patients' lives considerably (McCaffrey, 1993).

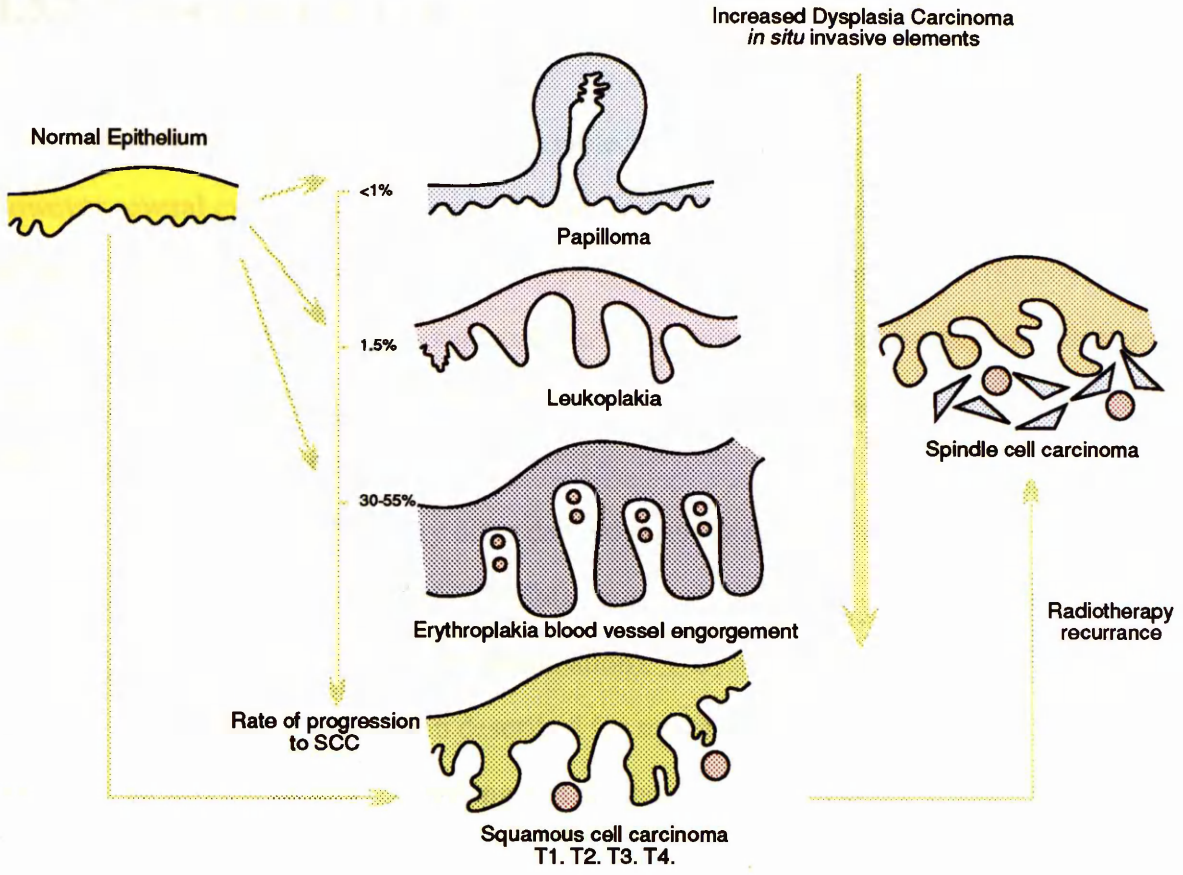


Figure 7 Stages of progression of oral squamous cell carcinoma. T1-T4 are clinical measures of tumour stage.

1.5.2 Cytogenetic alterations

Relatively little is known about the molecular events leading to SCC development, however several cytogenetic studies have been done. Common alterations include deletions of 3p, 8p, 10p, 11p, 18q and the short arms of the acrocentric chromosomes (Stacey *et al*, 1990, Cowan *et al*, 1992). Additional copies of 7p are also common. Breakpoints cluster to 1p11-32, 1q21-44, 2q31, 3p11, 4q35, 7p22, 11p15, 11q13, 12q24 and 17q25 (Jin *et al*, 1990, Jin *et al*, 1993, Patel *et al*, 1993).

The region 3p14-25 is very often deleted (Latif *et al*, 1992). Recently three discrete regions of deletion at 3p were identified (Maestro *et al*, 1993) in agreement with work in our group (O.Loughran, personal communication). They were 3p24-ter, 3p21.3, and 3p14-cen. These same regions have been described for squamous cell lung cancer, which shares the major etiological factors of smoking and alcohol (Hibi *et al*, 1992). Alterations at each of these sites have also been reported in many other cancers such as lung cancer, renal carcinoma, breast cancer, tesicular, ovarian and cervical cancers (Whang-Peng *et al*, 1982, Wang and Perkins, 1984, Lothe *et al*, 1989, Rabbits *et al*, 1989, Sato *et al*, 1990, Yamakawa *et al*, 1991, Jones and Nakamura, 1992).

11p13-14 is a site of non-random rearrangement in a subset of SCCs (Bradford *et al*, 1991a). This may be the same suppressor locus associated with Wilms' tumour and WAGR syndrome (Wilms' tumour, aniridia, genitourinary anomalies, mental retardation).

Work on primary head and neck SCCs at the Beatson has shown a high incidence of LOH at 3p, 5q, 9q, 11q, and 17p (Ah-See *et al*, in press). These results have been confirmed in cell lines derived from such tumours apart from 11q and 17p, which have not been investigated (O.Loughran, personal communication). Abnormalities of both 9p and 9q have been noted previously at the cytogenetic level (Patel *et al*, 1993, Worsham *et al*, 1993).

Chromosome abnormalities are ubiquitous in SCC and many changes are non-clonal (Jin *et al*, 1990). Interestingly, Jin *et al* (1993) have found that short-term cultures in

medium supplemented with fetal calf serum, cholera toxin and epidermal growth factor (EGF) had less complex karyotypes than those grown in serum-free medium. Apart from rearrangements of 1p22, which were only found in cultures in serum-containing medium, structural aberrations were similar.

1.5.3 Gene amplifications

Generally in head and neck SCC there are amplifications of the EGF receptor (Weichselbaum *et al*, 1989), C-MYC, BCL-1, INT-2, (Leonard *et al*, 1991) N-MYC, K-RAS and N-RAS (Saranath *et al*, 1989). BCL-1 & INT-2, N-MYC & C-MYC, and N-MYC & N-RAS may be coamplified, and multiple amplifications are common. L-MYC, TGF-β, C-MOS, C-ERB B2, C-ERB A2 and C-HA-RAS are not amplified. Amplification is not associated with degree of differentiation but Field *et al* (1986) have noted a significant difference between C-MYC expression in clinical stages one and two as compared with stages three and four of head and neck tumours. Other amplifications have also been associated with stages three and four (Saranath *et al*, 1989).

1.5.4 RAS mutations

In Indian cases of chewing tobacco-associated SCC, 35% have RAS mutations (Saranath *et al*, 1991). They are restricted to HA-RAS codons 12, 13 and 61. HA-RAS, KI-RAS and N-RAS mutations have been observed only rarely in oral cancer in the West (Chang *et al*, 1990, Warnakulasuriya *et al*, 1992).

It is not clear whether a RAS mutation in a keratinocyte gives it a growth advantage or not, since it renders the cell more responsive to epidermal growth factor (Henrard *et al*, 1990) but also allows amplification of differentiation as well as proliferation signals (Corominas *et al*, 1989). However RAS mutation enables a cell to secrete transforming

growth factor α which may then contribute to the clonal expansion of surrounding cells (Ozanne *et al*, 1980).

A recent study of all stages of progression of head and neck SCC has revealed that premalignant erythroplakias and papillomas do not carry activating RAS mutations (Clark *et al*, 1993). It was suggested therefore that since the incidence of RAS mutations was high in Indian SCC samples from patients who chewed betel quid, this tobacco may contain tumour promoters which allow the cells with RAS mutations to gain selective advantage. This was not occurring in Western patients who smoked because although the tobacco in the U.K. contains carcinogens causing H-RAS activation (Quintanilla *et al*, 1986), these tumour promoters were not present.

1.5.5 Tumour suppressor genes in head and neck SCC

1.5.5.2 *p53*

It is clear that *p53* mutation is an extremely common event in esophageal SCC (Hollstein *et al*, 1990, Sakai and Tsuchida, 1992, Wagata *et al*, 1993, Yin *et al*, 1993). In many cases mutation correlates with deletion of 17p (Wagata *et al*, 1993). This was also true of epithelial squamous cell carcinomas (Piercall *et al*, 1991, Moles *et al*, 1993) and immortal epithelial cell lines (Lehman *et al*, 1993). Work in our laboratory has shown over 80% of head and neck SCC cell lines have *p53* mutations and this correlates with the original tumours (Burns *et al*, 1993 and unpublished data). This is in agreement with other findings (Maestro *et al*, 1992, Sakai and Tsuchida, 1992).

Frequency of *p53* mutation may increase with progression of SCC (Boyle *et al*, 1993). One study has found stabilised p53 portein in precancerous esophageal lesions (Wang *et al*, 1993), but this contrasts with results for early epithelial lesions (Gusterson *et al*, 1991). Mutant p53 protein has additionally been found in tumour-distant epithelia of

head and neck cancer patients (Nees *et al*, 1993). Different mutations were found in different epithelia from the same patient in these cases.

1.5.5.2 RB-1

Approximately 50% of esophageal squamous cell carcinomas have loss of heterozygosity at the RB-1 locus (Boynton *et al*, 1991, Huang *et al*, 1992, Huang *et al*, 1993), but so far this has not been correlated with alterations in the Rb-1 protein so it is not clear what it represents.

1.5.5.3 Other tumour suppressor genes

Loss of heterozygosity has been reported at several other known suppressor loci in esophageal SCCs. These include APC, MCC and DCC (Boynton *et al*, 1992, Huang *et al*, 1992). Again this has not been correlated with mutations in these genes. It does appear that oral squamous cell carcinoma, like colon cancer, involves inactivation of multiple tumour suppressor genes.

Head and neck SCC is an excellent system for the study of the genetic alterations contributing to immortalisation and tumour progression and cancer in general. Progression takes place in clearly defined stages and immortal variants are frequent (Pindborg, 1985, Rheinwald and Beckett, 1981). There are very good tissue culture techniques available for growing all these stages and also normal keratinocytes (Rheinwald and Green, 1975a, 1975b, Sun and Green, 1976, Green, 1977). Furthermore, differentiation of keratinocytes takes place in culture via expression of many easily detectable markers, unlike fibroblasts.

1.6 Aims

The aims of the work in this thesis were firstly to establish cell lines from various stages of untreated head and neck SCCs and to characterise them in terms of differentiation status and cellular origin. The cell lines were to be derived under conditions which would allow us to determine if immortal populations existed in the tumours *in vivo*, and in such a way as to prevent to the best of our ability the selection of fitter variants. Defining the lifespans of the various cultures would enable us to correlate lifespan and genetic bakground. The original tumours were to be retained for comparison.

The system should be suitable for the study of inactivation of tumour suppressor genes. The genetics of the two-stage model systems (sections 1.3.2 and 1.3.3) for escape from senescence could then be analysed in naturally occurring immortal cells and their related tumours. Evidence for senescence genes at regions 1q, 4, 6q and 9 has been discussed in section 1.3.3.2.A. These areas in particular were to be analysed in the system to discover if they are altered *in vivo* in tumour progression. Finally, the cooperation of inactivation of the known tumour suppressor genes *p53* and RB-1 was to be investigated in oral squamous cancer.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Tissue culture

Gibco Europe Life Technologies Ltd, U.K.:

Dulbecco's Modified Eagles Medium
Keratinocyte SFM
Fetal calf serum
Sodium bicarbonate
Glutamine
Epidermal growth factor (recombinant)

Beatson Institute Central Services:

Amphotericin B
Penicillin
Streptomycin

Clonetics, U.S.A.:

Bovine pituitary extract

Becton Dickinson U.K. Ltd:

Plastic flasks and plates

A/S Nunc, Denmark:

Cryotubes
Chamber slides

Flow Laboratories, U.K.:

Mycoplasma Removal Agent
Donor calf serum

Worthington Biochemical Company, U.K.:

Trypsin

Unipath Ltd, U.K.:

Phosphate-buffered saline

Sigma Chemical Company Ltd., U.K.

Hydrocortisone

Swiss mouse 3T3 cells were obtained from laboratory stocks.

2.1.2 Immunocytochemistry

Sigma Chemical Company Ltd, U.K.:

Tween 80
Hydrogen peroxide
Diaminobenzoic acid
Bovine serum albumin fraction V
Hoescht 33258

Boehringer-Mannheim, Germany:

AE1/AE3 monoclonal anticytokeratin antibodies
Fluorescein-conjugated anti-mouse immunoglobulin

Vector Laboratories, U.K.:

Vectastain ABC kit

NRK cells were obtained from laboratory stocks.

2.1.3 Cloning

Bibby-Sterilin Ltd, U.K.:

Sterilin bacteriological plates

Gibco Europe Life Technologies Ltd.:

E.coli DH5α competant cells

DIFCO Laboratories, U.S.A.:

Bactotryptone
Agar

Beatson Institute Central Services:

L-broth

Beta Laboratories, U.K.:

Yeast extract

Sigma Chemical Company Ltd, U.K.:

Ampicillin
Chloramphenicol
Lysozyme
Ethidium bromide

Fisons Scientific Equipment, U.K.:

Magnesium sulphate
Sodium hydroxide
Isopropanol
Sodium acetate

Boehringer-Mannheim, Germany:

DNAse-free RNAse

2.1.4 DNA work

Sigma Chemical Company Ltd, U.K.:

β-mercaptoethanol
N-sodium lauryl sarcosine
Bromophenol blue
Xylene cyanol
Hepes
Calf thymus hexanucleotides
Salmon sperm DNA
TEMED
Poylvinylpyrrolidone
Dextran sulphate

Bethesda Research Laboratories, U.S.A.:

LMP agarose
Agarose
Protease K
1kb DNA ladder
ϕX 174 Hae III-digested DNA
Bacteriophage λ *Hind* III-digested DNA

Gibco Europe Life Technologies Ltd:

Bam H1
Eco R1
Hin f1
Hind III

Fisons Scientific Equipment, U.K.:

EDTA (sodium salt)
Sodium chloride
Sodium citrate

Fluka Chemica-Biochemika AG, Switzerland:

Guanidinium thiocyanate
Formamide

Boehringer-Mannheim, Germany:

Tris-HCl
Caesium chloride
Tris base

Aldrich Chemical Company, U.K.:

Ammonium chloride
Potassium carbonate

Pharmacia AB, Sweden:

NICK columns
Ficoll 400
dNTP set

James Burrough Ltd, U.K.:

Ethanol

Rathburn Chemicals Ltd, U.K.:

Phenol

Whatman International Ltd, U.K.:

Whatman 3MM filter paper

Amersham International PLC, U.K.:

$\alpha(^{32}P)$-dCTP, 3000Ci/mmol

Flowgen Instruments Ltd, U.K.:

NuSieve agarose

Northumbria Biologicals Ltd, U.K.:

Klenow enzyme
Xba 1

Biolabs, New England, U.S.A.:

Dde 1

ICN Biomedicals Ltd, U.K.:

Biotrans^{TM} nylon membrane

Perkin-Elmer Cetus, U.S.A.:

DNA PCR kit

Advanced Biotechnologies, U.K.:

Taq polymerase

Severn Biotech Ltd, U.K.:

40%(w/v) polyacrylamide

Schleicher and Schuell, Germany:

Nitrocellulose membrane

Technical Photo Systems, U.K.:

Fuji RX medical X-ray film

Eastman Kodak Company, U.S.A.:

XOMAT AR X-ray film
DUP1 duplicating film

Bio 101, Inc., U.K.

Geneclean 2^R kit

Harlan-Olac, U.K.

Nude mice (MF-1*Nu*)

All other chemicals were obtained from BDH Analar, U.K.

2.2 Methods

2.2.1 Tissue culture

2.2.1.1 Culture of human keratinocytes

Normal human epidermal keratinocytes (HEKs) were grown in Keratinocyte-SFM supplemented with bovine pituitary extract (50μg/ml) and recombinant epidermal growth factor (5ng/ml), which is necessary for growth of normal keratinocytes (Rheinwald and Green, 1977). They were kept in a moist atmosphere in a 37^{o}C incubator gassed with air containing 5% v/v CO_2.

SCC cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum, 3g/l sodium bicarbonate, 2M glutamine, 0.25mg/ml amphotericin B, 37.5μg/ml penicillin, 10mg/ml streptomycin and 0.4μg/ml hydrocortisone. Hydrocortisone makes the colony morphology more orderly and maintains proliferation at a slightly greater rate (Rheinwald and Green, 1975b). Cells were grown on a feeder layer of lethally irradiated Swiss mouse 3T3 fibroblasts at $1.5x10^4$ cells/cm^2 because keratinocytes require proliferation-controlled fibroblasts to initiate colony formation (Rheinwald and Green, 1975b), and were kept in incubators as described above.

Cells were passaged by rinsing with phosphate-buffered saline (PBS): 0.14M NaCl, 27mM KCl, 10mM Na_2HPO_4, 15mM K_2HPO_4 and removing cells from plates or flasks with trypsin solution (0.17% {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 (3000g, 5 min at room temperature) and aspiration of the supernatant. Cells were resuspended in fresh growth medium and replated.

Population doubling levels at each pass were determined using the formula:

population doubling level n= $3.32(\log_{10}N-\log_{10}N_0)$

where N= cell yield and N_0= number of colonies

The criterion for immortality was that cells could be passaged for at least 100 population doublings. A culture was designated as senescent when no colonies of more than thirty cells appeared on the dishes for four weeks and remaining cells were large, flat and terminally differentiated. Crisis was distinguishable by the presence of highly vacuolated cells and cell death appearing to balance cell division (cell number remaining constant).

All cells were frozen down for storage in liquid nitrogen by first trypsinising and removing trypsin as above, then resuspending in fresh serum-containing medium, pelleting cells and aspirating supernatant. Cells were suspended in 1ml DMEM, 10% serum per 10^6 cells plus 10% (v/v) dimethyl sulphoxide (DMSO). From this point onwards they were kept on ice. Cells were transferred to freezing ampoules and the ampoules wrapped in cotton wool and placed in a box at -20°C. After twenty minutes the box was transferred to -70°C overnight and then the ampoules were stored in liquid nitrogen.

Cells were thawed for replating by placing the frozen ampoules in water at 37°C, adding a large volume of prewarmed growth medium and centrifuging to pellet cells. After aspiration of the supernatant containing the DMSO the cells were resuspended in fresh growth medium and replated.

HeLa, W12 and SiHa cells were gifts from G.Sibbet and S.Cuthill.

2.2.1.2 Culture of Swiss mouse 3T3 feeder cells

These were grown in DMEM as in section containing 10% (v/v) donor calf serum and 0.23% sodium bicarbonate. They were passaged and frozen down as for keratinocytes except that the trypsin solution was o.17% (w/v) trypsin in PBS.

For irradiation cells were trypsinised and resuspended in fresh growth medium. They were subjected to 60Gy of γ-irradiation from a ^{60}Co source in order to prevent cell division.

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

2.2.1.3 Culture of human fibroblasts

Fibroblasts were grown in DMEM as for keratinocytes but containing 15% fetal calf serum. They were passaged as for mouse Swiss 3T3 fibroblasts and frozen down as for keratinocytes.

2.2.1.4 Derivation of BICR (Beatson Institute for Cancer Research) squamous cell carcinoma cell lines

A piece of the growing edge of the tumour was placed in Dulbecco's modified Eagle's growth medium (DMEM) containing 10% fetal calf serum (as described in section 2.2.2.1) immediately after surgical excision and kept on ice until explanted a few hours later. Almost all tumours collected had not been treated previously by chemo- or radiotherapy, and were from the head and neck regions. They were provided by Mr.D.Soutar at Canniesburn Hospital, Bearsden, Glasgow. Tumour staging information was provided by pathologists at the hospital, the presence of malignant SCC cells in each biopsy being confirmed independently by two pathologists. TNM (tumour, node,

metastasis) staging classifies the anatomical extent of disease in a clinical and histopathological manner and thus represents the state of progression of the tumour (UICC, 1987). To some extent it depends on the site but increasing tumour stage represents increasing size and/or local extent of the primary tumour. The node status is a measure of increasing involvement of regional lymph nodes, and metastasis status M_1 represents the presence of distant metastases.

To grow out explants the tumour was cut into pieces approximately $1mm^3$ and placed in 9cm dishes covered with a thin layer of fetal calf serum. The dishes were left in a dry incubator containing 10% CO_2 at 37^oC for forty-five minutes in order to evaporate moisture from the serum and allow the tumour fragments to adhere to the dishes. 5mls of DMEM, 5% fetal calf serum and containing 0.5µg/ml mycoplasma removal agent was then added gently to each dish, followed by a feeder layer of lethally irradiated Swiss mouse 3T3 fibroblasts. Low serum levels were used to select for growth of tumour versus normal cells (Rheinwald and Beckett, 1981). Otherwise these conditions are appropriate for growing normal keratinocytes and thus prevent selection of fitter variants by culture (Rheinwald and Green, 1975b, Rheinwald and Beckett, 1981). The feeder layer also prevents overgrowth of colonies by fibroblasts (Rheinwald and Green, 1975a)

The dishes were medium-changed twice a week and checked for cell outgrowth. When explants reached a size visible to the naked eye they were trypsinised as described in section 2.2.1.1 and plated in three T25 flasks in 2, 5 and 10% serum respectively, again with a feeder layer and mycoplasma removal agent, to see under which conditions they grew best. Media were changed twice a week as before and when colonies reached approximately 1cm in diameter they were trypsinised and half the cells frozen down as stocks, the other half expanded for preparation of genomic DNA.

2.2.1.5 Derivation of fibroblasts from human tumours

At the time of explantation half the dishes of tumour fragments were maintained in DMEM, 10% fetal calf serum with 0.5μg/ml mycoplasma removal agent but without a feeder layer in order to allow normal fibroblast outgrowth. The higher serum level selected for normal cell growth but was not high enough to sustain normal keratinocytes (Rheinwald and Green, 1975b). When fibroblast colonies had grown big enough to see with the naked eye they were trypsinised (section 2.2.1.2) and plated in T25 flasks, still in DMEM, 10% fetal calf serum. After this they were passaged and frozen down as described for the BICR lines.

2.2.1.6 Tumorigenicity assays in nude mice

Cells were grown up to appropriate levels and trypsinised, having removed the feeders, and washed twice with serum-free DMEM. $3x10^7$ cells were pelleted separately as 10^7 cells and each pellet resuspended in 200ml serum-free DMEM before subcutaneous injection into the flank of a four week old nude mouse. A pilot experiment showed that this site is better than the back for SCC tumour formation. SCC 12B (Rheinwald and Beckett, 1981) was used as a positive control and HEK as a negative control. Mice were examined weekly for tumour presence and tumour volumes recorded by the animal house staff. When a progressively growing tumour remained for three months it was scored as positive.

2.2.1.7 Staining of cell lines

a).Staining for keratin

Cells were grown on glass chamber slides and fixed according to Sun & Green (1978).HEKs and fibroblasts were used as a positive controls respectively. Slides were

submerged in 10% formalin for ten minutes followed by 100% methanol for five minutes and 100% acetone for one minute. They were then rinsed in PBS three times. A mixture of mouse keratin monoclonal antibodies AE1 & AE3 (Woodcock-Mitchell *et al*, 1982) were applied at 1:1000 dilution in PBS, and the slides left in a humid box for two hours at room temperature. Slides were then rinsed in NaCl/Tween/PBS (0.05% {v/v} tween 80, 0.15M NaCl in PBS), three times for ten minutes each, and the second antibody (fluorescein-conjugated anti-mouse immunoglobulin) applied at 1:30 dilution in PBS. The slides were incubated at 37^{o}C in the dark in a humid box to visualise the antibody by fluorescence. After rinsing as above with NaCl/PBS/Tween the cells were mounted in non-fade mountant (1g/ml p-phenylenediamine in 10% {v/v} PBS, 90% {v/v} glycerol) and coverslips were applied and sealed with nail varnish. They were stored at -20^{o}C in the dark.

As a further control the procedure was carried out as described using HEKs but omitting the primary mixture of antibodies to show staining was due to the presence of keratins.

b).Staining for involucrin

Cells were again grown on chamber slides with HEKs and fibroblasts as positive and negative controls respectively but were fixed using 50:50 acetone:methanol for ten minutes and allowed to air dry for forty minutes. They were then treated with H_2O_2 diluted 1:10 in PBS for ten minutes and rinsed three times in PBS for ten minutes each. Goat blocking serum from the rabbit peroxidase Vectastain kit at 1:10 in PBS/BSA (0.1% {w/v} bovine serum albumin fraction V in PBS) was added for twenty-five minutes at room temperature, followed by rabbit anti-involucrin antibody (a gift from F.Watt) at 1:5000 in PBS,BSA. This was left overnight at 4^{o}C in a humid box.

The following day the antibody was rinsed away with NaCl/Tween/PBS (as before), three times for ten minutes each and goat anti-rabbit second antibody from the kit applied (1:200 in PBS/BSA), one hour at room temperature. The rinses in NaCl/Tween/PBS were

repeated and then kit ABC reagent applied for one hour at room temperature. The PBS/NaCl/Tween rinses were repeated again and then diaminobenzoic acid (0.6mg/ml in PBS) containing 0.017% (v/v) H_2O_2 was added to each slide for 7.5 minutes at room temperature, in the dark, to visualise the antibody by the peroxidase reaction. Slides were rinsed thoroughly in water, mounted in aquamount and sealed.

An additional control was carried out in that HEKs were also "stained" using normal rabbit serum at the same dilution as the involucrin antibody. This showed that staining was due to the antibody and not the blocking serum.

2.2.2 DNA work

2.2.2.1 Preparation of genomic DNA from cell lines

This was carried out essentially as described in Maniatis *et al*, 1989.

Medium was aspirated from early passage cells growing on 9cm dishes and the cells rinsed with PBS. Cells were lysed on the plates using 5M guanidinium thiocyanate, 50mM Tris-HCl (pH 7.0), 50mM EDTA & 5% (v/v) β-mercaptoethanol, 1ml per plate. N-sodium lauryl sarcosine was added to a concentration of 2% (w/v). Lysates could be stored at -20°C at this point.

DNA was isolated using centrifugation (106,400g, 17°C, 48 hours) through a 5.7M CsCl, 50mM EDTA step gradient (upper layer CsCl, RI=1.3925, lower layer CsCl, RI=1.4025). The DNA collects at the interface between the two CsCl solutions.

DNA was precipitated with three volumes of 70% ethanol and spooled out. It was then washed in 70% ethanol & 100% ethanol, dried and dissolved in TE buffer (10mM tris-HCl, pH 7.5, 1mM EDTA), 0.5% SDS. This solution was made up to 150mM NaCl, 10-50mM EDTA & 100µg/ml proteinase K, and incubated at 37°C for 2 hours.

The resulting solution was extracted twice with an equal volume of phenol/chloroform (1:1, phenol:chloroform) and once with an equal volume of chloroform.

Phases were separated by centrifugation at 3000g at room temperature. DNA was precipitated with three volumes 100% ethanol, dried and resuspended in TE buffer (pH 7.5). Concentrations were determined by measuring absorbance at 260 nm, using the fact that an absorbance of 1 unit is equivalent to a concentration of 50µg/ml.

2.2.2.2 Preparation of lymphocyte DNA from blood samples

a).Erythrocyte lysis method

One to five volumes of erythrocyte lysis buffer (0.83% {w/v} ammonium chloride, 37mg/l EDTA, 1g/l potassium carbonate) were added to whole blood and the mixture kept on ice for ten minutes. Mixture was centrifuged (3000g, 5 minutes) and supernatant discarded. Cells were resuspended in PBS and lysis was repeated until a pellet of clean lymphocytes was obtained. The pellet was finally washed twice in PBS by centrifugation and aspiration of the supernatant as above and could be stored at -20^{o}C.

b).Salting out method for extracting DNA

This was carried out essentially as described by Miller *et al*, 1988. Cells were lysed in 10mM Tris-HCl, 400mM NaCl, 2mM EDTA, pH 8.0, approximately 3mls of lysis buffer for lymphocytes obtained from 10mls whole blood. Cell lysates were digested overnight at 37^{o}C with 0.2mls 10% (w/v) SDS & 0.5mls of protease K solution (1mg protease K in 1% {w/v} SDS, 2mM EDTA). After digestion 1ml of approximately 6M NaCl was added and the tube shaken vigorously for fifteen seconds, followed by centrifugation (3000g, 5 minutes) to pellet the protein. The DNA was precipitated from the supernatant with two volumes of absolute ethanol, spooled out, dried and resuspended in 100-200µl TE, pH 7.5. Concentration was measured as described above.

2.2.2.3 Transformation of bacterial cells with DNA

25ng of plasmid DNA was used to transform 20µl *E.coli* DH5α competant cells. DNA and cells were mixed thoroughly and left on ice for 30 minutes. Cells were then heat-shocked for 45s at 42°C and returned to ice. 80µl of SOC rich growth medium (2% {w/v} bactotryptone, 0.5% {w/v} yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose) were added and the cells incubated with shaking for one hour at 37°C. Cells were spread onto two plates each (10µl & 90µl) containing ampicillin (50µg/ml) and the plates incubated upside-down overnight at 37°C.

2.2.2.4 Preparation of small amounts of plasmid DNA

Minipreparations were carried out using the alkaline lysis method as described by Maniatis *et al*, (1989).

Single colonies of bacteria containing the plasmid of interest were inoculated into 10mls L broth containing 50µg/ml ampicillin and incubated overnight with shaking at 37°C. Bacteria were harvested from 1.5mls overnight culture by microcentrifugation (30s, room temperature). The pellet was resuspended in 100µl solution 1 (50mM glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0). 200µl fresh solution 2 was added (0.2N NaOH, 1% {w/v} SDS) and mixed gently. Tubes were stored on ice for one minute followed by addition of 150µl ice-cold solution 3 (3M potassium, 5M acetate) and vortexing. Tubes were then stored on ice for five minutes before centrifuging for 5 minutes. Finally, DNA was precipitated from the supernatant by mixing with two volumes of ethanol, standing for two minutes at room temperature and microcentrifugation for five minutes. The resulting pellet was rinsed with 1ml 70% ethanol at 4 C and air-dried before dissolving in 50µl TE containing DNAse-free RNAse (20µg/ml). DNA was stored at -20°C.

2.2.2.5 Large-scale isolation of plasmid DNA

This is a scaled-up version of the mini-preparation described in the previous section.

5mls of overnight culture were transfered to 500mls of L broth containing 50µg/ml ampicillin and shaken at 37 C until absorbance at 600nm reached 0.6. Chloramphenicol was then added to 100µg/ml and the cultures grown overnight at 37^{o}C with shaking.

Cells from the 500ml cultures were pelleted at 4225g, 4^{o}C, rinsed in ice-cold 50mM Tris-HCl pH 8.0 and resuspended in 25mls lysis solution 1 (as section 2.2.2.4) with the addition of 0.5g/100ml lysozyme just before use) before leaving thirty minutes at room temperature. 40mls of fresh solution 2 (section 2.2.2.4) was added and mixed well, and the tubes left on ice for fifteen minutes. 20mls acetate (solution 3 as section 2.2.2.4) was then added and the tubes mixed by five inversions and left on ice for another fifteen minutes. The flocculates were then spun at 10,810, 0^{o}C, 5 min. The supernatant was filtered through gauze. 0.6 volumes of isopropanol at -20 C was added to precipitate the DNA and the mixture spun immediately at 10,810g, room temperature, 5 min. After discarding the supernatant the pellet was left to drain for ten minutes and then resuspended in 5ml TE pH 8.0.

Plasmid DNA was then isolated by centrifugation. 7.5g CsCl were added and dissolved followed by 5mg ethidium bromide. The refractory index was adjusted to 1.386-1.39 with TE pH 8.0, and the solution centrifuged at 146,600g, 40 hours, 20^{o}C. The plasmid band was extracted with water-saturated isobutanol until all ethidium bromide had been removed, and one volume of water added. two volumes of ethanol were used to precipitate DNA at room temperature for fifteen minutes followed by centrifugation (11,950g, 4^{o}C, 15 min). The pellet was resuspended in 1ml deionised water and reprecipitated with two volumes of ethanol & 0.1 volumes 3M NaOAc at -20 C for two hours. The pellet was spun down again as above, rinsed with 70% ethanol, freeze-dried and

resuspended in the appropriate volume of TE. DNA concentration was measured using absorbance at 260nm as described in section 2.2.2.1 and the DNA stored at -20 C.

2.2.2.6 Restriction enzyme digestion of DNA

Plasmid DNA was incubated with 5-10 units of enzyme/µg DNA under the conditions specified by the supplier, for one to two hours. Eukaryotic DNA for use in Southerns was digested with five units of enzyme/µg DNA under the conditions specified by the supplier, overnight. Digestion of amplified DNA was carried out with 10-20 units of enzyme/µg DNA, also under the conditions specified by the supplier, overnight.

2.2.2.7 Agarose gel electrophoresis

Flat bed apparatus was used. 0.6-4% agarose (w/v) gels were cast in 1x TAE buffer (40mM Tris base, 16mM acetic acid, 1mM EDTA, pH 8.0) and contained 0.5µg/ml ethidium bromide. Low melting point agarose was used to isolate plasmid inserts for use as probes and NuSieve agarose for checking PCR reactions. Gels were submerged in 1x TAE, 0.5µg/ml ethidium bromide and samples were loaded in the wells in bromophenol blue loading buffer (50% {w/v} glycerol, 0.25% {w/v} bromophenol blue). Gels for Southern blots were run at 30-50V, 20-28 hours, and low melting point (LMP) agarose gels at 80V, 1.5 hours. All other gels were run at 100V, 1/2 -1 hours. The appropriate molecular weight markers were run on each gel as a guide in order to achieve good separation of the experimental DNA. After electrophoresis the DNA was visualised by illumination with short wave (312nm) ultraviolet light and photographed through a red filter using Polaroid type 57 high speed film.

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

3-6µg of plasmid DNA was digested to completion and the insert separated from the plasmid by electrophoresis in low melting point agarose. The insert band was excised after visualising and photographing the DNA, and stored at 4 C in the agarose before radioactive labelling using the random priming method. Concentrations were estimated to be 2-3ng/µl.

2.2.2.9 Generation of random-primed radio-labelled probes

Low melting point agarose containing the DNA of interest was melted at 60 C for ten minutes in order to remove a volume containing approximately 50ng. Deionised water was added to 30µl. DNA was denatured by boiling for seven minutes and labelled in 50µl of a solution of 50mM Tris-HCl pH 7.5, 5mM $MgCl_2$, 0.4% (v/v) β-mercaptoethanol, 70µM each of dATP, dGTP, & dTTP, 200mM Hepes, 6 OD units/ml calf thymus hexanucleotides and 80µg/ml bovine serum albumin. Labelling was with 50µCi (α ^{32}P)-dCTP and 4-5 units Klenow enzyme, for five hours at room temperature.

Unincorporated nucleotides were removed using a NICK column containing Sephadex G-50, DNA grade. The agarose reaction was melted and made up to 100µl with TE before running through the column. The probe was washed into the column with 400µl TE and then eluted with the same. 1µl of the probe solution was counted Cerenkov with a scintillation counter to ensure 10^8 cpm/µg DNA before use. The probe solution was boiled for five minutes to denature the DNA and used immediately

2.2.2.10 Southern blotting

10-20µg DNA was digested as described and separated by agarose gel electrophoresis as described. Blotting used Biotrans^{TM} nylon membrane and was carried out essentially as described by Rigaud *et al*, 1987.

The gel was denatured for thirty minutes in fresh 1.5M NaCl, 0.5M NaOH and then neutralised for thirty minutes in 3M NaOAc pH 5.5. The gel was then placed upside-down on a raised platform covered with a wick of Whatman 3MM paper soaked in transfer buffer (generally 20x SSC: 3M sodium chloride, 0.3M sodium citrate), such that the ends of the filter paper reached into a reservoir of transfer buffer. The gel was surrounded by clingfilm to ensure the buffer moved only through the gel. The Biotrans^{TM} membrane was then placed on the gel and covered with two sheets of 3MM paper, a stack of paper towels and a weight. Transfer occurred overnight, after which the membrane was baked between sheets of 3MM paper for one hour at 80 C. Membranes could be stored dry at room temperature, wrapped in foil.

Where blots were to be used for hybridisation to minisatellite probes the gels were treated slightly differently. Gels were denatured in 1.5M NaCl, 0.5M NaOH twice for twenty minutes each, and neutralised for one hour in 1M NaOAc, 0.02M NaOH. The tranfer set-up was as above except that the transfer buffer was 10x SSC and transfer took place onto nitrocellulose membrane. This membrane was soaked in distilled water followed by 10x SSC prior to placing on the gel. After transfer the nitrocellulose was washed in 6x SSC, five minutes, blotted dry and baked for two hours at 80 C. Filters were stored in sealed bags in the dark.

2.2.2.11 Hybridisation of radioactively labelled probes to Southern blots

a).Single copy probes

The pMUC 7 probe consists of a mucin cDNA insert in the *Eco R1* site of pBS-SK. The sequence is published in and the plasmid was a gift from S.Gendler.

Filters were prehybridised at 42^{o}C in a shaking water bath in sealed plastic bags containing approximately 15mls prehybridisation solution (50% {v/v}

formamide,100µg/ml denatured salmon sperm DNA, 5x SSC, 20mM $NaPO_4$ pH 6.5, 1x Denhardt's {0.02%, [w/v], each of Ficoll type 400, polyvinylpyrrolidone and bovine serum albumin fraction V}, 10% {w/v} dextran sulphate, 0.1% SDS), for at least three hours. Probes were labelled by the random priming method was then added such that there were at least 10^6 cpm/ml hybridisation solution. Filters were hybridised overnight, also at 42°C.

To remove non-specific binding filters were rinsed after hybridisation in 2x SSC, 0.1% SDS and then washed for fifteen minutes at room temperature with shaking in the same. They were then washed once in 0.1x SSC, 0.1% SDS for fifteen minutes at room temperature, and four times for thirty minutes each at 65°C in the same in a shaking water bath. The washes at 65°C were repeated until background radioactivity levels on the filter were low (5-10 cps). Filters were then blotted damp, wrapped in clingfilm and exposed to photographic film (either Kodak X-OMAT AR or X-OMAT S, or Fuji RX) with intensifying screens at -70°C.

b).Minisatellite probes

The minisatellite probes used were 15.1.11.4, the core minisatellite from phage λ 33.15, and 6.3, the core from 33.6 (Jeffries *et al*, 1985). They were cloned into the ampicillin-resistant plasmid T3/T7-18 in an *Eco* R1/*Hind* 111 site and were gifts from K.Brown.

For fingerprinting the DNA was digested with *Hinf1* and run on a 0.6% agarose gel.

In this case prehybridisation took place in three stages, all at 65°C in sealed plastic bags in a shaking water bath. The first step was 1/2 hour in 1x Denhardt's. This was followed by 1/2 hour in a filter mix consisting of 1x Denhardt's, 1x SSC, 0.1% SDS, 20ng/ml single-stranded salmon sperm DNA. Salmon sperm DNA was boiled for five minutes to denature it before it was added. This was finally followed by fifteen minutes in fresh filter mix including 6% (w/v) polyethylene glycol (PEG) 6000.

For hybridisation the radio-labelled probe was added to the filter mix + 6% PEG 6000 such that there were at least 10^6 cpm/ml solution, and the filter left overnight at 65°C in the shaking water bath.

After hybridisation the membrane was rinsed briefly in 1x SSC, 0.1% SDS and washed in the same for ten minutes at room temperature followed by five minutes at 65 C. Washes at 65°C were repeated until cps at the top of the membrane were approximately five. The filter was then blotted damp, wrapped in clingfilm and exposed as for single-copy probes.

2.2.2.12 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 pH 8.0.

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 then detritylated using an Applied Biosystems oligonucleotide purification cartridge as follows.

5ml of acetonitrile was passed through the column to waste at a rate of 1 drop/s using a syringe. This was followed by 5ml of 2M triethylammonium acetate. The oligonucleotide ammonia deprotection solution was then diluted with an equal volume of distilled water and the diluted solution passed through the cartridge in 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 half of 5ml of 2% (v/v) trifluoroacetic acid through the column, letting stand for 5 min and passing the other half through to waste. The cartridge was flushed with 10 ml distilled water. The

oligonucleotide was eluted drop-by-drop with 3ml 20% (v/v) acetonitrile, freeze-dried overnight and dissolved in 250ml TE pH 8.0. Its concentration was determined by measuring absorbance at 260nm and using the standard that a solution of 24µg/ml has an absorbance of one OD unit at this wavelength. Primers were stored at -20^{o}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 purification procedure involved desalting the primers by precipitating them with butan-1-ol, 1ml of butanol/150µl primer solution, and centrifuging (13,000g, 20 min at room temperature). The butanol was removed by centrifuging under vaccuum and the primer redissolved in 150µl TE pH 8.0. Primer concentration was determined as above.

2.2.2.13 DNA amplification by the polymerase chain reaction (PCR)

a).PCR to detect human papilloma viruses types 16 and 18

This was carried out essentially as described by Yeudall & Campo, (1991). As described in the reference the primers led to amplification of a sequence spanning the E6 and E7 genes in each virus. The final reaction volume was 50µl consisting of 0.5µg DNA to be screened, 10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.001% (w/v) gelatin, 200µM each of dATP, dCTP, dTTP, dGTP, 0.5µg of each of the four HPV amplimers and 80nM HPRT amplimers. The reaction was overlaid with 75ml paraffin oil and heated to 94^{o}C for five minutes to inactivate DNAses and ensure all DNA duplexes were melted. While at 94^{o}C 2.5 units of thermostable DNA polymerase from *Thermus aquaticus* (*Taq* polymerase) were added. The reaction was then subjected to 30 cycles of 94^{o}C for one minute , 51^{o}C for one minute to allow annealing of amplimers and 72^{o}C for one minute to allow extension of amplimer sequences. This was followed by a seven minute extension at 72^{o}C and cooling to 4^{o}C. Thermocycling was acheived with a Perkin-Elmer Cetus type 480 programmable heating block.

The HGPRT amplification in the same tube acted as an internal control to show that the DNA was of sufficient quality in cases where no other amplification was observed. A pilot experiment showed that the HGPRT primers were compatible with those for HPV 16 and 18 (data not shown but see figure 13, lanes for W12 and SiHa). Amplification of bacteriophage λ DNA with primers from the Perkin-Elmer Cetus DNA PCR kit was carried out at the same time under the same conditions for each reaction as an additional control for all the components of the reaction apart from DNA and primers. Positive controls for HPV sequences were HeLa DNA, containing HPV 18, W12 DNA, containing HPV 16, and SiHa DNA, also containing HPV 16.

PCR products were analysed by polyacrylamide gel electrophoresis (see section 2.2.2.15).

b).PCR of regions of DNA containing restriction fragment length polymorphisms (RFLPs)

PCR was carried out basically as described in the previous section, with slightly varying conditions according to the original references for the primers. The RFLPs used were one within the AT III gene (Dale and Perry, 1990) and the *Xba 1* polymorphism in the RB-1 gene (McGee *et al*, 1990). PCR products were analysed by agarose gel electrophoresis as described in section 2.2.2.7.

c).PCR of DNA containing microsatellite sequences

All PCR of microsatellite sequences was carried out the same way and not according to the original reference for the primer pair in question, although the primer sequences were those described in the references (table 2). Total reaction volumes were 25µl containing 200ng DNA, 10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.001% (w/v) gelatin, 10% (v/v) DMSO, 350ng of each amplimer, 200µM each dNTP and 1µl of

Table 2.

List of polymorphic microsatellite markers used in this thesis

D1S243	1p36.3	Weissenbach *et al*, 1992
APO A2	1q21-23	Weber and May, 1989
D1S104	1q21-23	Weber *et al*, 1990
pMUC 7	1q21	1988
D1S242	1q25	Weissenbach *et al*, 1992
D1S212	1q25	Weissenbach *et al*, 1992
D1S103	1q32	Weber *et al*, 1990
D1S229	1q42	Weissenbach *et al*, 1992
D1S245	1q42	Weissenbach *et al*, 1992
D1S102	1q42-43	Weber *et al*, 1990
D4S174	4p15-p11	Weber *et al*, 1990
GABRB1	4p13-12	Dean *et al*, 1991
D4S175	4q21-qter	Personal communication by J.L.Weber to the Human Genome Mapping Project, 1990
D6S87	6q22.3-23.1	Weber *et al*, 1990
IGF SR	6q25-27	Goto *et al*, 1992

List of microsatellite markers. continued

D9S54	9p23	Personal communication by J.L.Weber to the Human Genome Mapping Project, 1991
D9S199	9p23	Graw and Kwiatkowski, 1993
D9S168	9p23-22	Weissenbach *et al*, 1992
D9S43	9p21	Weber and May, 1990
D9S165	9p21	Weissenbach *et al*, 1992
D9S50	9p21	Wilkie *et al*, 1992
D9S55	9p12	Sharma *et al*, 1991
GSN	9q33	Kwiatkowski and Perman, 1991
ABL	9q34.1	Kwiatkowski, 1991
D17S520	17p12	Personal communication by J.L.Weber to the Human Genome Mapping Project, 1991

Chromosome 1 marker map positions were taken from the Human Genome Mapping Project and Engelstein *et al*, 1993. Chromosomes 4 and 6 marker positions were taken from the Human Genome Mapping Project, and chromosome 9 marker postions from Kwiatkowski *et al*, 1993.

$\alpha(^{32}P)$dCTP diluted 1 in 30 in deionised water. This solution was heated to 94°C for five minutes then cooled to 85°C at which point 2.5 units of *Taq* polymerase was added. Reactions were then subjected to 6 cycles of 94°C for 30s and 60°C for 30s followed by 28 cycles of 94°C, 30s, 55°C, 30s, 72°C, 30s. After this was completed the reactions underwent further extension of seven minutes at 72°C and were cooled to 4°C. Thermocyclers were Perkin-Elmer Cetus type 9600. In some cases conditions were varied slightly to give a better yield or more specific DNA amplification.

In all cases cell line and normal DNA were amplified at the same time in an experiment for comparison.

Prior to polyacrylamide gel electrophoresis the success of the reaction and the size of the products were checked by electrophoresis on a NuSieve agarose gel along with molecular weight markers in a similar size range (section 2.2.2.7).

2.2.2.14 Purification of amplified DNA for restriction enzyme digestion

DNA containing an RFLP amplified by PCR was purified to remove excess primers and nucleotides and recovered in a small volume of distilled water in order that it could be digested and separated by electrophoresis. A Geneclean 2^{R} kit was used according to the manufacturer's instuctions.

Three volumes of 6M NaI was added to the contents of the PCR reaction tube followed by 5µl glassmilk^{R}, a suspension of silica matrix in water which binds single and double-stranded DNA without binding contaminants. The mixture was vortexed and left on ice for five minutes to allow binding. The glassmilk was pelleted by brief centrifugation and washed by mixing with 300µl NEW wash (a Tris & EDTA-buffered solution of NaCl, ethanol and distilled water), pelleting the glassmilk and removing the supernatant. This was repeated three times. Finally half of the desired final volume of distilled water was added to the clean pellet and the pellet heated to 55 C for two minutes. The glassmilk was again

pelleted and the supernatant containing the DNA removed. This was repeated to elute the rest of the DNA.

2.2.2.15 Polyacrylamide gel electrophoresis

Polyacrylamide gels were used to separate radiolabelled amplified DNAs differing in size by approximately 4-20 base pairs. 5-8% (w/v) polyacrylamide gels were cast in 1x TBE (90mM Tris base, 90mM boric acid, 2mM EDTA) buffer and polymerised using 420ml 10% (w/v) ammonium persulphate and 42ml TEMED (N,N,N',N'-tetramethylethylenediamine). The ratio of polacrylamide to bisacrylamide was 29.1:0.9. Immediately after reagents were mixed the gel was poured between glass plates separated by 0.4mm spacers and a well-former was inserted. When the gel had polymerised it was placed on a vertical apparatus with each end submerged in a reservoir of 1x TBE.

DNA samples were loaded in a 30% (v/v) glycerol, 0.25% (w/v) bromophenol blue and 0.25% (w/v) xylene cyanol loading buffer. Gels were of the appropriate percentage such that the PCR products ran halfway between the bromophenol blue and xylene cyanol in order to achieve maximum separation over the length of the gel and so the position of the products on the gel were known. They were run at 25 watts for the appropriate length of time (4-6 hours) until the bromophenol blue reached the end of the gel, removed from the apparatus, backed with Whatman 3MM paper, covered in clingfilm and exposed to Kodak X-OMAT AR or X-OMAT S film with intensifying screens at -70 C.

This type of electrophoresis was also used to analyse the results of screening for human papilloma viruses by PCR (section 2.2.2.13a), with slight modifications. Amplified regions differed in size by 60 base pairs so a smaller gel was used and run at 50mA, 1.5 hours. PCR samples were not radioactive so the DNA was visualised by soaking the gel in a solution of 0.5µg/ml ethidium bromide for five minutes, illuminating with short wave ultraviolet light and photographing as has been previously described.

2.2.2.16 Scoring of loss of heterozygosity

LOH was scored by observing the number of allele bands in the cell line DNA as compared with normal constitutive DNA (lymphocyte or fibroblast). Where two bands were clearly visible on the autoradiograph in the normal DNA a cell line was marked as informative. If one of these bands was clearly consistently missing in the cell line DNA over the course of at least two to three reactions the cell line was said to have lost heterozygosity. If in any one of these reactions the cell line showed two bands it was not marked as LOH and was not marked as informative in the results. When cell line DNA was clearly heterozygous it was marked as such and the reaction was not repeated. Normal DNAs and cell lines showing only one allele band were marked as homozygous. If normal DNA samples were not either clearly heterozygous or homozygous they were not included in the results.

Chapter 3

Results

3.1 Derivation of BICR cell lines

Seven immortal cell lines were established (table 3) and one culture which enters crisis (BICR 7). None of the immortal lines went through a period of slow growth or crisis so the immortal cells isolated were present in the original tumour and had not arisen in culture. Figure 8 shows phase contrast micrographs of a range of cultures as grown on a 3T3 feeder layer. The erythroplakia looks very similar to the HEKs in that the cell edges are slightly blurred due to stratification. Cultures from later stage tumours grow in monolayers and have sharp edges.

Ease of isolation of immortal cells increased with the clinical stage of progression of the original tumour (figure 9). Only one cell line was isolated out of three stage T2 tumours which explanted successfully i.e. viable cells grew out. However 4/5 stage T4 tumours and 2/2 metastases which successfully explanted gave rise to cell lines. Four premalignant oral lesions, erythroplakias, (BICR E1, E2, E4, E5) cultured by E. K. Parkinson also did not give rise to continuous lines when grown under the variety of conditions described in section 2.2.1.4. Thus it appears that within the limitations of tissue culture the immortal phenotype arises late in the transition from a premalignant to a malignant SCC.

Immortality is an extremely common phenotype in late-stage, recurrent and metastatic head and neck SCC but is not a prerequisite for continuous tumour growth since BICR 7 enters crisis.

Cell lines, BICR 31, 56, 63, 66 and 68, were subsequently derived by E.K Parkinson in the group using the same techniques and have been included along with the erythroplakia cultures in the experimental work described in the following sections.

Culture	Site	Previous treatment	TNM staging[a]	Proliferative fate
Early stage primary tumours				
BICR 1	tongue	none	$T_2N_0M_0$	senescent
BICR 2	tongue	none	$T_1N_0M_0$	senescent
BICR 3	alveolus	none	$T_2N_{2b}M_0$	immortal
Late stage primary/ recurrent tumours				
BICR 6	hypopharynx	none	$T_4N_1M_0$	immortal
BICR 7	tongue	none	$T_4N_{2b}M_0$	crisis
BICR 10[b]	buccal mucosa	surgery & DXT	$T_4N_0M_0$[c]	immortal
BICR 16[b]	tongue	surgery & DXT	$T_2N_0M_0$[c]	immortal
BICR 19	epidermis	none	d	immortal
Lymph node metastases				
BICR 18	larynx	none	$T_4N_1M_0$	immortal
BICR 22	tongue	none	$T_4N_3M_0$	immortal

a: TNM= tumour, node, metastasis staging
b: Recurrent tumour
c: Staging at recurrence
d: Large and aggressive epidermal SCC. TNM staging not applicable

Table 3. Origin, previous treatment, clinical stage and proliferative fate in culture for those tumours which explanted successfully (tumour adhered to flask and keratinocytes grew)

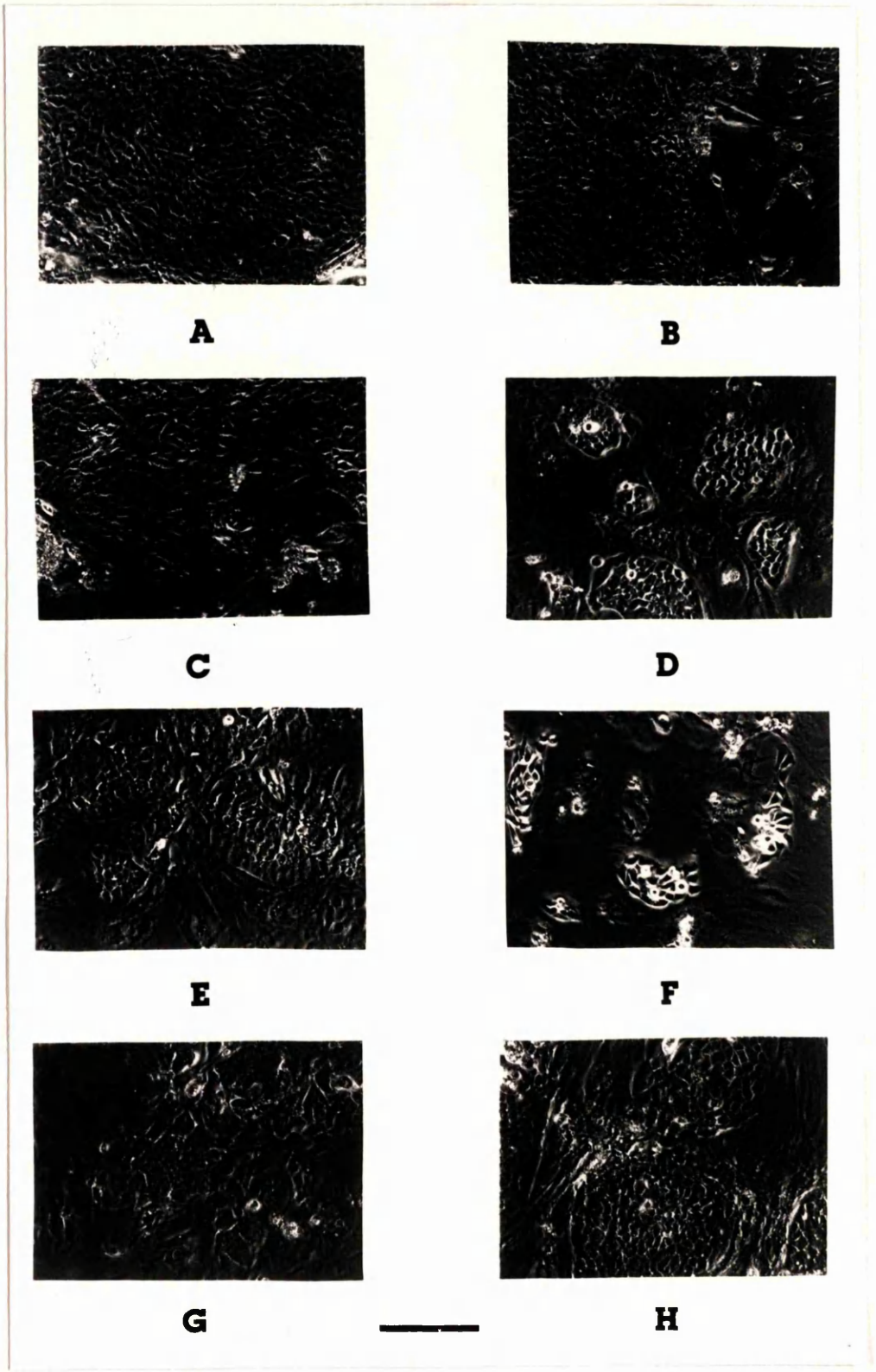


Figure 8. Phase contrast micrographs of BICR cultures grown on 3T3 feeder layers. A= normal human epidermal keratinocytes, B= premalignant erythroplakia BICR E4, C= premalignant erythroplakia BICR E5, D= BICR 3 (T2), E= BICR 6 (T4), F= BICR 19 (epidermal), G= BICR 18 (lymph node metastasis), H= BICR 22 (lymph node metastasis). Bar= 500 μm.

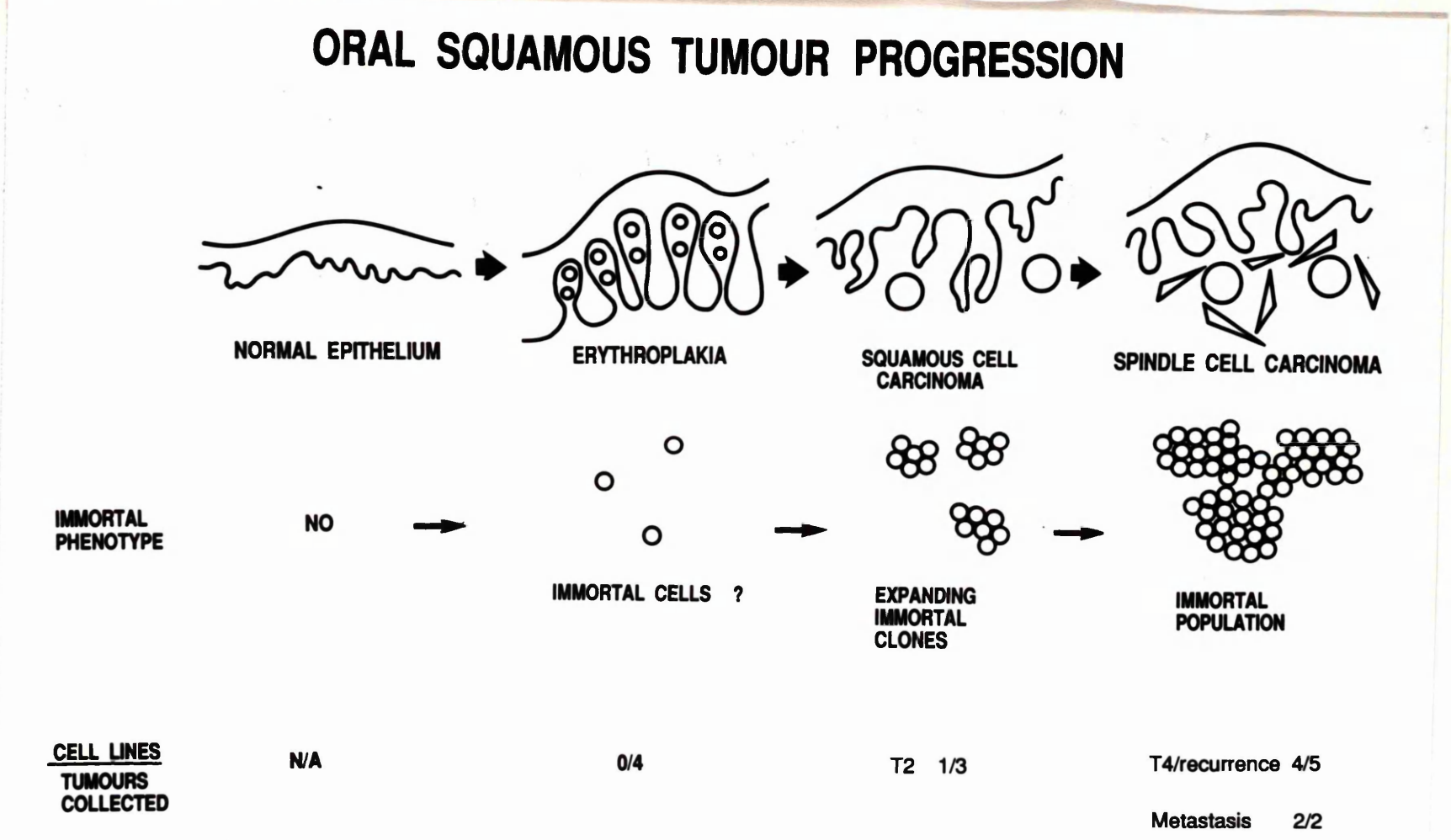


Figure 9. Diagram showing numbers of immortal lines obtained from tumours collected at each stage of progression. T2= early clinical stage, T4= late clinical stage, N/A= not applicable.

3.1.1 Immunocytochemistry

All cultures reacted with monoclonal antibodies recognising stratified epithelial keratins (figure 10 A,C,E). HEKs act as a positive and fibroblasts as a negative control. Staining is cytoplasmic and the nucleus appears largely unstained. Varying levels of keratins are expressed indicating the varying extent of differentiation of the different lines.

In addition the cell lines express the cornified envelope precursor protein involucrin, characteristic of stratified epithelium, although at much lower levels than their normal counterparts (figure 10 B,D,F). Involucrin is also a cytoplasmic marker. As with keratin different cell lines express different levels according to their differentiation status. Positive cells are at the centres of colonies, consistent with the differentiation pattern of keratinocytes in culture (Rheinwald and Green, 1975b)

3.1.2 Electron microscopy

Figure 11 shows electron micrographs of the BICR lines. This work was done in collaboration with Dr.L.Coggins and Mrs.M.O'Prey.

As indicated in the figure, the ultrastructure of BICR E5 resembles that of human epidermal keratinocytes with the exception of reduced numbers of strata in the colonies. All cultures did stratify to an extent though and contained tonofilaments and desmosomes, also characteristic of squamous epithelium. The free cell surface of BICR E5 has a slightly thickened plasmalemma indicating cornification.

Taken together, the ultrastructural and immunocytochemical data support the conclusion that the cell cultures originated in squamous epithelium.

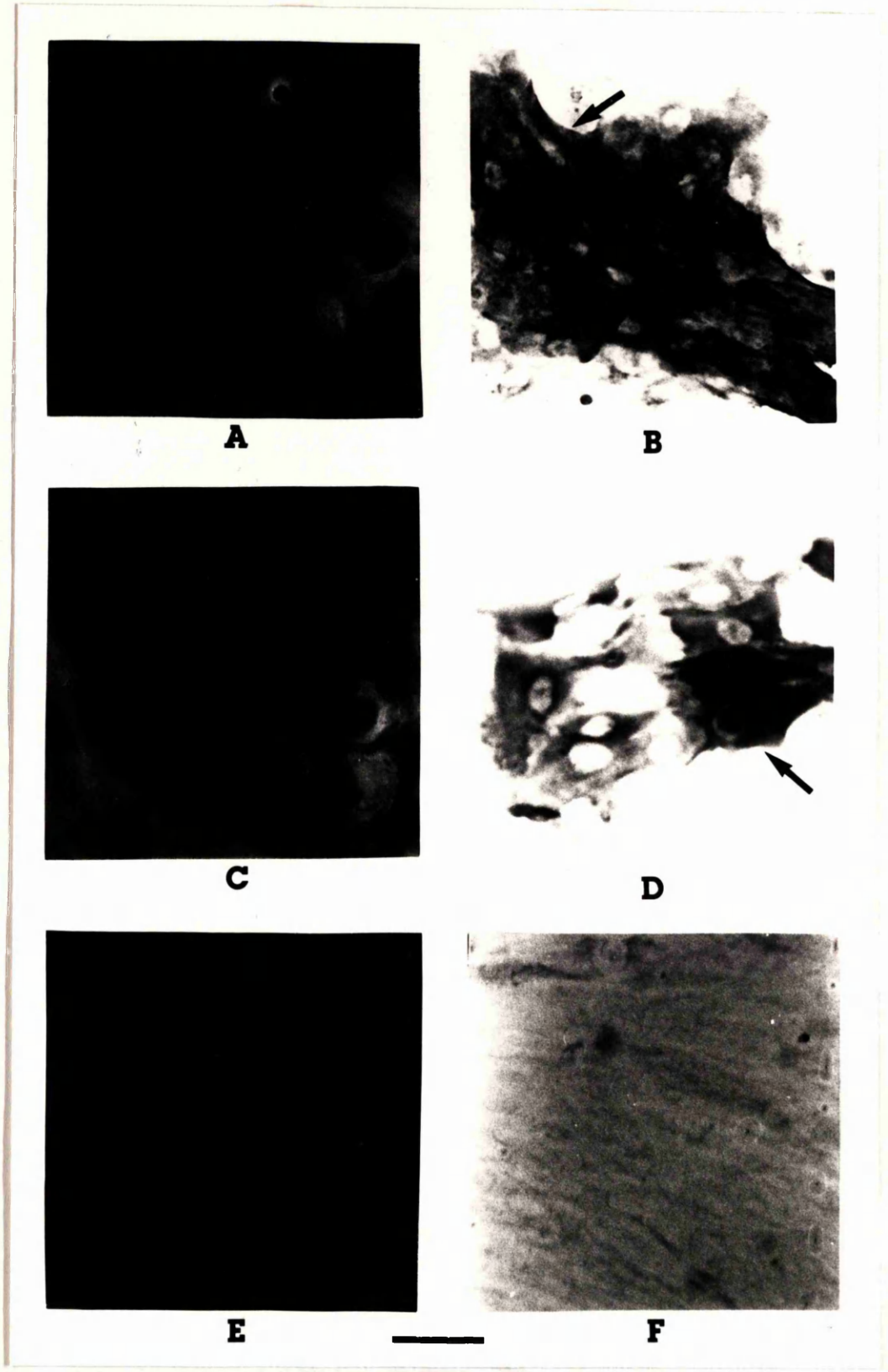


Figure 10. Detection of keratin and involucrin in premalignant erythroplakia keratinocytes. A, C, E: immunofluorescence detection of keratin. A= normal human epidermal keratinocytes (positive control), C = BICR E4, E= normal human fibroblasts (negative control). B, D, F: immunoperoxidase detection of involucrin. B= normal HEK (positive control), D= BICR E5, F= normal human fibroblasts (negative control). Arrows point to strong involucrin staining at the centres of colonies.

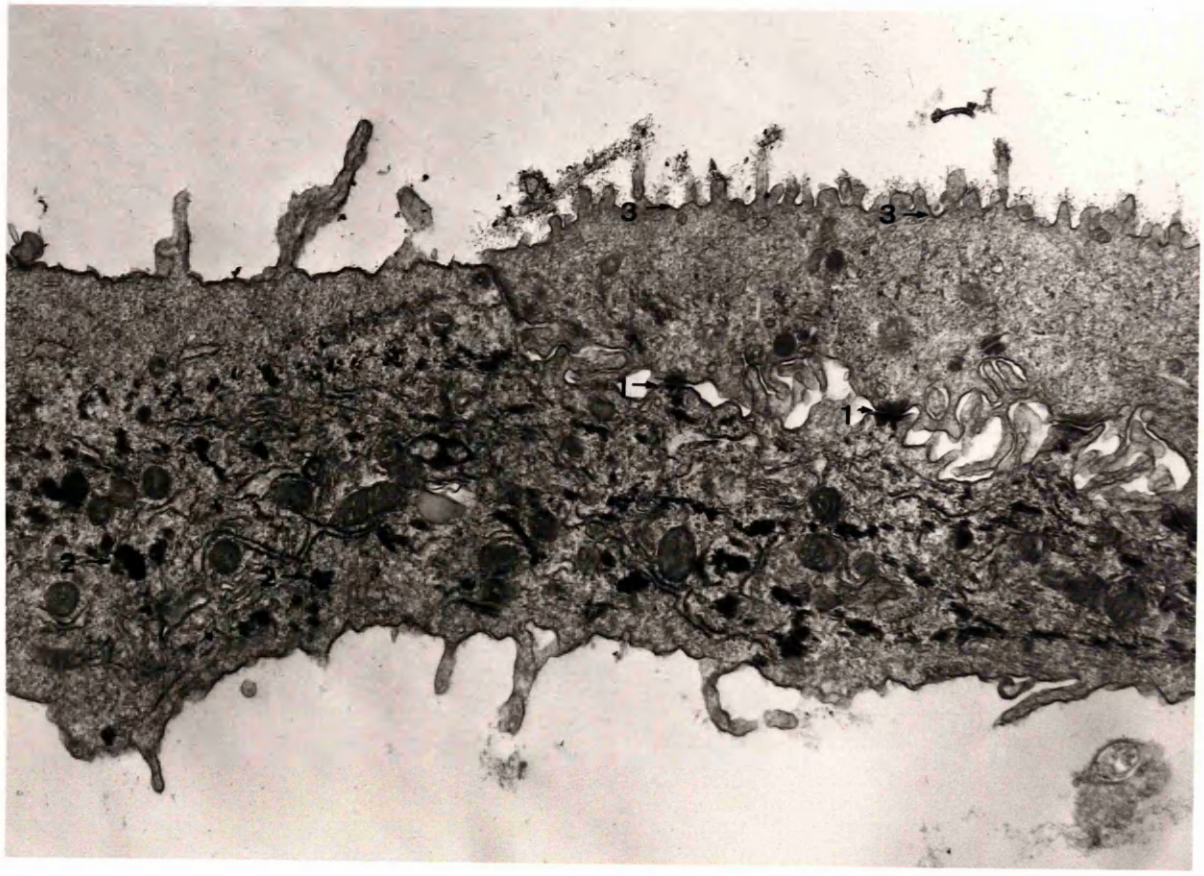


Figure 11. Electron micrograph (magnification 5600X) showing ultrastructural features of premalignant erythroplakia BICR E5. Two stratifying cells are shown. Arrows (1) indicate desmosomes, (2) indicate tonofilaments and (3) cornification.

3.1.3 Tumorigenicity in nude mice of BICR cell lines

This work was done in collaboration with E.K.Parkinson. The data are shown in table 4.

The BICR cell lines do not consistently form tumours in nude mice unless they were derived from tumours of stage T4 or later.

Keratinocytes from early stage carcinomas are either non-tumorigenic (BICR 66) or form slow-growing tumours which eventually regress (BICR 3 & BICR 63). BICR 68, a late-stage tumour, also formed tumours which regressed. However all the other T4, recurrent and metastatic lines produced large, progressively growing tumours which reached a volume of $1cm^3$ within 71-102 days. Normal HEKs, BICR E4 & BICR E5 gave rise to squamous cysts which disappeared within 2 weeks, but there were no tumours after 216 days.

3.1.4 DNA fingerprinting

The BICR lines were to be used for studies on inactivation of candidate tumour suppressor genes in the tumours as compared with normal situation. It was crucial then to show that the normal DNA obtained from each patient was matched correctly to the cell line. DNA fingerprinting is useful for this type of analysis as described in Jeffreys *et al*, 1985. Results are shown in figure 12 for all the cultures where the method was applicable for cell line and lymphocyte and/or fibroblast DNA.

Firstly it is clear that the patterns of the different patients are unique, showing that the cell lines are separate and unique and have not been confused or combined. Secondly there are some bands which appear to be lost in the cell lines as compared with the lymphocytes and fibroblasts. This has been noted before (Matsamura & Tarin, 1992) and could result from large chromosomal deletions or rearrangements in the unstable tumour cells. There are also some increases in band intensities which could be explained by

Keratinocyte culture	Latent period in days (to form a tumour of 1 cm^3)	Latent period in days (to form a tumour of 0.125 cm^3)	Number of mice with tumours/ Number of mice alive after 90 days
Normal keratinocytes			
HEK34	NT 216	NT 216	0/6
SCC (T2)			
BICR 3	NT 163	NT 163	1[b]/5
BICR 63	NT 108	34	0[c]/3
BICR 66	NT 90	NT 90	0/1
SCC (T4 and recurrent)			
BICR 6	101	N.D.	5/6
BICR 10	98	16	3/3
BICR 16	84	16	3/3
BICR 19	78[a]	13	4/4
BICR 31	71	20	2/2
BICR 56	77[a]	35	2/2
BICR 68	NT 108	34	0[c]/5
SCC (metastases)			
BICR 18	90	N.D.	2/4
BICR 22	102	80	2/4

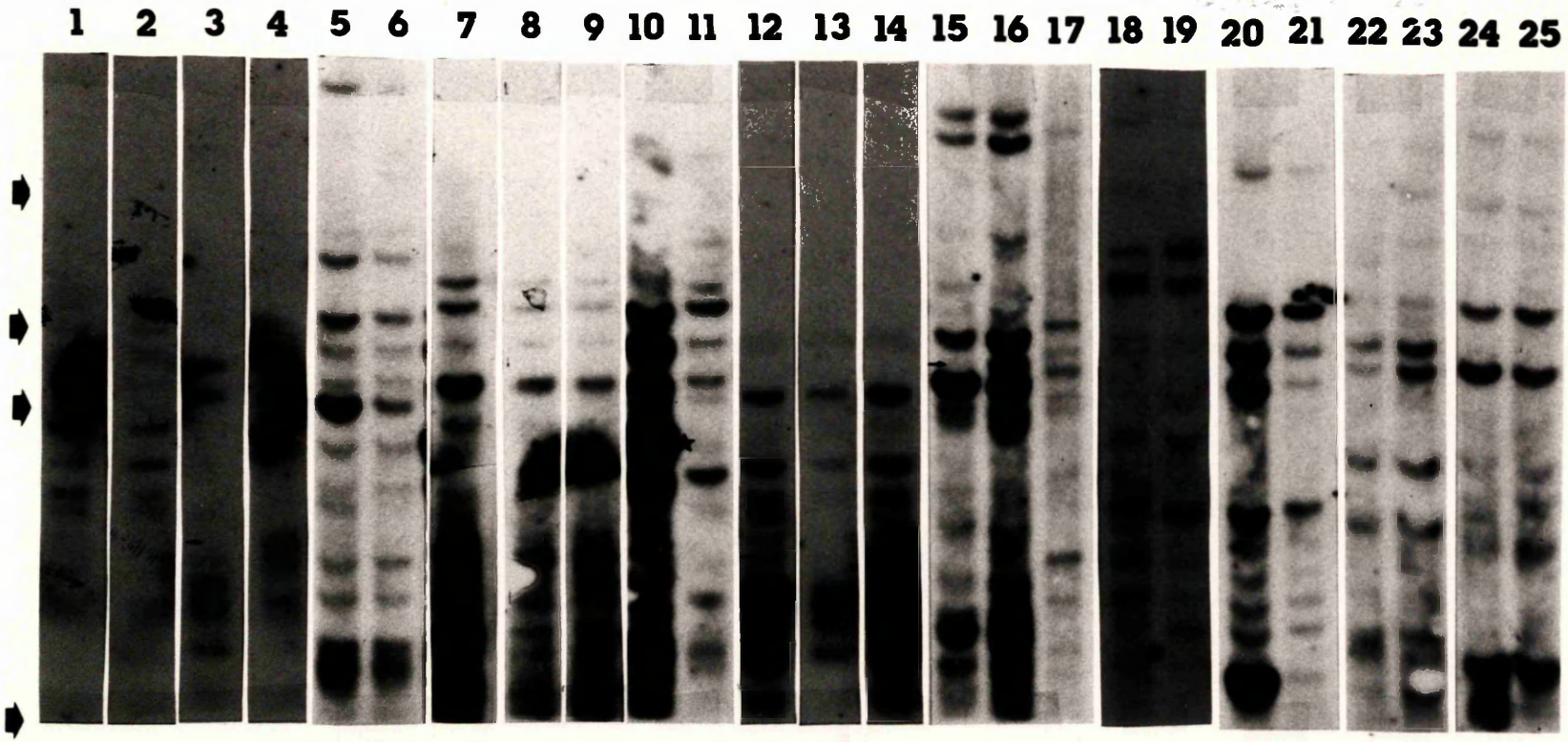
a Mice died bearing tumours>0.125 cm^3 but <1 cm^3
b Maximum size of tumour 0.064 cm^3, but it regressed after 134 days
c Maximum size of tumours 0.27 cm^3, but they regressed after 70 days

Table 4. Tumorigenicity in nude mice of BICR cultures. NT= no tumours at the number of days stated.

Figure 12. DNA fingerprinting of BICR cultures and their patient-matched lymphocytes and/or fibroblasts.

Lane 1, BICR E4; 2, BICR E4 lymphocytes; 3, BICR E5; 4, BICR E5 lymphocytes; 5, BICR 3; 6, BICR 3 fibroblasts; 7, BICR 6; 8, BICR 6 fibroblasts; 9, BICR 6 lymphocytes; 10, BICR 18; 11, BICR 18 lymphocytes; 12, BICR 22; 13, BICR 22 fibroblasts; 14, BICR 22 lymphocytes; 15, BICR 31; 16, BICR 31 fibroblasts; 17, BICR 31 lymphocytes; 18, BICR 56; 19, BICR 56 fibroblasts; 20, BICR 63; 21, BICR 63 fibroblasts; 22, BICR 66; 23, BICR 66 fibroblasts; 24, BICR 68; 25, BICR 68 fibroblasts.

l (*Hind* III-digested) markers are depicted by the large arrows and from top to bottom are 23.1kb, 9.4kb, 6.6kb, and 4.4kb. Small arrows indicate bands present in normal DNA samples but absent in tumour DNA. The star indicates a band present in the tumour but not in the normal DNA.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

chromosome duplication since these cell lines are aneuploid (Edington *et al*, submitted for publication). Finally there are rare bands appearing in the cell lines but not in the normal DNA. An increase in the size of a band might occur in regions of DNA amplification, and smaller bands would result again from deletions and rearrangements.

It was not possible to fingerprint the erythroplakias BICR E1 & E2 because the DNA from these was obtained from cultures of limited lifespan so was not available in sufficient quantities. Similarly there was not enough lymphocyte DNA from BICR 10 and 16. However polymorphism analysis acts as a fingerprint when several markers are used. In the cases of E1, E2 and BICR 10 the allele sizes were consistent between normal and cell culture DNA so these cultures were thought to be suitable for analysis.

In all cases except E4 and E5 fingerprinting was repeated, generally both with probe 6.3 and with 15.1.11.4. For BICR 19 the fingerprint was repeated twice with the same probe. In an effort to conserve the limited DNA available for E4 and E5 repetition was not thought to be necessary given the high quality of the first fingerprint (see figure 11).

3.1.5 The BICR cell lines do not contain human papillomaviruses types 16 and 18.

HPV DNA of various types has been detected in normal oral mucosa, benign proliferative lesions and esophageal SCC, with HPV 16 being the most common (Adler-Stolz *et al*, 1986, Maitland *et al*, 1989, Yeudall and Campo, 1991, Benamouzig *et al*, 1992). Additionally HPVs 18 and 31 have been infrequently detected in head and neck SCCs (Bradford *et al*, 1991b). High risk HPV types, most commonly HPV 16, 18, 31 and 33, are present in 80% of cervical cancers (reviewed in Howley, 1991) and facilitate immortalisation of keratinocytes (Kaur and McDougall, 1988, Woodworth *et al*, 1988, Schlegel *et al*, 1988). We therefore screened the BICR cell lines for the presence of the most common and most immortalising HPVs, that is HPV 16 and 18.

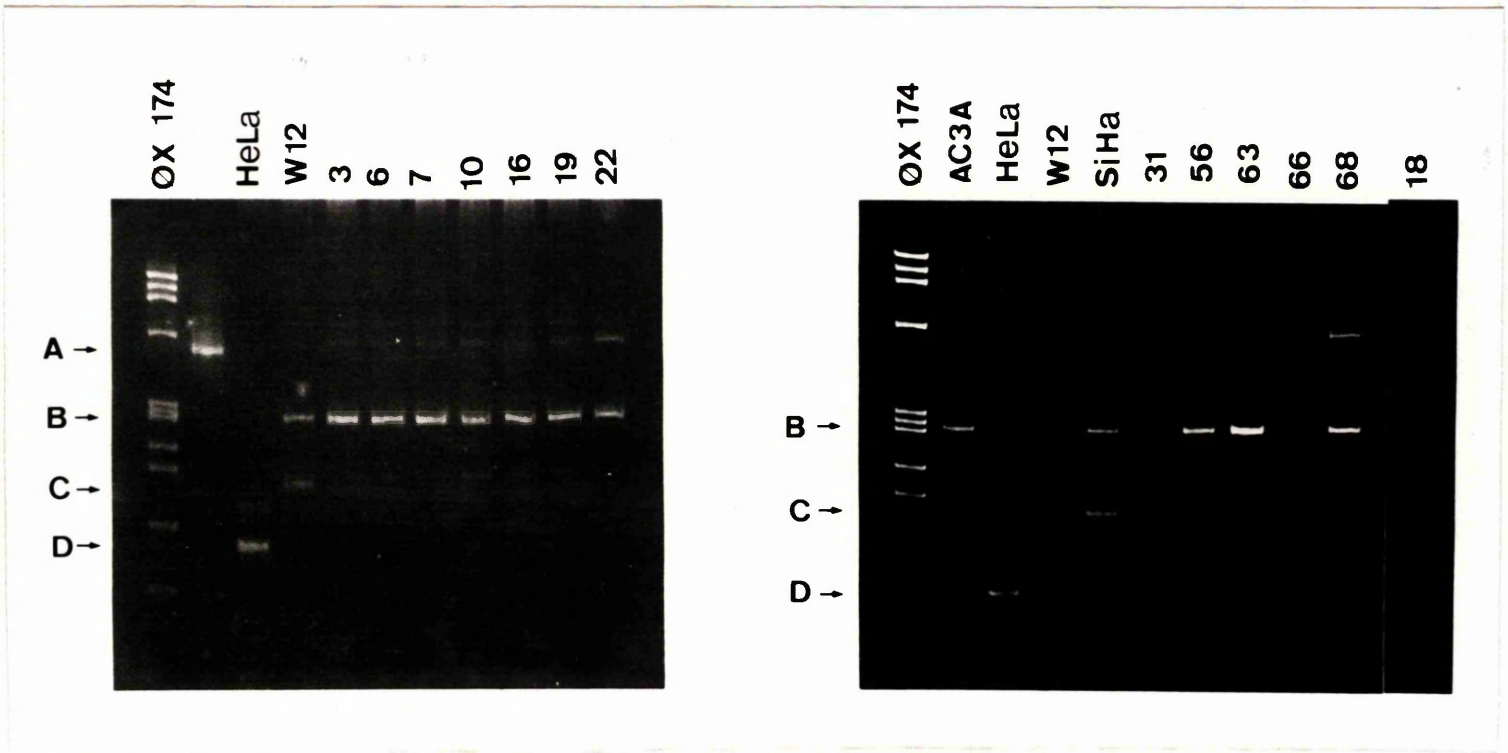


Figure 13. Screening for human papillomaviruses (HPV) types 16 and 18 in the BICR cultures stated. Photographs of DNA amplified by the polymerase chain reaction and separated by polyacrylamide gel electrophoresis. A= amplified λ DNA (kit control), B= product of amplification of the HPGRT gene (internal control), C= band representing HPV 16 DNA, D= band representing HPV 18 DNA. Markers are ϕx 174 (*Hae* III-digested) DNA and HeLa, W12 and SiHa DNA samples are positive controls for HPV 18, HPV 16 and HPV 16 respectively. AC3A is a separate SCC cell line.

Results are shown in figure 13. The band representing the hypoxanthine guanosine phosphoribosyl transferase (HGPRT) gene controls for the success of the PCR reaction (these primers being added to the same tube as the HPV amplification reaction) since in all cases other than HeLa, W12 & SiHa there are no other DNAs which have amplified. Faint band which may be visible were not of the predicted product size and in any case were not reproducible. Screening of all lines was repeated several times. Therefore within the sensitivity limits of the reaction HPV 16 & 18 are not present in the cell lines.

SiHa cells contain one or two copies of HPV 16 per cell (El Awady *et al*, 1987) which was detected easily in the assay used. In a sensitivity experiment where HeLa DNA was diluted with 5BR DNA (known not to contain any HPVs: McKeran *et al*, 1974) a band representing HPV 18 was clearly visible on the gel at a 1/10 dilution (data not shown). Since HeLa cells contain approximately 50 copies of HPV 18 per cell the dilution is equivalent to 5 copies per cell (Schwarz *et al*, 1985). The cell lines tested therefore have less than one copy of HPV 16 and under five copies of HPV 18 per cell. Thus HPV 16 and 18 E6 and E7 are not continually required for the immortal phenotype in these cell lines.

A "hit and run" action is not ruled out by this result. Papillomavirus DNA may be lost from cells over time, especially if it is episomal and the cells are placed in tissue culture (DiLorenzo *et al*, 1992). The DNA used in the screening assay was extracted from very early passage cells (approximately pass 3) so it is unlikely that HPV 16 or 18 DNA was present in the original tumours. It could have had an effect early in the tumours' development.

HGPRT reproducibly does not amplify in HeLa DNA. It is not clear why this should be so. One possibility is that high levels of HPV 18 DNA compete heavily for dNTPs during the PCR.

3.2 Genetic analysis of the BICR cultures

3.2.1 The karyotype of BICR 6

G-band karyotyping was carried out by Mrs.M.Fitchett of the department of Medical Genetics, Churchill Hospital, Oxford. A representative karyotype is shown in figure 14. Cells were hyperdiploid with chromosome numbers ranging from 56 to 70. Consistent abnormalities were noted including a deletion of the short arm of one chromosome 8 (8p-), extra material of unidentified origin on the short arms of chromosomes 8, 9 and 21 (8p+, 9p+ and 21p+ respectively) and on the long and short arm of chromosome 14 (14p+, q+), a Robertsonian translocation {t(14q14q)}, an isochromosome of the long arm of chromosome 5 {i(5q)}, and a translocation between the short arm of chromosome 11 and the long arm of chromosome 13 with breakpoints at p15 and q14. This latter event may be affecting the RB-1 gene. There were several other marker chromosomes (12mar).

3.2.2 Loss of heterozygosity at the *p53* locus

Figure 15 shows the state of the alleles at the D17S520 locus at 17p12, close to *p53* at 17p13. This work was done to complement immunostaining and PCR-direct sequencing work on the *p53* status of the BICR cell lines by other group members. The LOH studies show that BICR 3, 18 & 31 have reduction to homozygosity of D17S520, and BICR 56, 66, 68, and E4 & E5 retain heterozygosity. These results agree with previous work (Burns *et al*, 1993) in that lines 3 and 31 have *p53* mutations as shown by PCR and direct sequencing (see table 5). BICR 3 has a base change in codon 282, causing an arginine to proline amino acid change. Normal sequence was not detected by Burns *et al*. BICR 31 has a three base pair deletion in codons 173-174, causing the amino acid sequence valine, arginine to become glycine. Again no normal sequence was detected. As yet no mutations have been

Representative karyotype:

66, X, +1, -3, -6, +7, +8, +10, -11, +12, -15, +16, +17, +20, -22, +i(5q), +8p-, +8p+, +9p+, +der(11)t(11;13)(p15;q14), t(14q14q), 21p+, +12mar.

Figure 14. Representative karyotype of BICR 6.

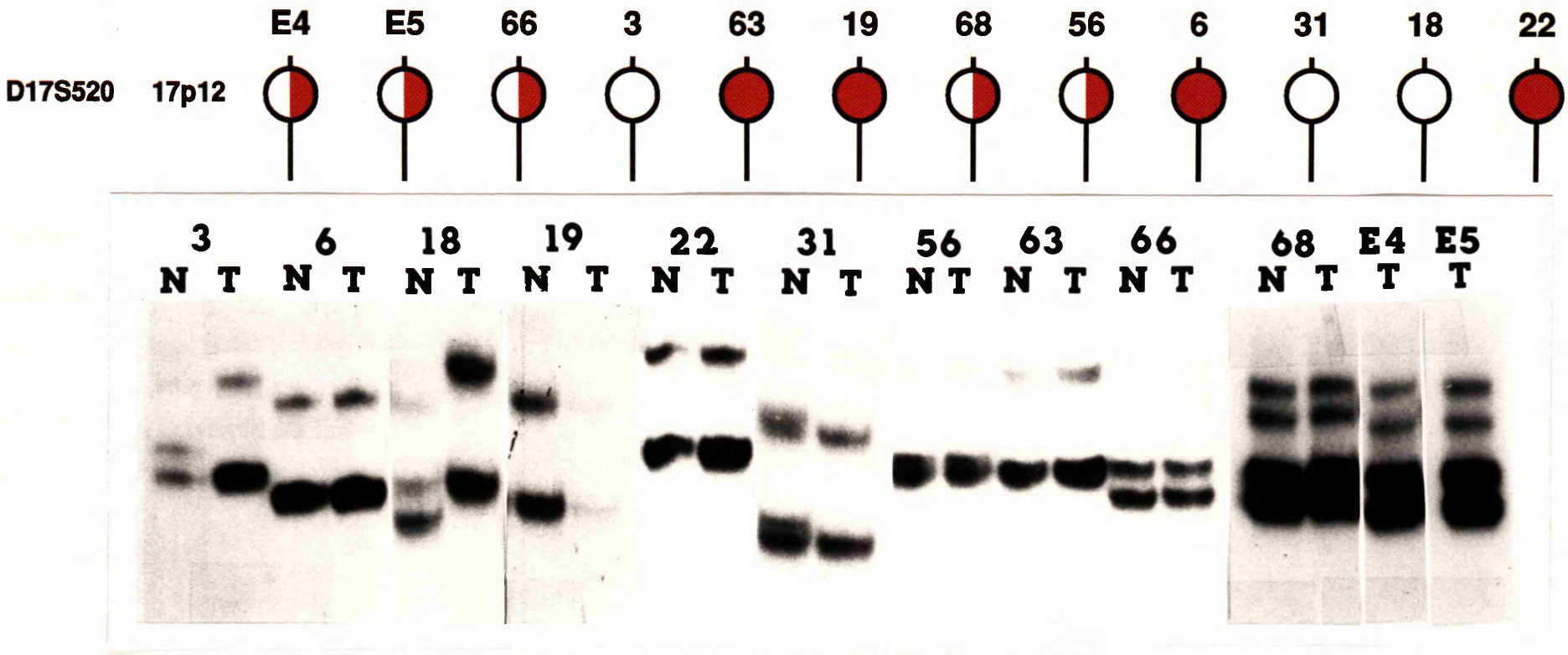


Figure15 Loss of heterozygosity at D17S520 in the BICR cultures stated.

Culture	E1	E2	E4	E5	66	3	63	19	68	56	6	31	10	18	22
LOH data			het	het	het	LOH	n/i	n/i	het	het	n/i	LOH		LOH	n/i
p53 staining	+/-	+/-	+/-	+/-	+/-	+	+	-	+	+	-	+	-	-	-
***p53* mutation**	-	-	-	-	nd	+	nd	+	nd	+	+	+	nd	nd	+
Presence of normal sequence	n/a	n/a	n/a	n/a	nd	-	nd	+	nd	-	-	-	nd	nd	-

Table 5. Comparison of *p53* loss of heterozygosity (LOH) data with immunocytochemistry and sequencing data in the BICR cultures. Sequencing and staining data are taken from Burns *et al*, 1993. Het= heterozygous, n/i= non-informative, LOH= loss of heterozygosity, +/-= normal staining (occasional positive cells), nd= not done, n/a= not applicable.

found in BICR 18; the hotspot regions of codons 2-96, 171-335, and 342-393 were sequenced but there could be a mutation outside these regions.

There is a mutation in BICR 56 and the normal allele sequence was not detected by Burns *et al.* Retention of heterozygosity at D17S520 could be explained by the distance between this marker and the *p53* locus. The tumour from which BICR 56 was derived shows LOH using a microsatellite marker within the *p53* gene (K.Ahsee, personal communication). This marker was not used in the BICR lines because it did not give satisfactorily clear results when tested in these cell lines.

BICR E4 and E5 have normal *p53* sequence, E4 being heterozygous due to one allele having a proline-proline polymorphism at the RNA level (Burns and Clark *et al*, submitted for publication). *p53* has not yet been sequenced in BICR 66 & 68, but immunocytochemistry work seems to show that BICR 66 does not have elevated levels of p53 protein.

BICR 68 does have increased levels (Berry and Parkinson, unpublished data). Elevated p53 protein levels are a sign of a dominant negative or a stabilising type of mutation, therefore BICR 68 may well have a dominant negative mutation in one copy and retain heterozygosity. Alternatively D17S520 may again be too far from the gene to reflect any LOH.

3.2.3 The status of the RB-1 locus

It was important to investigate the RB-1 locus in the BICR lines because inactivation of the RB tumour suppressor may be one step in the immortalisation process (see section 1.3.3.1.B). pRb-1 staining in the BICR lines shows normal nuclear localisation (E.K.Parkinson, personal communication). Previously no alteration in RB mRNA size had been found by Northern analysis in other SCC lines (J.Burns, unpublished data), and no change in protein size or phoshorylation status of pRb has been found by Western analysis in the BICR lines (A.Malliri, personal communication). To date mutations in RB-1 have

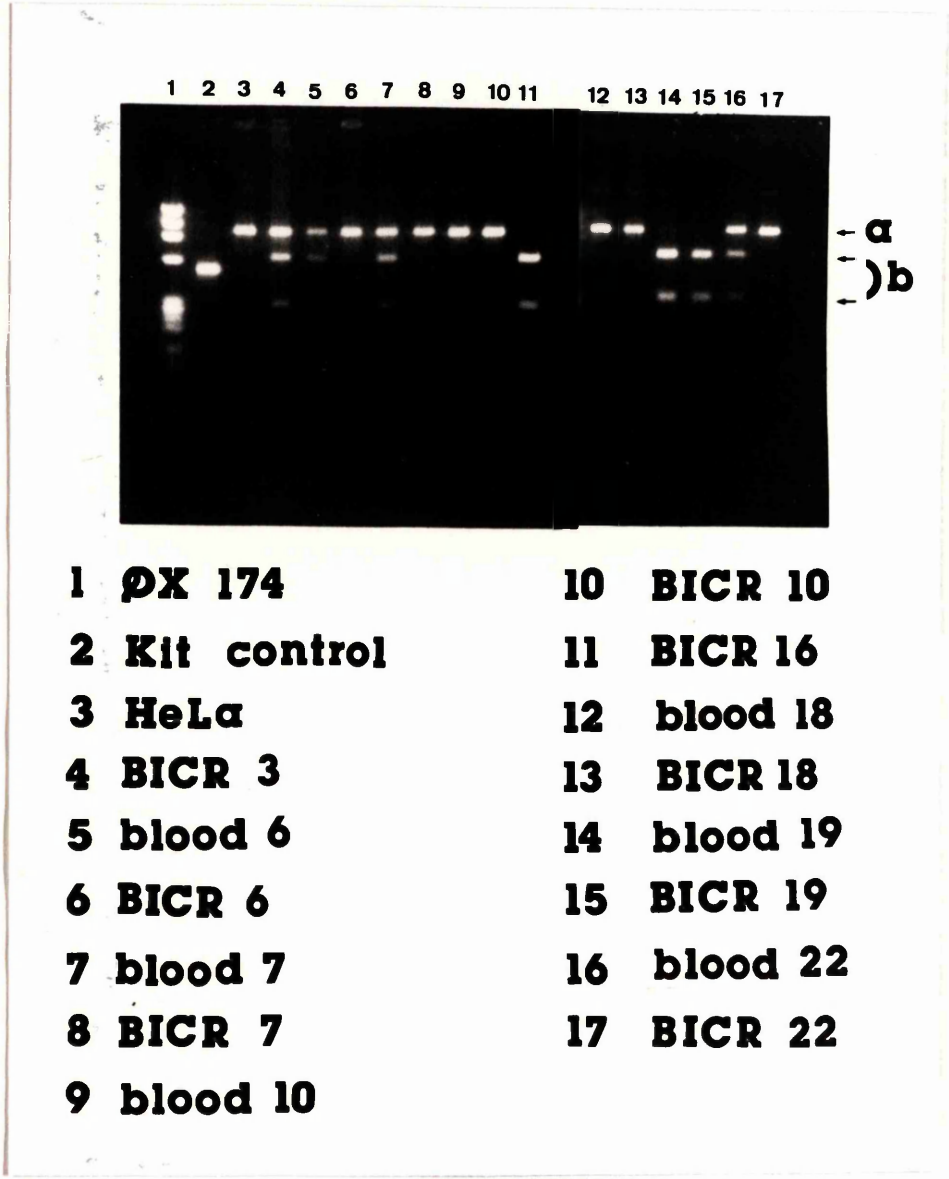


Figure 16. Digested PCR products of the *Xba* I restriction fragment length polymorphism at RB in the BICR cultures stated and their matched normal DNAs. a= allele without restriction site, b= allele containing restriction site. Informative cultures show three bands in the normal DNA samples. There is loss of heterozygosity in BICR 6, BICR 7, and BICR 22. The kit control was λ DNA and ϕx 174 (*Hae* III-digested) DNA were the markers.

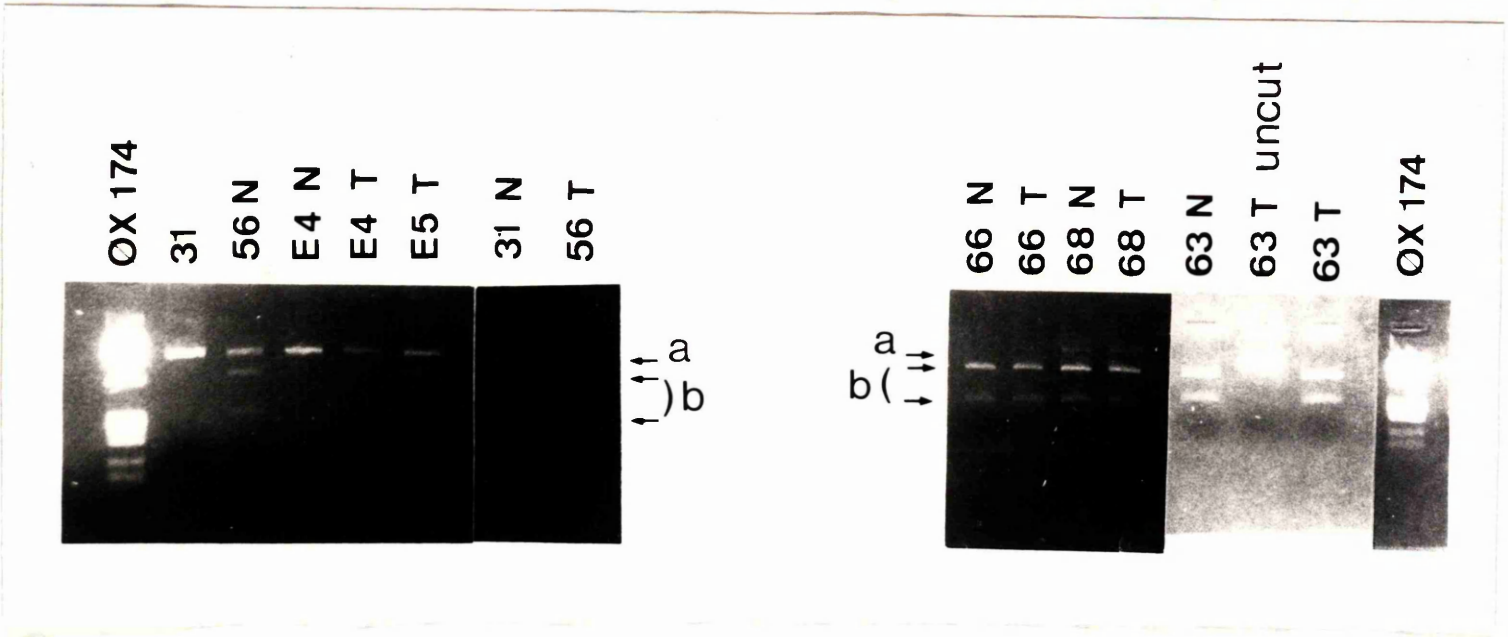


Figure 17. Digested PCR products of the *Xba* I restiction fragment length polymorphism at RB in the BICR cultures stated and their matched normal DNAs. N=normal DNA sample, T= tumour cell line DNA sample. a= allele without restriction site, b= allele with restiction site. Markers were ϕx174 (*Hae* III-digested) DNA. All cultures shown were either heterozygous or non-informative. BICR 68 has partially digested in this case but in other digestions was non-informative.

usually affected either protein size or phosphorylation status (Horowitz *et al*, 1990, Templeton *et al*, 1991), but in these SCC lines it appears neither of these are altered. However as can be seen in figures 16 & 17, amplification of an *Xba 1* restriction fragment length polymorphism within the seventeenth intron of the RB-1 gene itself shows there is loss of heterozygosity in three (BICR 6,7 and 22) of six informative cases. This reaction was repeated three times for all cell lines.

Apart from the idea that there may be a novel kind of RB mutation in SCC which is not detectable by Northern or Western analysis, one other explanation could be that there is another tumour suppressor gene close to the RB locus, and this other gene is the target for the loss of material. Hawthorn *et al*, 1993, mapped deletions in B-cell leukaemia at 13q14 close to the RB locus and found that in 1/5 cases the RB gene was still present but in all cases the locus named D13S25 (just distal to RB at 13q14.3) was deleted. Part of the locus can be amplified by PCR so we can tell if at least one allele is present. From figure 18, BICR 22 retains the amplified region of D13S25 so, at least in this case, there is a deletion involving RB and possibly some material proximal to RB. Any tumour suppressor gene at D13S25 which is inactivated by deletion in B-cell leukaemia is retained in BICR 22 although the possibility remains that it contains a point mutation or is truncated in some way not affecting the amplified fragment. This and one other possible explanation involving cyclin D1 will be fully discussed in section 4.2.3.

It is interesting that in all three cases of loss of heterozygosity the allele with the restriction site is the allele which is lost. In all cases digests were repeated using at least 20 units of enzyme activity per μg amplified, purified DNA. This is four times the recommended amount of enzyme so it is unlikely that incomplete digestion could have been the cause of this result.

One further possibility is that the RB-1 locus becomes methylated. This idea stems from the fact that *Xba 1* does not cleave DNA when the 3' adenosine residue or the cytosine residue of its recognition site are N^6-methyladenine or 5-methylcytosine respectively (Well and McClelland, 1989). Thus loss of the allele containing the restriction site in the cell line

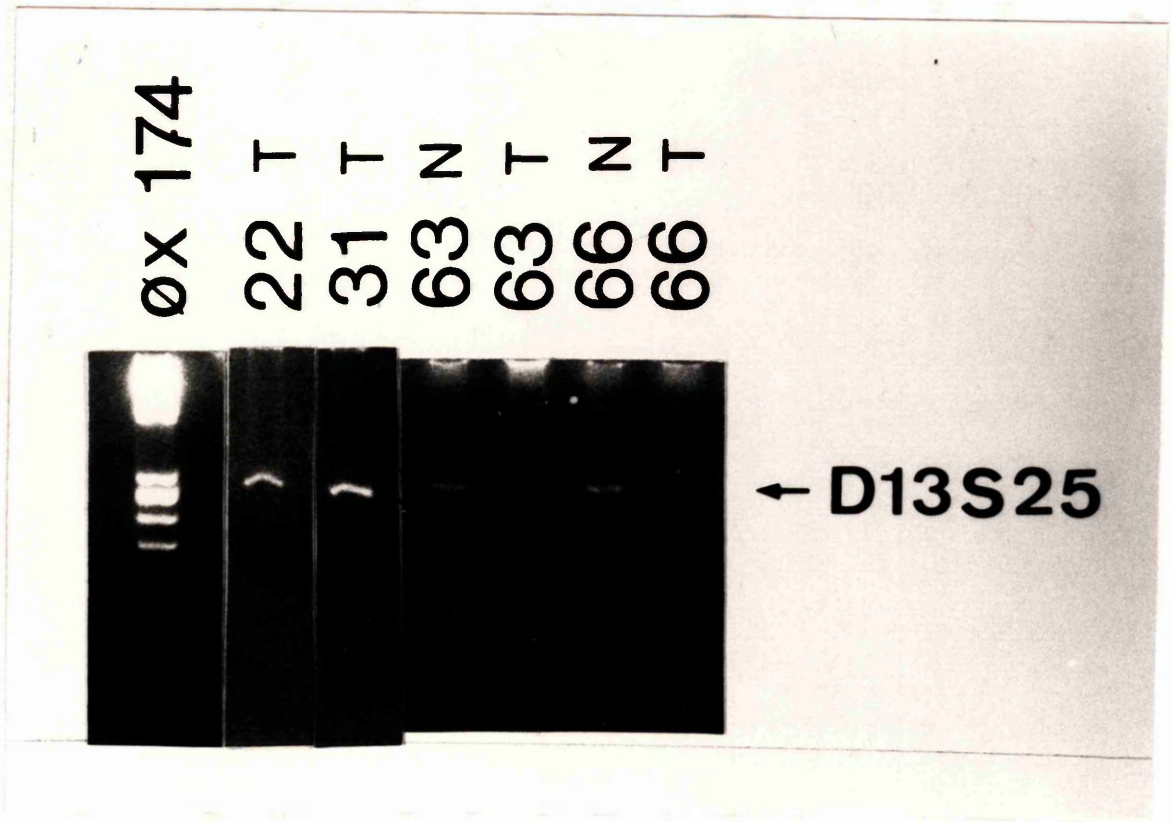


Figure 18. PCR of the region D13S25 at 13q14, just distal to RB-1, in the BICR cultures stated. T= tumour cell line DNA samples, N= matched normal DNA samples. Markers are ϕx174 (*Hae* III-digested) DNA.

could simply represent methylation of the site. This could be distinguished from LOH by treating BICR 6, 7 or 22 cells in culture with 5-azacytidine to demethylate the DNA and repeating the reaction. However it is likely that DNA amplified by PCR is not methylated anyway.

3.2.4 Chromosome 1q

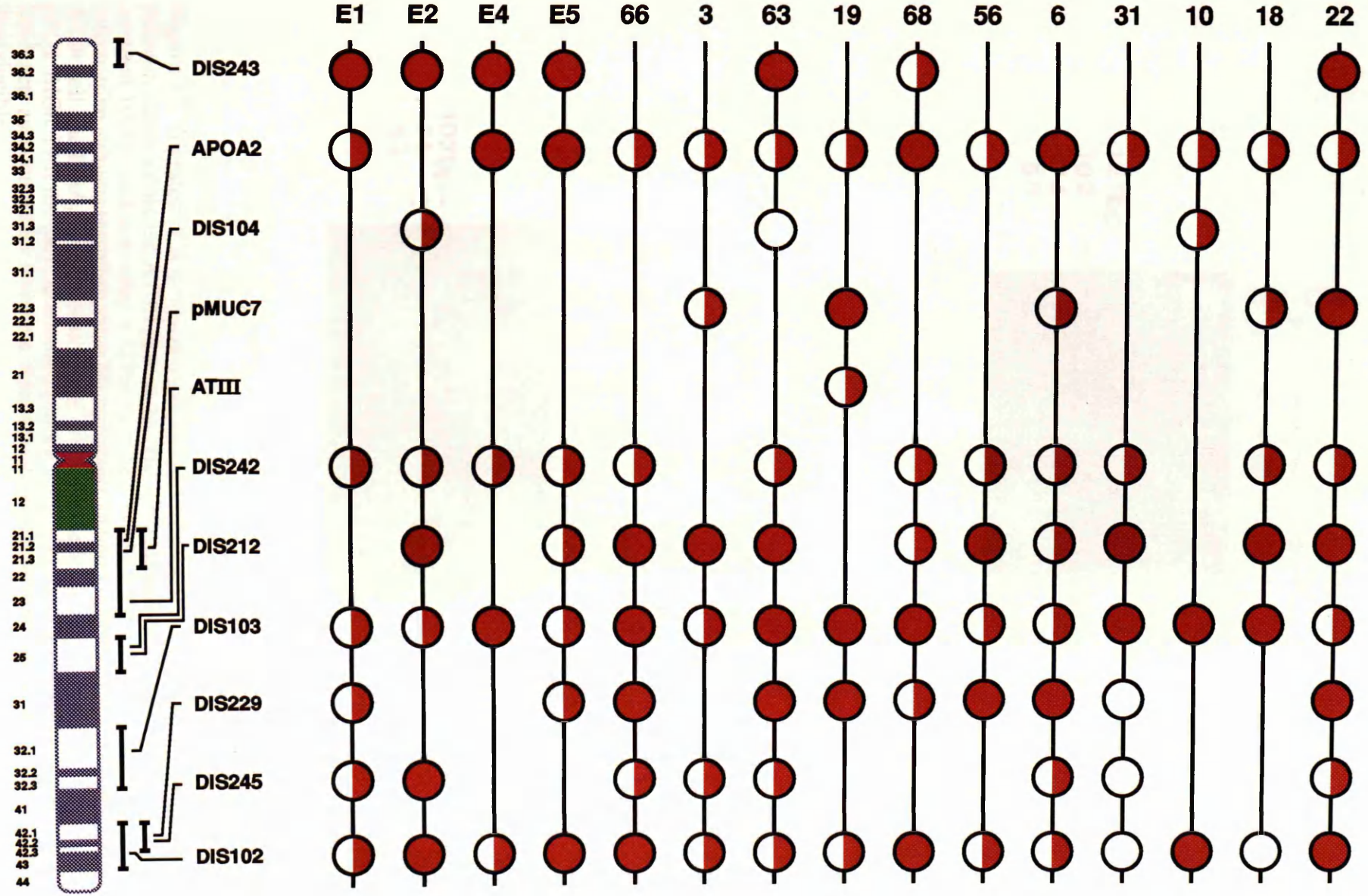
Polymorphism analysis of chromosome 1 concentrated on the long arm for reasons discussed in section 1.3.3.2.A. A summary of data obtained is shown in figure 19 and results are illustrated in figures 20-22, 33 and 34.

Loss of heterozygosity was not common on chromosome 1q. Only three of fifteen cultures tested had loss of material, and this was at two different loci (1q21-23 and 1q42-43) so is less significant. This is confirmed in view of the comparatively high frequencies of LOH at 9p and 9q (section 3.2.7). 0/15 informative cases at 1q25 and 2/11 at 1q42-43 suggest these regions are not major contributors to the immortal phenotype. Either D1S102 has shown LOH in two BICR lines by chance or the losses here contribute in some cases to SCC development but are not the most common targets, since these losses are appearing against a background of heterozygosity.

A microsatellite on 1p (D1S243) was used as a control for loss of whole chromosomes but was not very informative and in view of the low frequency of LOH on 1q was not necessary.

The "shadow" bands appearing at constantly larger increments are common in PCR of microsatellites (for example, see figure 21, marker D1S245). It is unclear what they represent or how they may be eliminated, but one hypothesis is that where there is a vast excess of primers the shadows are amplifiifed, partially denatured product with extra primer bound at the denatured molecule ends (D.Black, personal communication)

Figure 19. Summary of loss of heterozygosity data for chromosome 1 for the BICR cultures stated. Cultures are arranged in order of increasing clinical stage of the original tumours. Markers are ordered according to their map positions on the chromosome.
● = non-informative, ◑ = heterozygous, ○ = loss of heterozygosity.


E1
E2
E4
E5
66
3
63
19
68
56
6
31
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ATIII
DIS242
DIS212
DIS103
DIS229
DIS245
DIS102

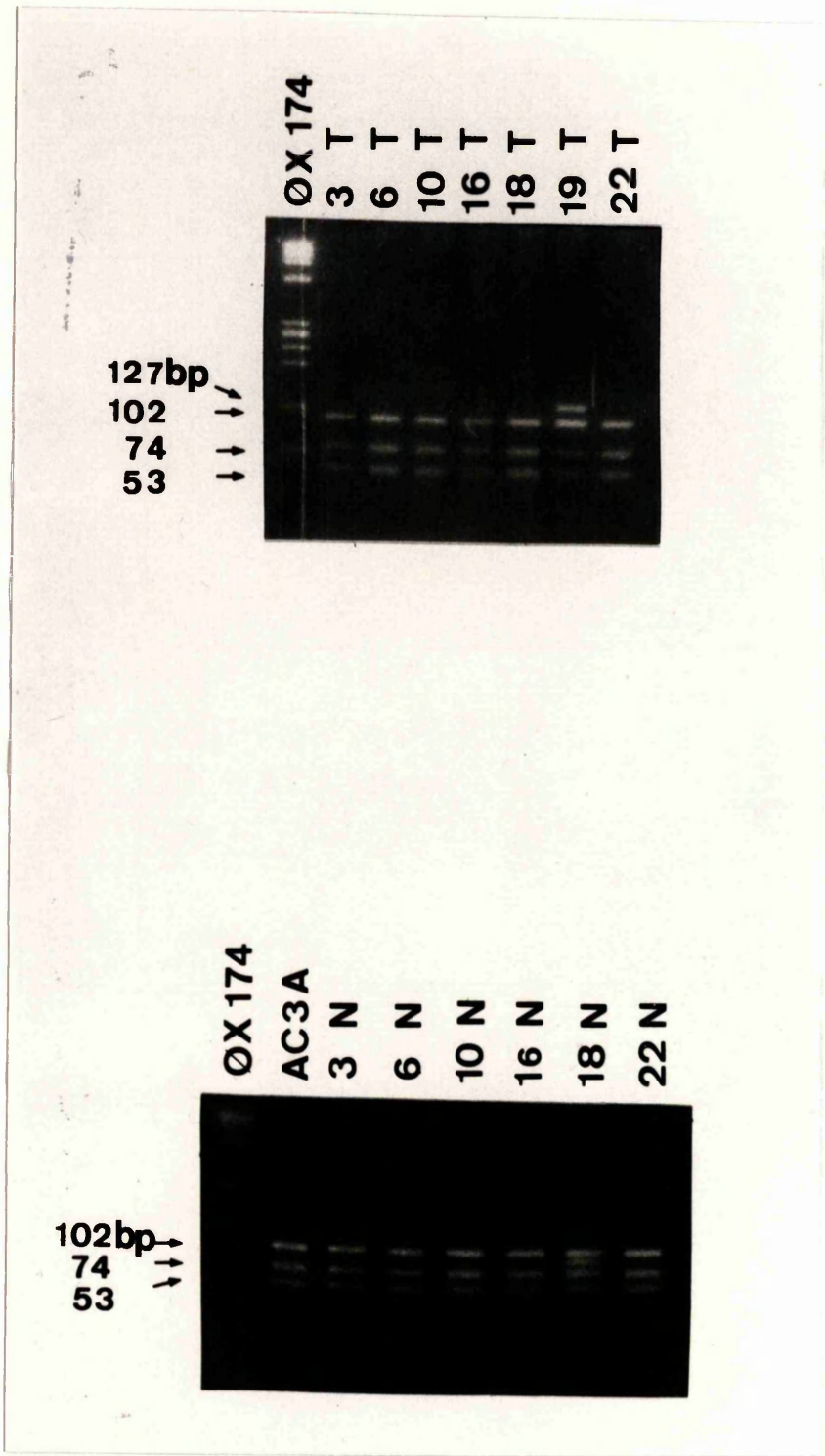


Figure 20. Digested PCR products of the *Dde* I restriction fragment length polymorphism within the AT III gene at 1q. The 222bp initial product is digested to a constant 102bp band and either a 127bp band or a 74 and 53bp pair of bands. Informative cultures therefore show four products and non-informative show three.T= BICR cell line DNA, N= matched normal DNA.Markers are ϕx174 (*Hae* III-digested) DNA. BICR 19 is the only informative culture and has not lost heterozygosity. AC3A DNA acts as a control for complete digestion. += spurious band which was not reproducible.

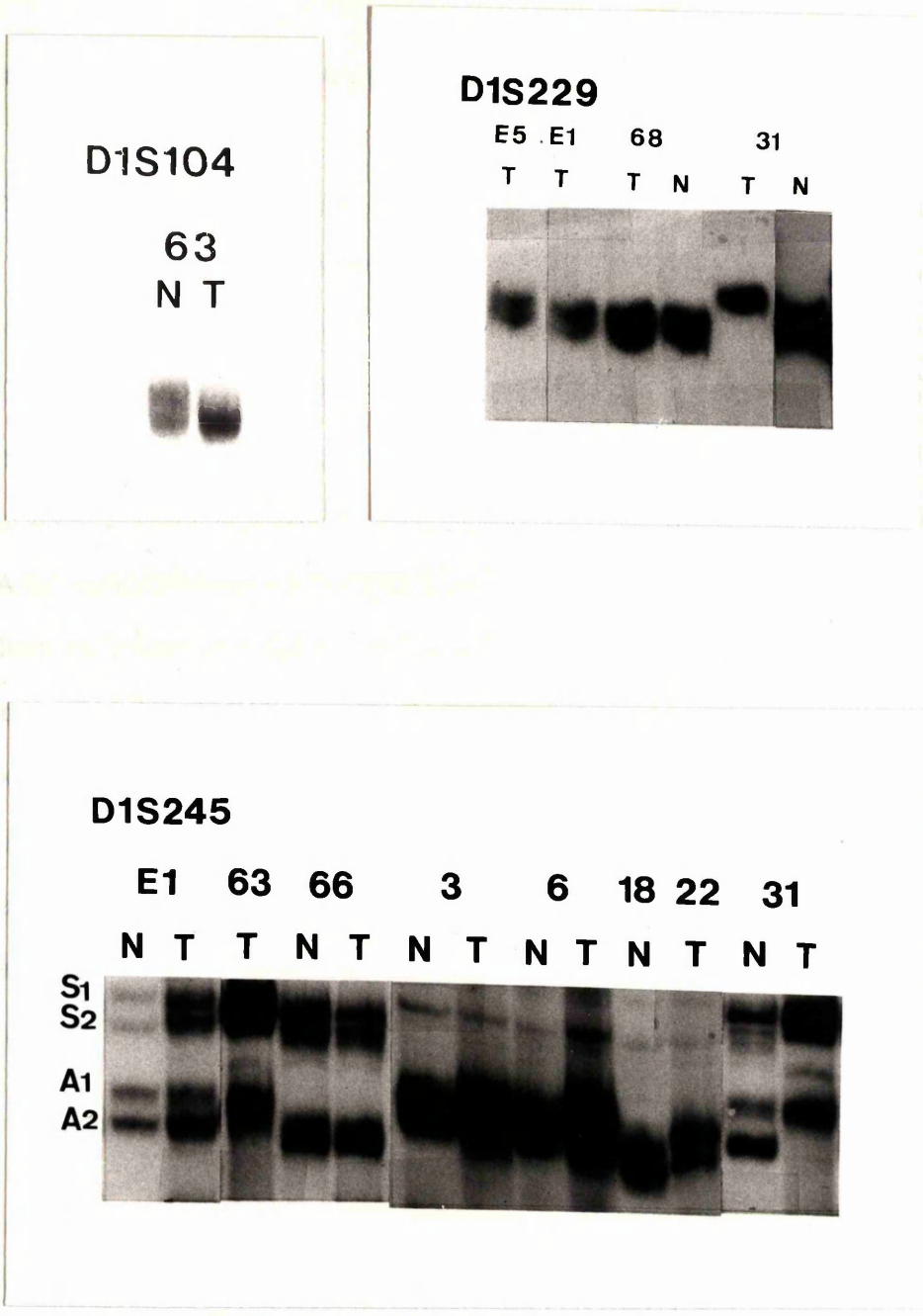


Figure 21. Autoradiographs of polyacrylamide gel electrophoresis of amplified microsatellite sequences on chromosome 1q. T= BICR culture DNA samples, N= matched normal DNA sample.
D1S104 maps to 1q21-23 and shows loss of heterozygosity (LOH) in BICR 63.
D1S229 maps to 1q42 and shows a loss in BICR 31.
D1S245 exhibits shadow bands, labelled S1 and S2 in BICR E1, in addition to the allele bands A1 and A2. There is LOH in BICR 31.

3.2.4.1 Imbalances in allele copy number

There is some evidence for loss of heterozygosity within some clones of these polyclonal cell lines. For example in the Southern blot in figure 22, BICR 18 has an imbalance of alleles. BICR 31 and 63 show imbalances using the microsatellite marker at APO A2 (figure 22B). Finally, BICR 63 also shows an imbalance at D1S102 (figure 22C).

These differences in allele copy numbers are reproducible from reaction to reaction over at least three separate experiments. It is unlikely that they are caused by contamination of the DNA by normal keratinocyte or fibroblast DNA since cell lines are relatively pure and the culture conditions selected for tumour cell growth. Experiments indicate that contamination of up to 10% by normal DNA does not affect the end result (Clark et al, 1993). Making this assumption one would expect alleles to appear with equal intensity on an autoradiograph. Probes hybridising to a Southern are equally radioactive and bind the same sequence in each allele, so they should hybridise in equal numbers and signals would be of comparable intensity. Similarly in a PCR reaction although one allele is bigger so has more radioactivity incorporated, the smaller allele should amplify more times to balance the effects. Thus the differences in intensity seen here are probably due to differences in allele copy numbers, a result of either amplification, duplication and/or loss of selected regions, bearing in mind that the cell lines are aneuploid.

It is harder to assign a mechanism to a result like this, but the situation could be clarified at least in terms of whether there is simply loss of heterozygosity in a proportion of clones by doing some single-cell cloning and investigating those markers which showed the imbalance. Otherwise fluorescence *in situ* hybridisation would show if amplification and/or duplication had occurred at a specific region.

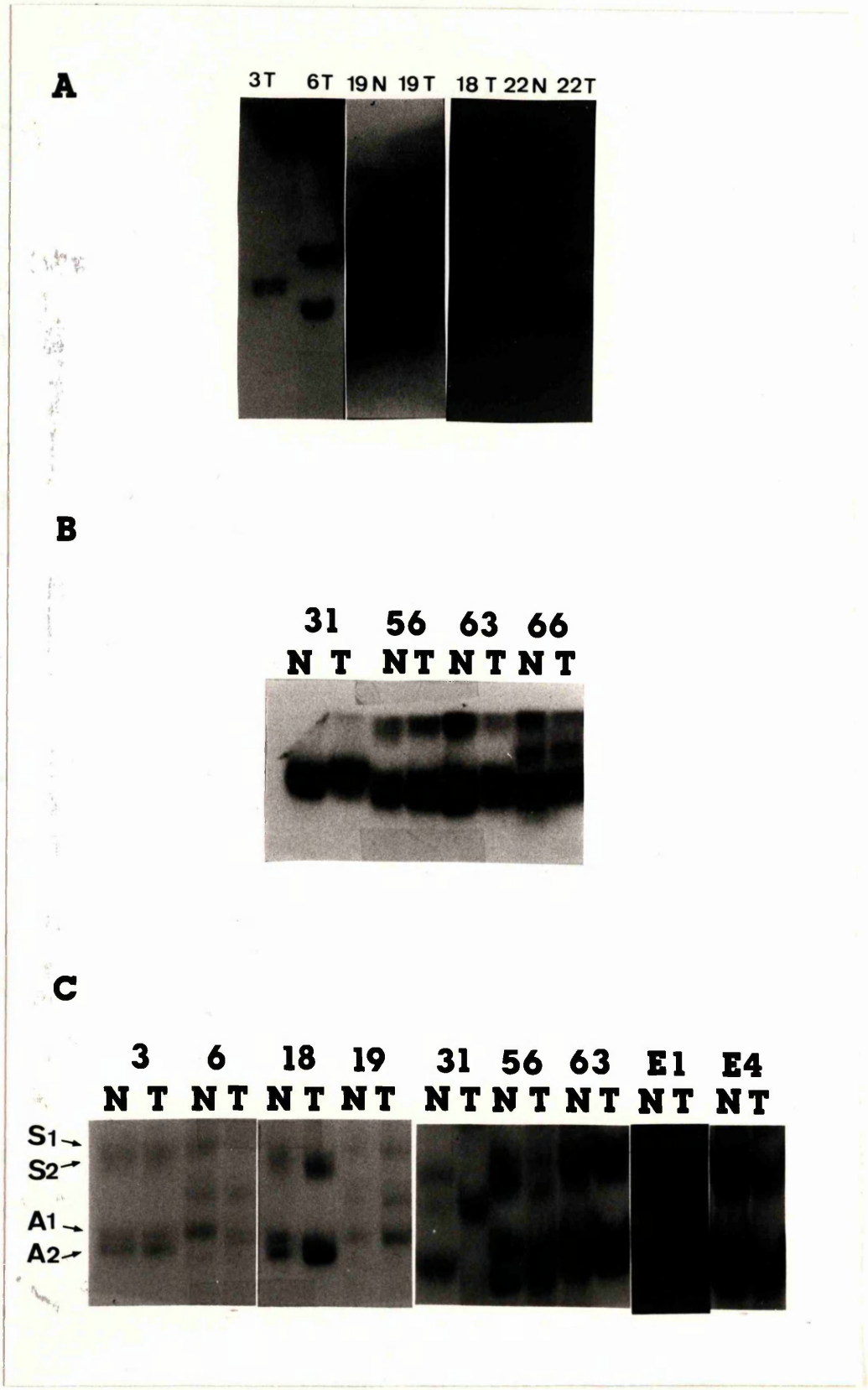


Figure 22. Illustration of imbalances in allele copy number in BICR cultures. T= BICR DNA, N= matched normal DNA.
A. Autoradiograph of Southern blot hybridised with probe pMUC7 mapping to 1q21. BICR 18 exhibits a reproducible imbalance. BICR 3 and 6 are heterozygous and 19 and 22 are not.
B. Autoradiograph showing microsatellite sequences at APO A2 (1q21-23).BICR 31 and 63 exhibit imbalances. A shadow band is visible at the top of the gel.
C. Microsatellite at D1S102. BICR 31 and 18 show loss of heterozygosity and BICR 63 shows imbalance. Alleles are A1, A2 and shadows S1 and S2.

3.2.5 Chromosome 4

A summary of data for chromosome 4 is shown in figure 23 and results are illustrated in figure 24. Unfortunately there are few documented markers on 4q, of which only D4S175 worked as shown.

Again it appears that losses of material on 4p are uncommon in SCC at any stage, since 18 informative cases retained both alleles. There could however be microdeletions which have not been located where the markers used have mapped. These would possibly become clear with the use of many more markers.

It would be important to utilise any further markers which become available on 4q to find how frequent the observed loss at D4S175 is.

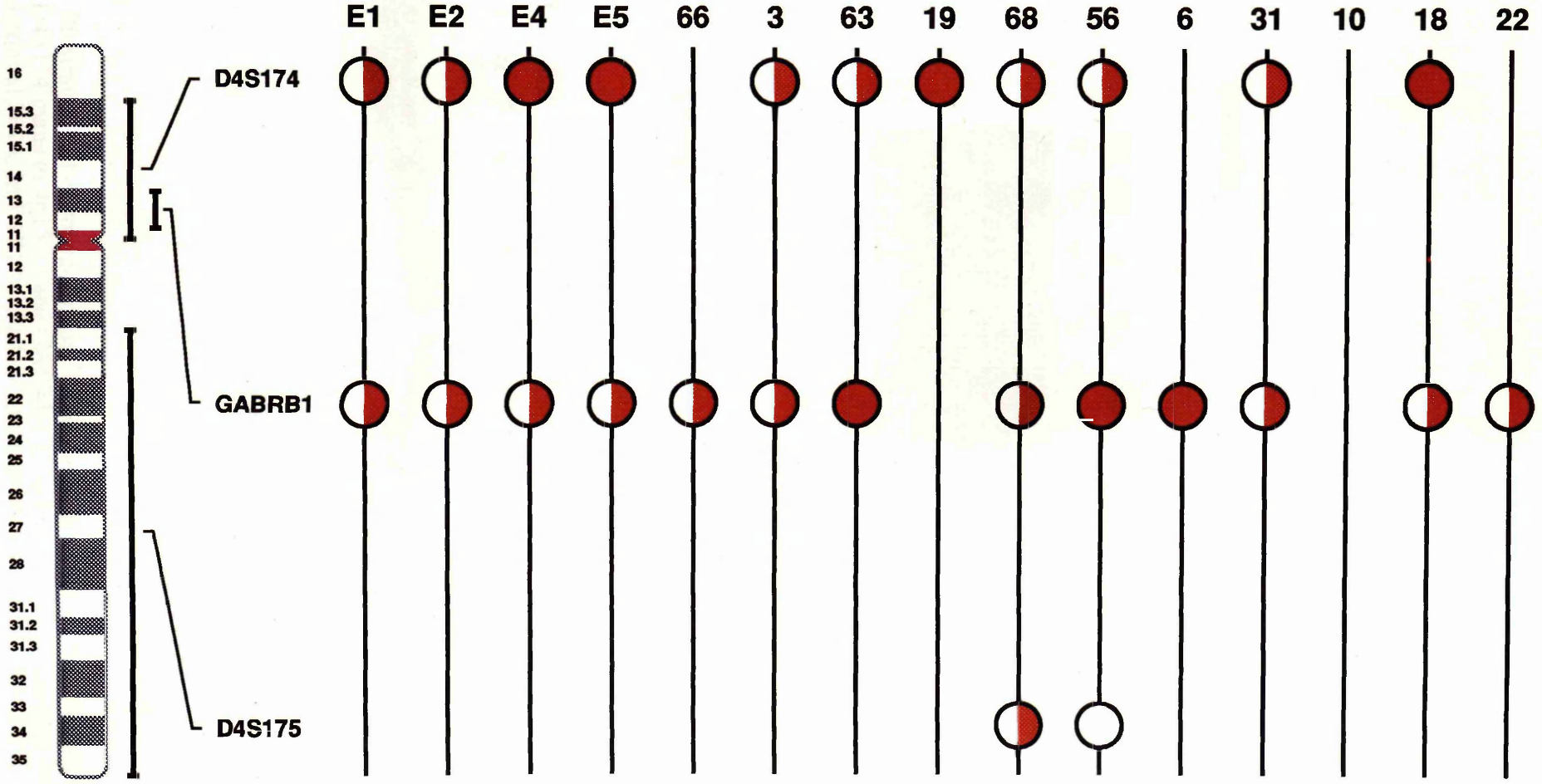
3.2.6 Chromosome 6q

Analysis of chromosome 6 concentrated on the long arm for reasons described previously (section 1.3.3.2.A). Data are summarised in figure 25 and illustrated in figure 26.

Using two markers mapping to the long arm of chromosome 6, there were no cases of LOH in 23 informative cases. Any tumour suppressor genes located on this chromosome arm are not inactivated by large deletions in SCC. They could be inactivated by point mutations in both alleles, however this is not a common mechanism for losing function of tumour suppressor genes. Usually one allele is lost by non-disjunction or a large deletion (Cavanee *et al*, 1983)

The lack of LOH at 4p and 6q show that background LOH levels are lower in SCC than in colon cancer (Fearon and Vogelstein, 1990). Thus regions where LOH does occur are likely to be important.

Figure 23. Summary of loss of heterozygosity data for chromosome 4 for the BICR cultures stated. Cultures are arranged in order of increasing clinical stage of the original tumours. Markers map to the chromosomal positions shown.
● = non-informative, ◑ = heterozygous, ○ = loss of heterozygosity.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31.1
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34
35
D4S174
GABRB1
D4S175

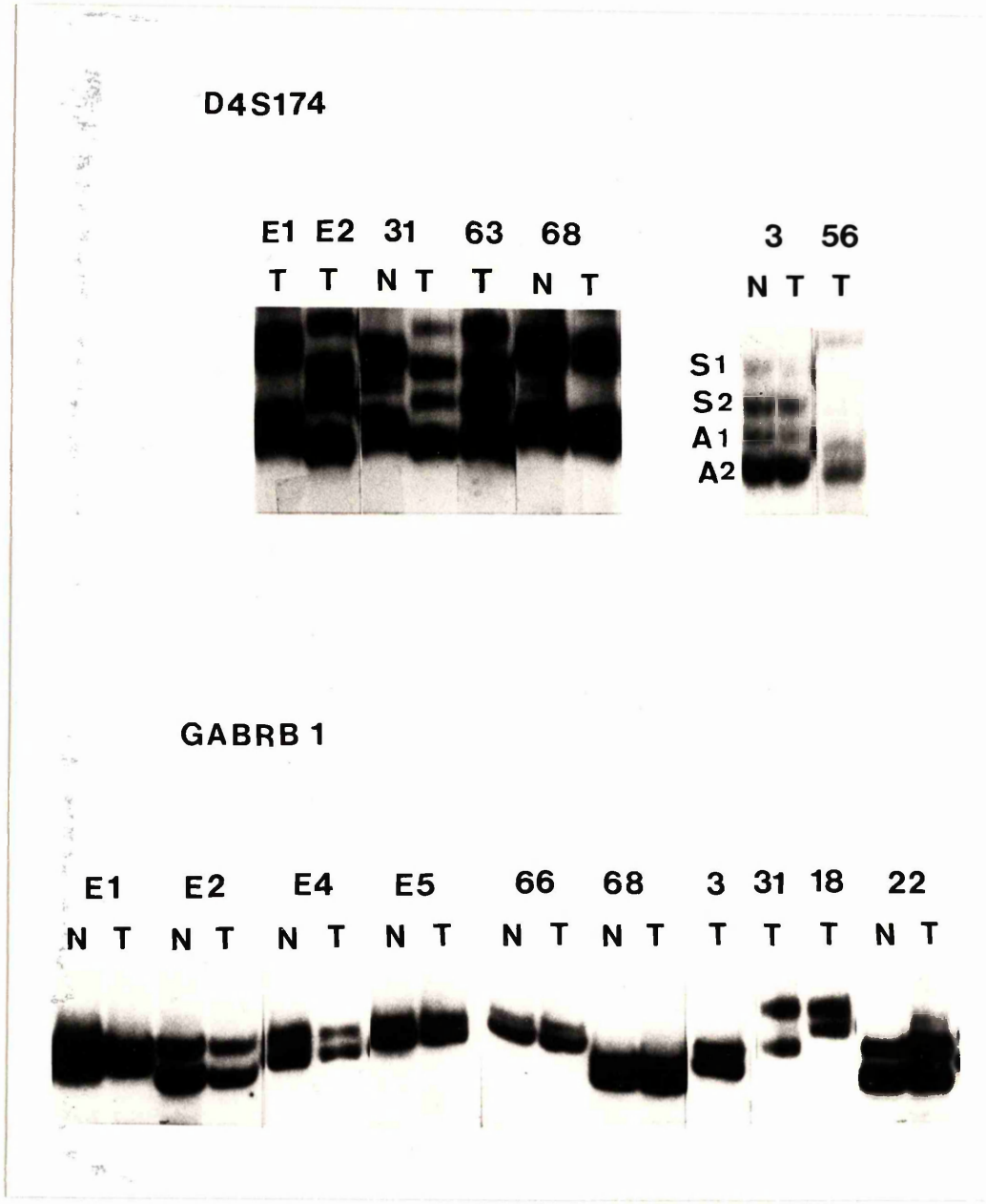
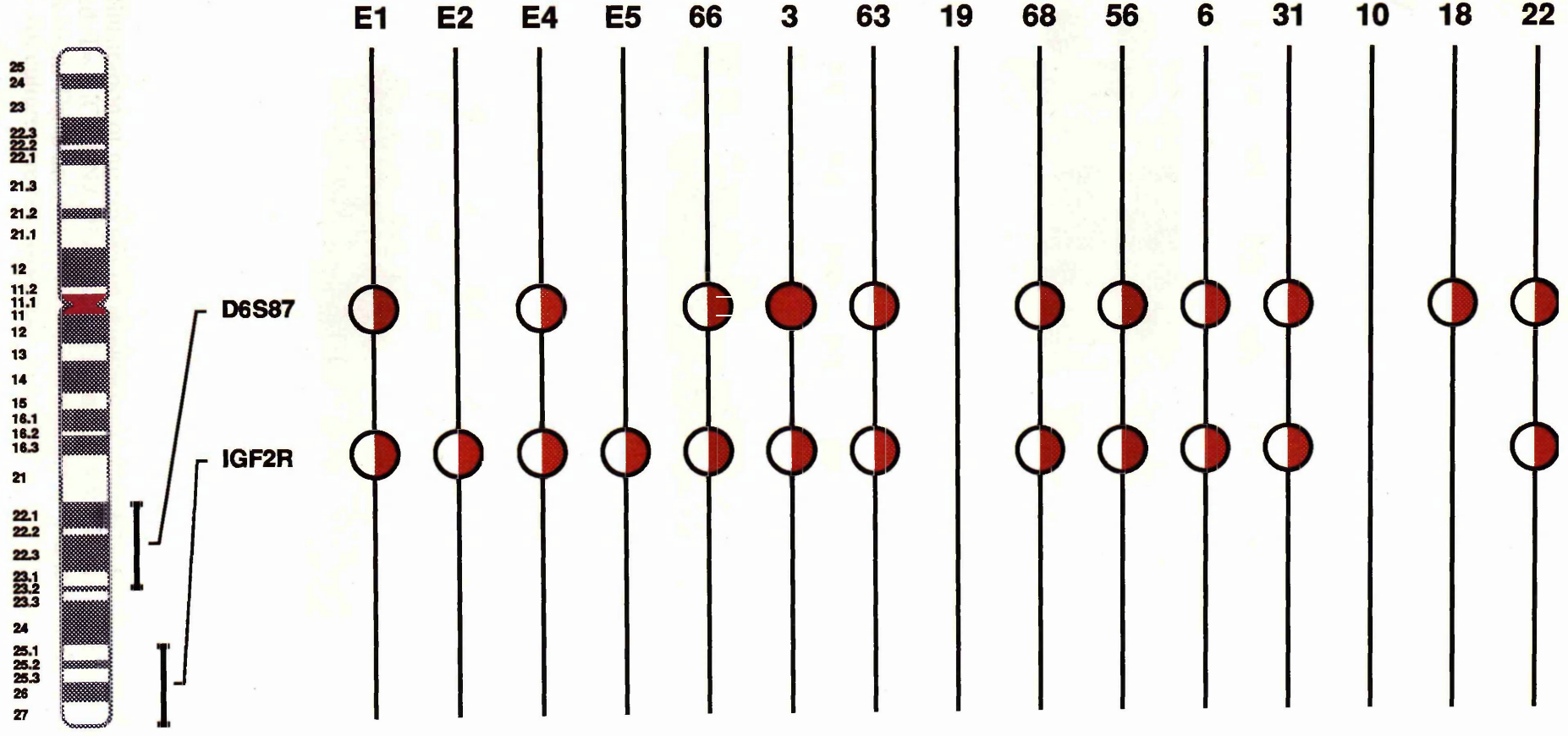


Figure 24. Illustration of heterozygosity on chromosome 4p as shown by microsatellite sequences. D4S174 maps to 4p15-12 and GABRB1 to 4p13-12. T= BICR culture DNA, N= matched normal DNA. In D1S174, shadow bands are labelled S1, S2 and allele bands A1, A2 in BICR 3 as an example.

Figure 25. Summary of loss of heterozygosity data for chromosome 6 for the BICR cultures stated. Cultures are arranged in order of increasing clinical stage of the original tumours. Markers map to the chromosomal positions shown.
● = non-informative, ◑ = heterozygous, ○ = loss of heterozygosity.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26
27
D6S87
IGF2R

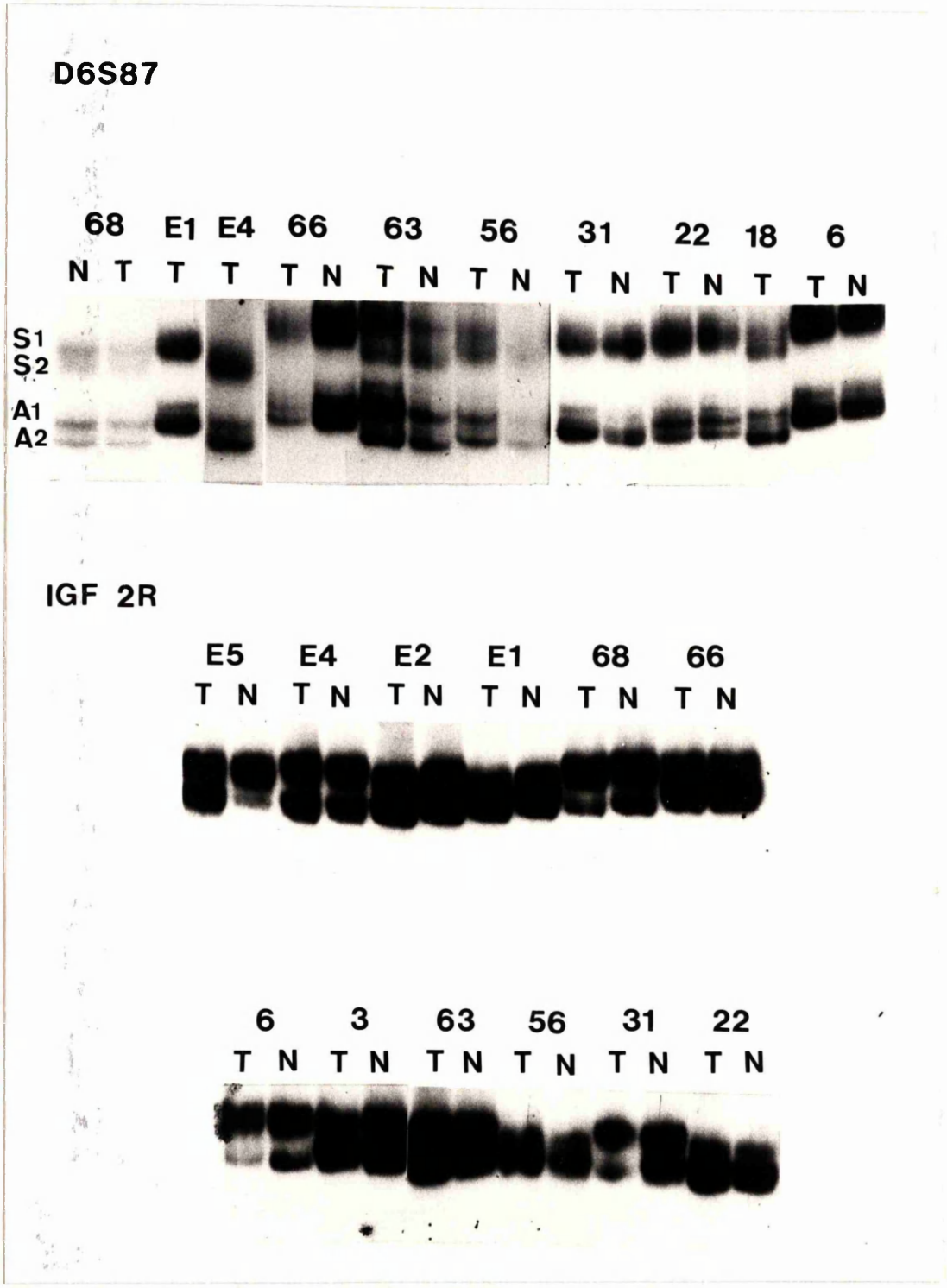


Figure 26. Illustration of microsatellite sequences on chromosome 6q in the BICR cultures stated. T= BICR DNA, N= matched normal DNA. D6S87 exhibits shadow band amplification. These are labelled S1, S2 in contrast with allele bands A1 and A2 for BICR 68. All cultures are heterozygous.

3.2.7 Chromosome 9

Data for chromosome 9 are presented in figures 27-30.

Loss of material on 9p is very frequent (11/26 informative cases).

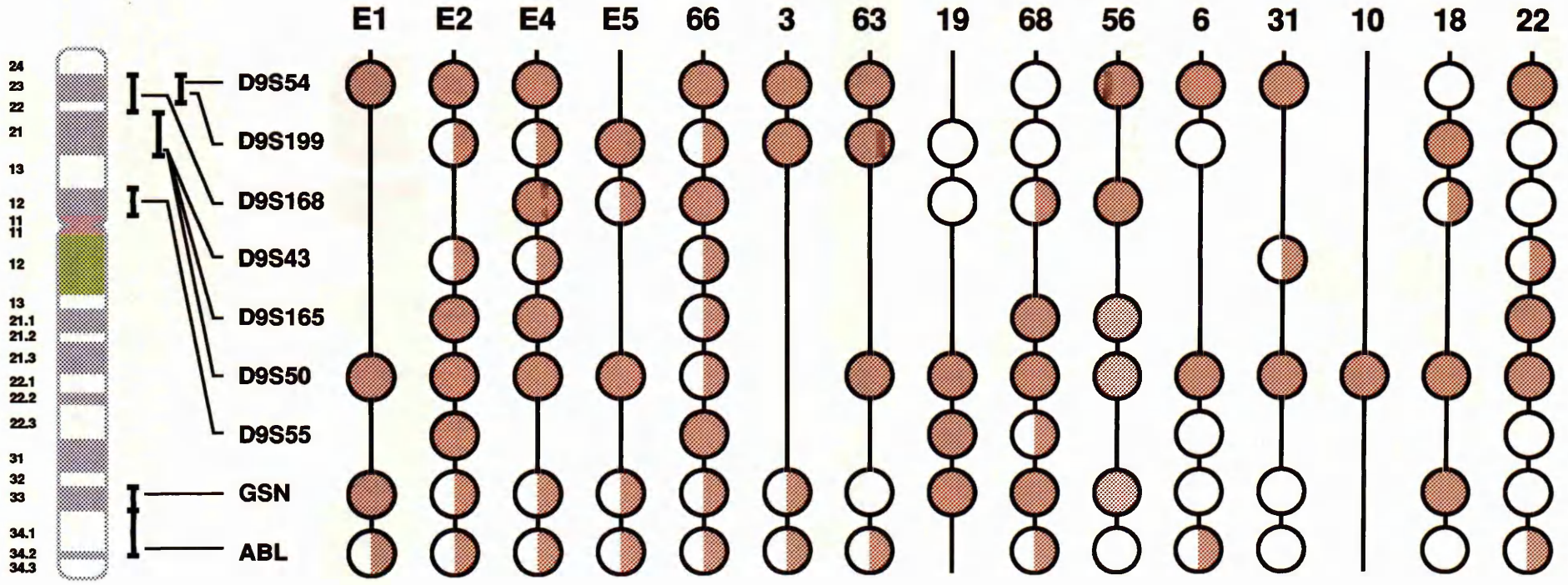
Chromosome 9 is interesting in that it shows separate LOH on 9p and 9q (figure 27). This can be concluded because in lines 18, 22 31 and 68 there is a region of heterozygosity between regions of LOH at 9p and 9q. The marker showing the highest frequency of LOH is D9S199 at 9p23, with four out of four immortal informative lines having loss. Based on the markers used to date, we can tentatively assign a tumour suppressor gene locus to 9p22-23 or distal to this (see figure 31).

It is possible that there are two regions of interest on 9p since BICR 22 has loss of material at 9p22-23 (markers D9S199 and D9S168), retains heterozygosity at 9p21 (D9S43), and has another region of loss at 9p12 (D9S55). There is a lower frequency of loss here but tumour suppressor genes located nearer to a centromere are less likely to be lost. This region may or may not be linked to LOH on 9q (figures 27 and 31).

LOH on 9q is also frequent, occurring in 7/22 informative cases (figure 27). Porterfield *et al* (1992) showed that hybrids of melanoma or leukaemia cells and chromosome 9 senesced when they contained an intact 9p arm but were not affected when 9p was not intact. It was not mentioned whether 9q was intact or not, so it is still formally possible that there are senescence genes on both arms. Other groups have seen senescence upon transfer of whole chromosome 9 in different cell lines (table 1). Figure 32 shows graphically that loss of both 9p and 9q did not occur in any cultures which were not immortal, implying these alterations a possible causative role in escape from senescence. Due to lack of time however the 9q analysis was continued by O.Loughran in the group.

The very high levels of LOH on 9p and separately on 9q relative to the other chromosomal regions which were investigated imply that 9p and 9q are extremely important in SCC development.

Figure 27. Summary of loss of heterozygosity data for chromosome 9 for the BICR cultures stated. Cultures are arranged in order of increasing clinical stage of the original tumours. Markers are ordered according to their map positions on the chromosome.
● = non-informative, ◑ = heterozygous, ○ = loss of heterozygosity.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33
34.1
34.2
34.3
D9S54
D9S199
D9S168
D9S43
D9S165
D9S50
D9S55
GSN
ABL

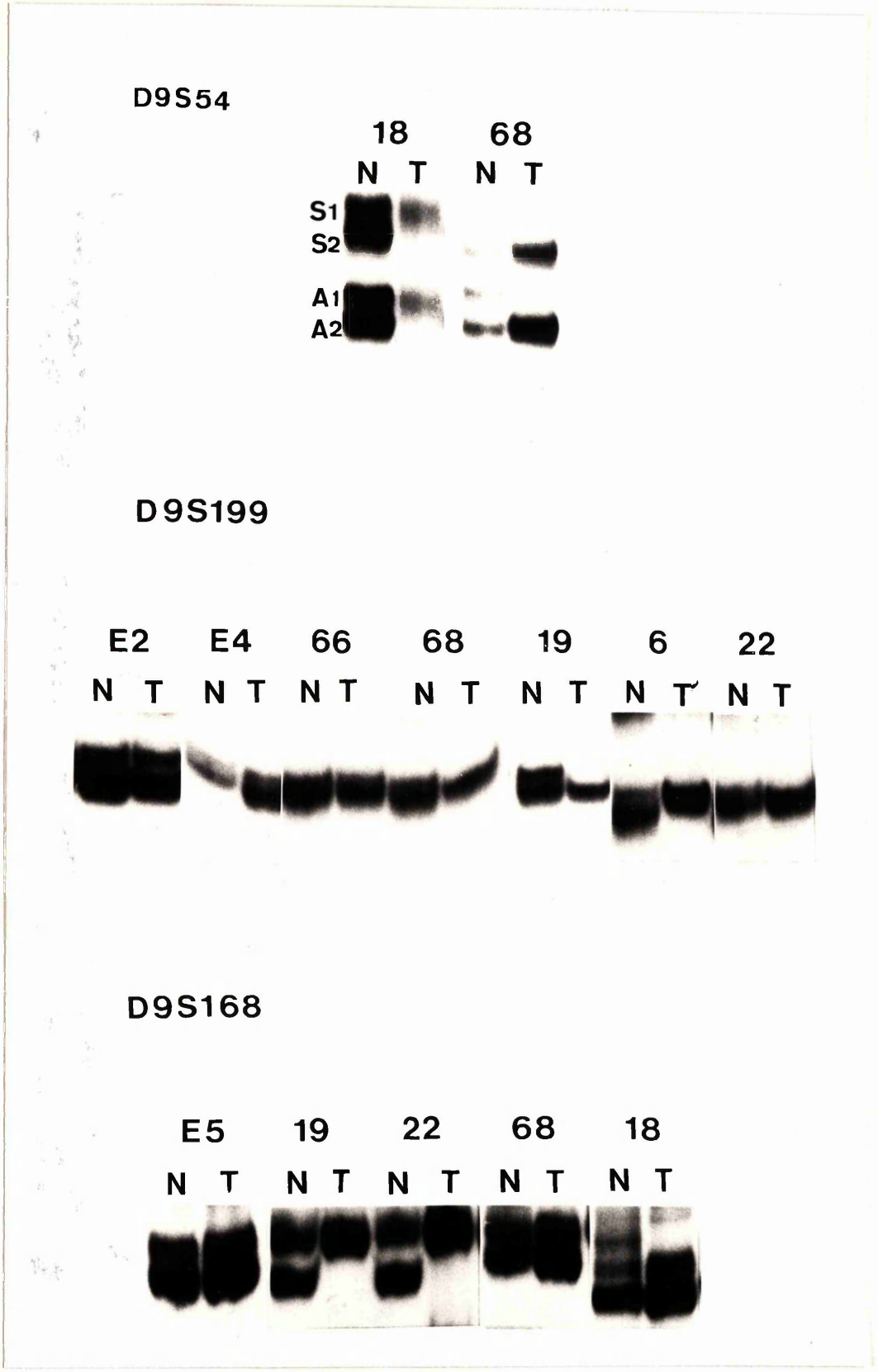


Figure 28. Illustration of microsatellite sequences on chromosome 9p. T= BICR culture DNA, N= matched normal DNA.
D9S54 at 9p23 shows loss of heterozygosity (LOH) in BICR 18 and 68. Shadow bands are labelled S1, S2 and allele bands A1 and A2 in BICR 18.
D9S199 at 9p22-23 shows LOH in BICR 19, 68, 6 and 22.
D9S168 maps to 9p22-23 and shows LOH in BICR 19 and 22.

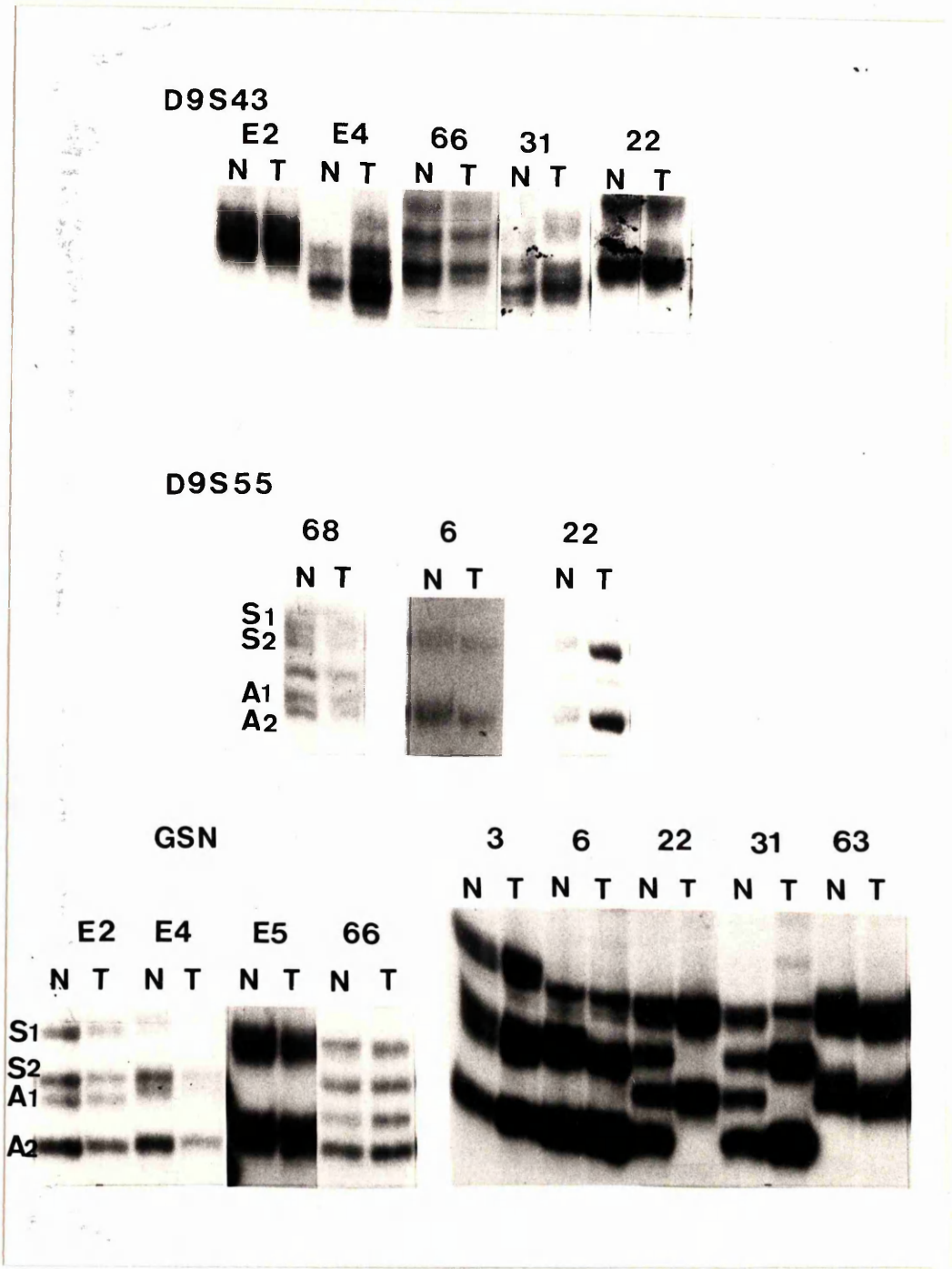


Figure 29. Illustration of microsatellite sequences on chromosome 9. T= BICR culture DNA, N= matched normal DNA. A1, A2= allele bands; S1, S2= shadow bands.
D9S43 maps to 9p21 and all cultures shown are heterozygous. The two lower bands represent the alleles but some shadow bands are visible at the top of the autoradiograph.
D9S55 maps to 9p12. There is loss of heterozygosity (LOH) in BICR 6 and 22 although the two allele bands at the bottom of the autoradiograph in the normal DNA samples are very close together. BICR 68 is heterozygous.
GSN maps to 9q33 and BICR 63, 6, 31 and 22 exhibit LOH.

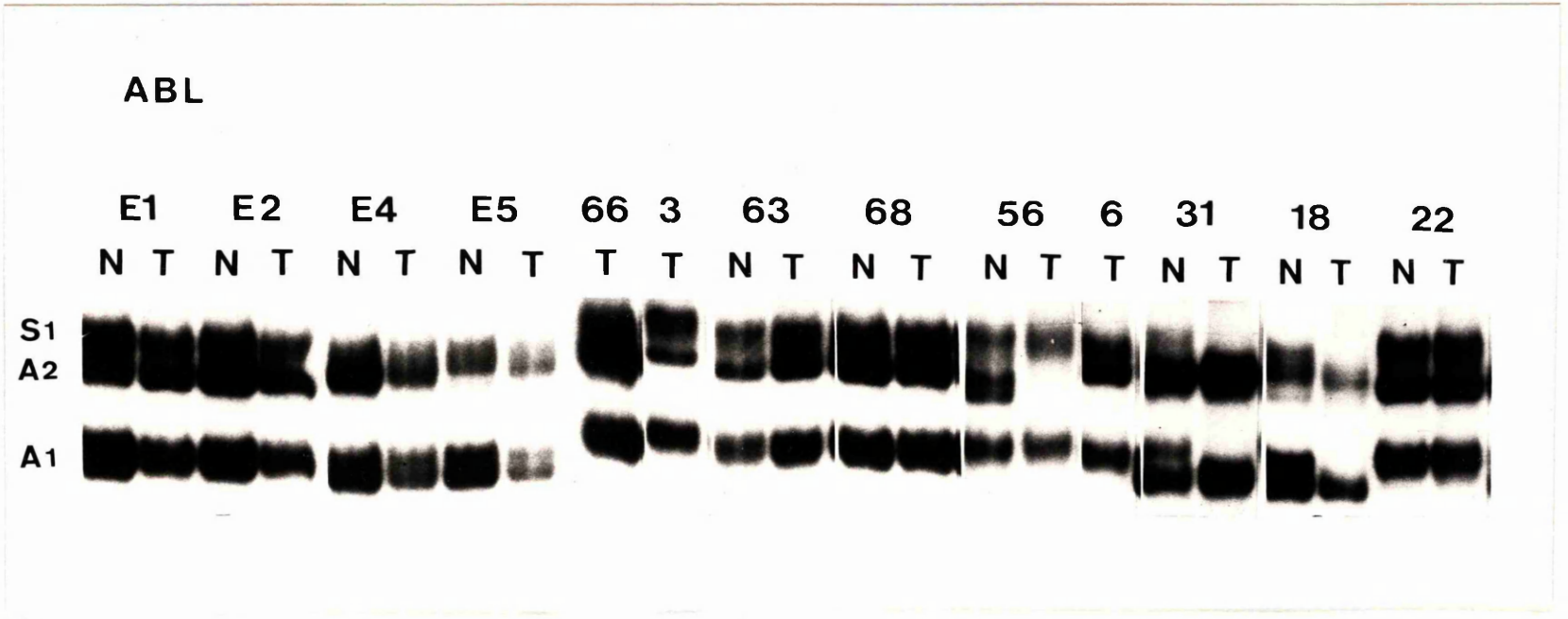


Figure 30. Illustration of microsatellite sequences on chromosome 9q. T= BICR culture DNA, N= matched normal DNA. ABL maps to 9q34.1-34.2, and there is loss of heterozygosity at this locus in BICR 56, 31 and 18. One or two shadow bands are visible in all lanes and are marked in BICR E1 (S1). Alleles are labelled A1, A2 for BICR E1.

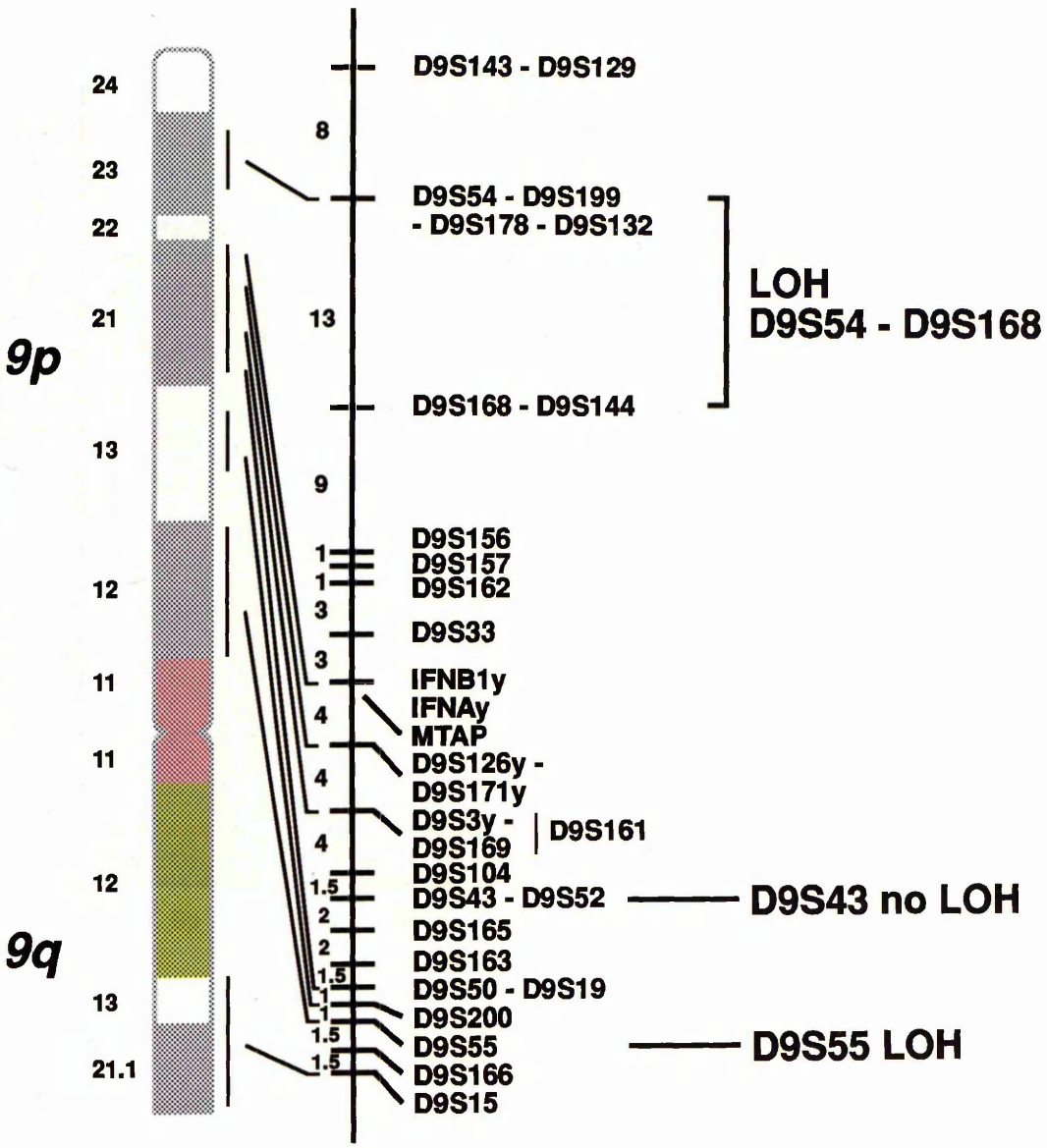


Figure 31 Map of chromosome 9p showing regions of loss of heterozygosity (LOH) in the BICR cell lines.

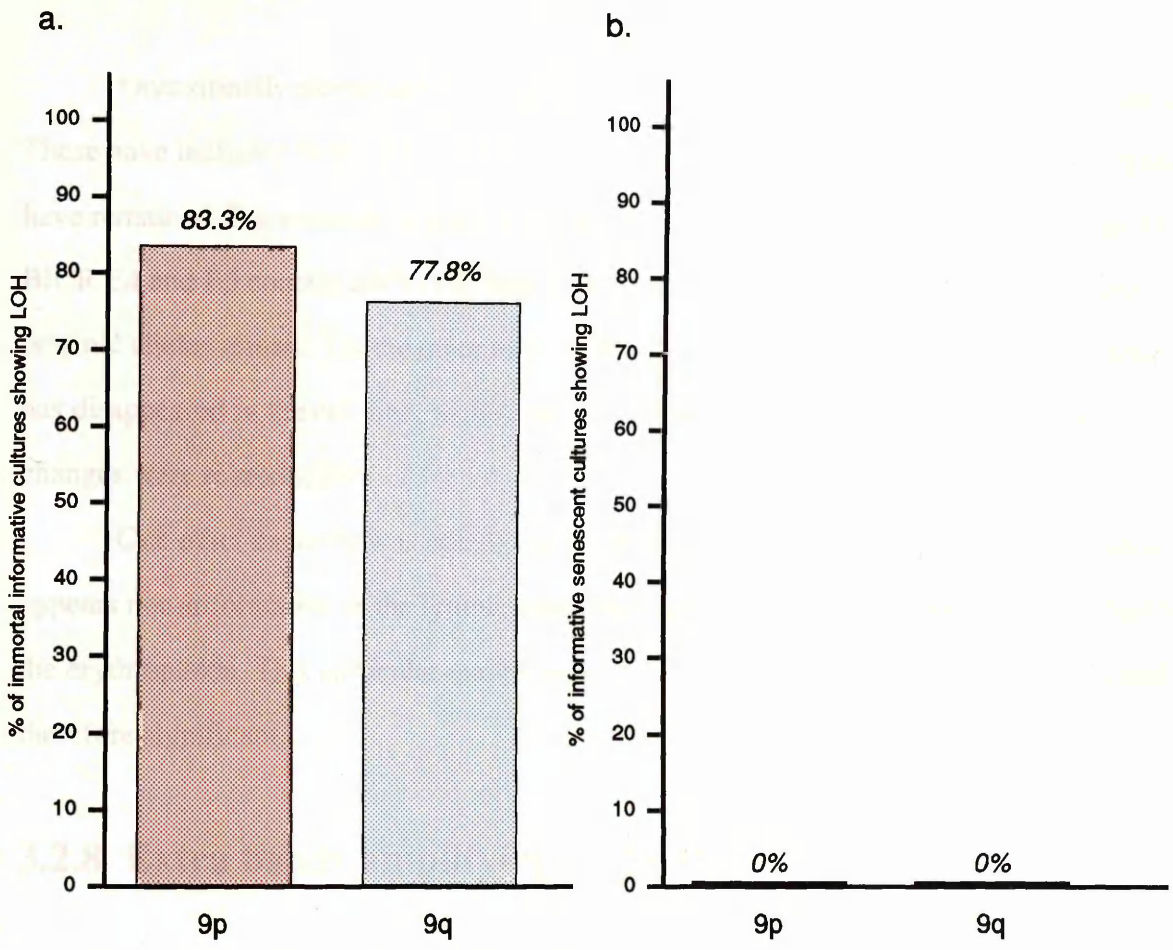


Figure 32 Comparison of alterations on chromosome arms 9p and 9q in senescent and immortal cultures.
a. Proportions of informative immortal cultures showing loss of heterozygosity (LOH) on 9p and 9q.
b. Proportions of informative senescent cultures showing loss of heterozygosity on 9p and 9q.

3.2.7 Allele size changes in tumour cell lines

Occasionally alterations in the sizes of alleles have been seen in the BICR cell lines. These have included increases and decreases in length, but in most cases the normal alleles have remained. For example, using the microsatellite marker located at D1S245 (figure 33), BICR E4 and E5 contain additional larger bands relative to lymphocyte DNAs while the original alleles remain. On the other hand in BICR 68 the fibroblast DNA has a band which has disappeared in the cell line, while another smaller band has appeared. Both these changes were reproducible.

One other example was noted: that of BICR E1 at D1S243 (figure 33B).The marker appears non-informative in the lymphocyte DNA but a second, larger band has amplified in the erythroplakia. This particular marker was not repeated so may not be reproducible and therefore significant.

3.2.8 Extra bands amplifying by PCR

Other groups have reported seeing bands over and above those representing alleles after amplification by PCR (Hauge and Litt, 1993, Murray *et al*, 1993). These were distinct from "shadow" bands and in the course of this work have been observed in the cases of some microsatellite markers but not others and not where a site of restriction fragment length polymorphism was amplified. The two main cases are shown in figure 34. D1S103 reproducibly shows faint bands approximately one or two bases smaller than the main allele (see lines 18, 22 and 31). A different result showed for D6S87, where one and sometimes two larger faint bands appeared above the alleles. These may be one to four bases larger in size and matched in normal and tumour samples. It has been suggested that they are due to slippage of *Taq* DNA polymerase during amplification (Litt, 1991, Murray *et al*, 1993).

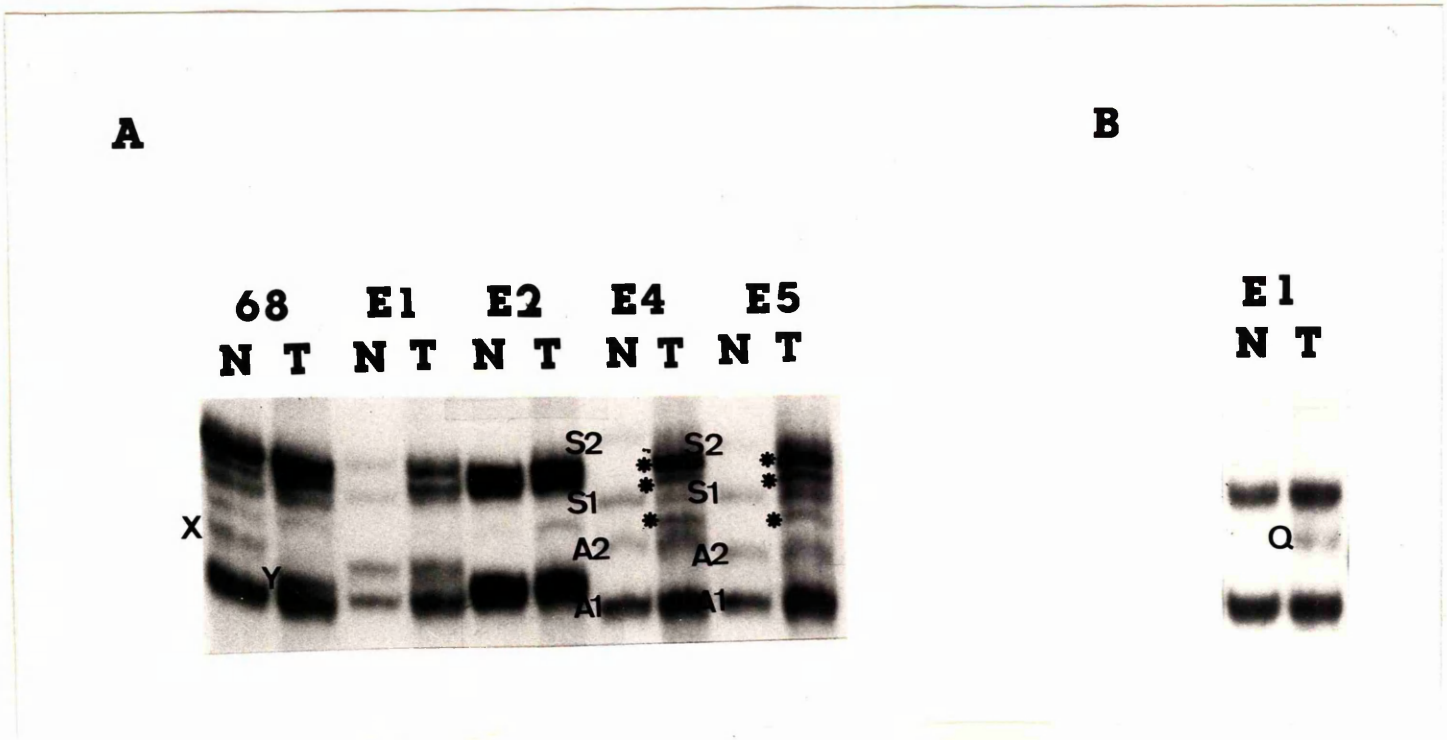


Figure 33. Allele size changes in the BICR cultures. T= BICR culture DNA, N= matched normal DNA. A1, A2= allele bands; S1, S2= shadow bands.
A: Autoradiograph of microsatellite sequences at D1S245 (1q42). BICR E4 and E5 have extra larger bands in the tumour culture relative to the normal DNA marked by stars. BICR 68 loses band X in the tumour cell line but gains band Y.
B: Microsatellite sequences at D1S243 (1p36.3) in BICR E1. Band Q is gained in the tumour culture.

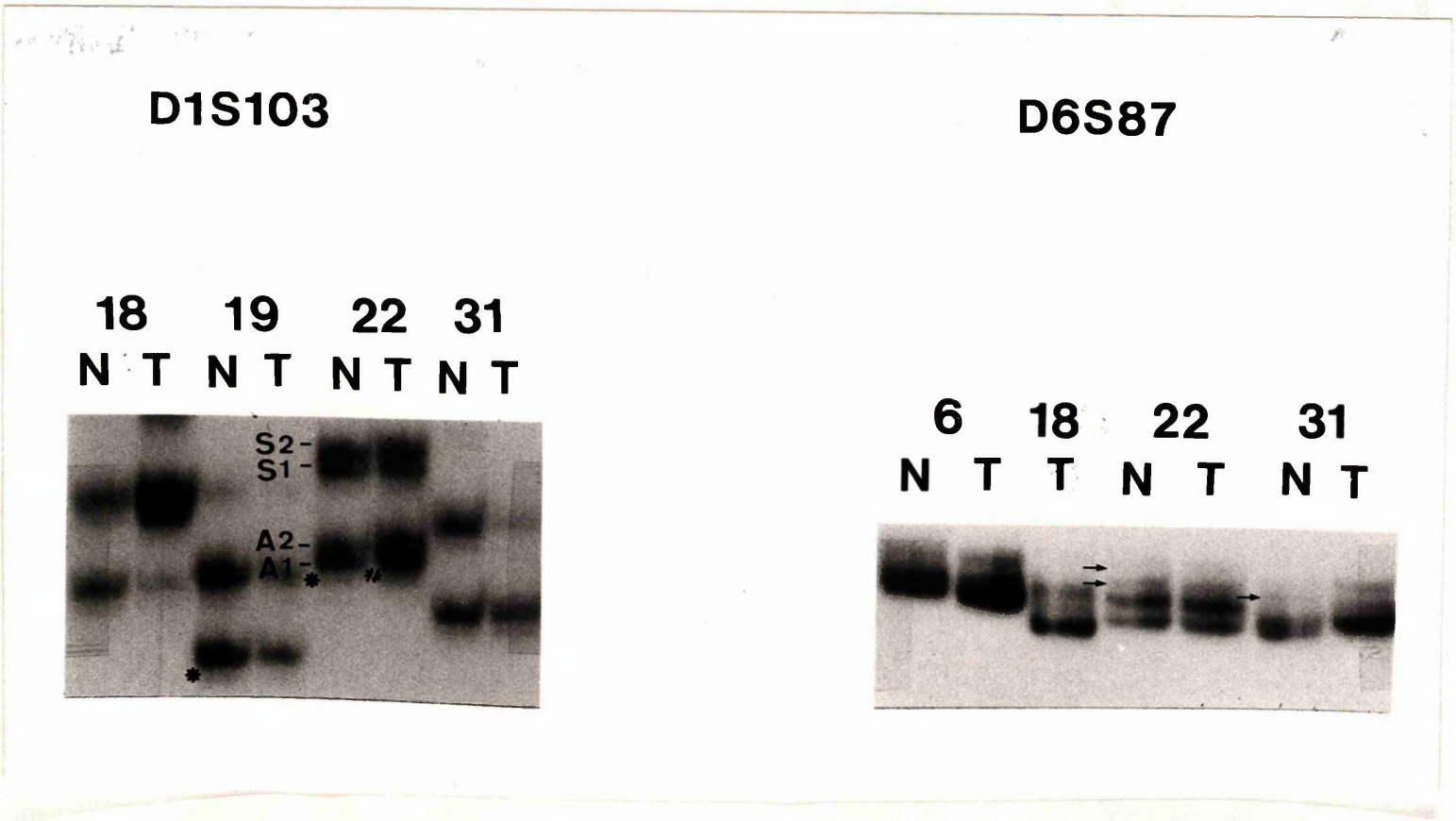
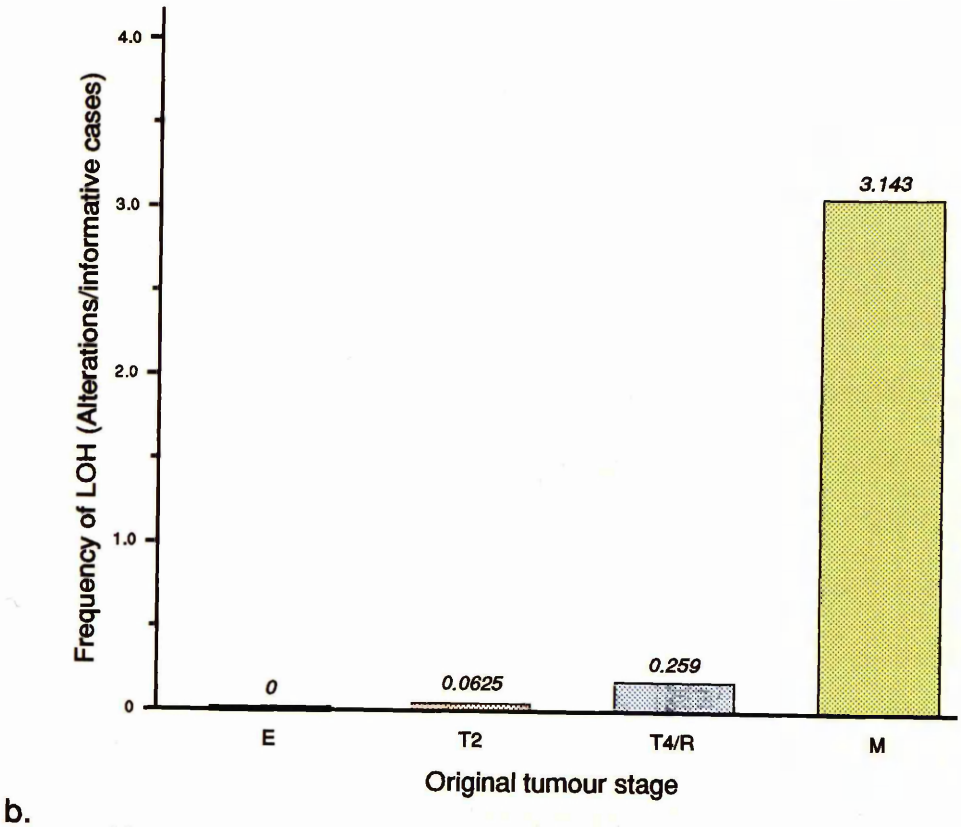


Figure 34. Illustration of extra bands amplifying by PCR along with microsatellite sequences. T= BICR culture DNA, N= matched normal DNA. S1, S2= shadow bands; A1, A2= allele bands. Marker D1S103 (1q32) gives additional bands of one or two base pairs less than the alleles (marked by stars). D6S87 (6q22) exhibits extra bands one or two base pairs larger than the alleles (marked by arrows).

Throughout the PCR work we did not observe any homozygous deletions. These would be notable by allele amplification in the normal DNA in contrast with no observable amplification in the cell line DNA, and would also be a symptom of tumour suppressor genes.

Figure 35a shows total numbers of genetic alteration in cultures derived from the stages shown. LOH increases dramatically with increasing stage of progression. This would be expected since larger tumours would be likely to be more highly evolved, and again supports the idea that the cultures represent the tumours from which they were derived.

Figure 35b summarises the frequency of LOH on the different chromosome arms studied for comparison.



b.

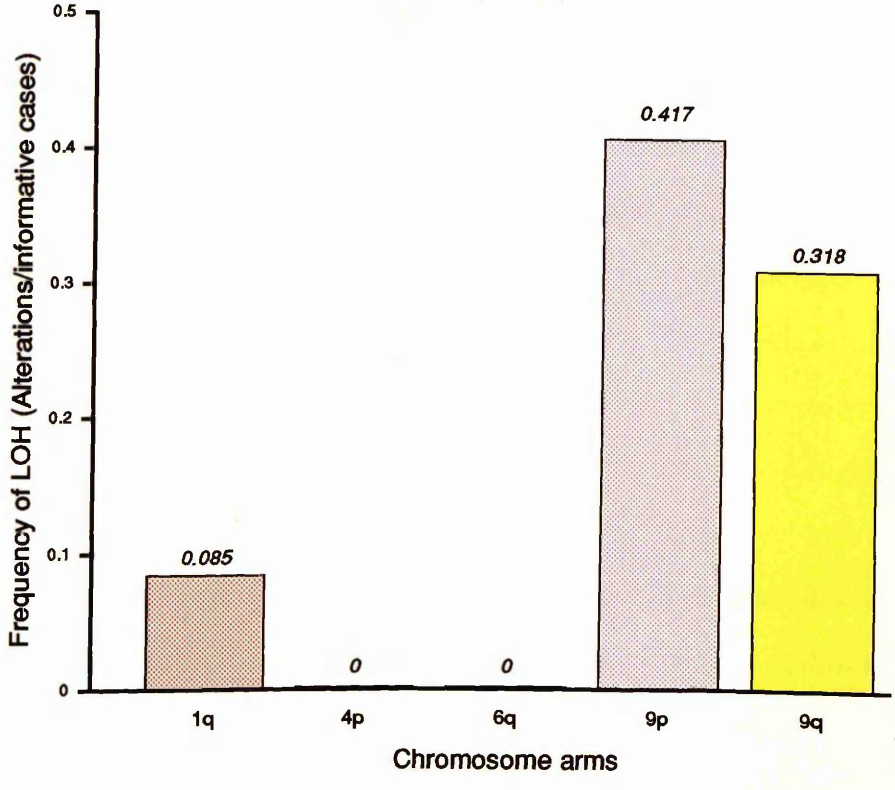


Figure 35 a. Histogram showing the frequency of loss of heterozygosity (LOH) in SCC cultures at different stages of progression. E = erythroplakia, T2 = early stage carcinoma, T4/R = late stage or recurrent carcinoma, M = metastasis.
b. Histogram showing frequency of loss of heterozygosity on the chromosome arms stated.

Chapter 4

Discussion

4.1 The keratinocyte as a cell type for the study of senescence and immortalisation.

The keratinocyte is an extremely useful cell type in which to study the genetic events leading to the immortal phenotype as well as other events in carcinogenesis.

Like other normal cells it exhibits a limited lifespan in tissue culture (Rheinwald and Green, 1975b). Unlike other cells including fibroblasts its *in vitro* differentiation as compared with its *in vivo* differentiation pattern is well understood, both being very similar. The process takes place in defined compartments: keratinocytes leave the proliferative basal layer, increase in size and express involucrin as they lose viability. Finally they assemble a cornified envelope, become squames and are sloughed off (Rheinwald and Green, 1975a, 1975b, Sun and Green, 1976, Green, 1977). This process is useful since immortalisation may involve a partial block in differentiation.

In addition, immortal variants are frequent in squamous cell carcinoma, the malignant form of the keratinocyte (Rheinwald and Beckett, 1981).

Oral cancer has been chosen due to the availability of different types of lesions from different stages of cancer development (Pindborg, 1985).

4.1.1 A new *in vitro* head and neck SCC system

A series of cell lines has been derived from untreated head and neck squamous cell carcinomas of varying stages of progression. Matched blood samples and/or normal fibroblasts together with cultures of premalignant erythroplakias and the ability to grow normal epithelial keratinocytes make this a powerful system to study inactivation of tumour suppressor genes and activation of oncogenes in oral SCC, and to assay functionally for these gene products. Previously little has been discovered of the genetic events causing SCC other than alterations in the *p53* tumour suppressor gene (see section 1.5).

Malignant head and neck SCCs have frequently been reported to contain immortal variants which give rise to established cell lines (Easty *et al*, 1981a, Easty *et al*, 1981b, Rheinwald and Beckett, 1981, Virolainen *et al*, 1984, Rupniak *et al*, 1985, Sacks *et al*, 1988). Most of these lines were derived from recurrent or irradiated tumours however so alterations present in the established lines may not have given rise to the original tumours. Additionally, with the exception of Rheinwald and Beckett (1981), they were derived under conditions which are sub-optimal, namely without the use of a lethally irradiated Swiss 3T3 feeder layer. This renders selection of fitter variants likely (Rheinwald and Beckett, 1981). Finally these cell lines are not ideal for looking at inactivation of tumour suppressor genes by allelic loss since matched normal DNA samples are not available.

The cell lines described in this thesis were established on feeder layers and their serum requirements were determined early. Thus it was relatively easy to obtain a large number of cell lines (seven lines from ten successful explants). The lines were shown to be of squamous epithelial origin by keratin (Sun and Green, 1978) and involucrin (Rice and Green, 1979, Watt and Green, 1981) immunocytochemistry and by electron microscopy. In most cases keratinocytes from the lesions have also been confirmed as transformed by their delayed ability to become post-mitotic and form cornified envelopes when suspended in methylcellulose (Edington *et al*, submitted for publication). Normal keratinocytes rapidly and irreversibly lose colony-forming ability under these conditions and assemble cornified envelopes by cross-linking involucrin, among other soluble precursor proteins (Sun and Green, 1976, Rice and Green, 1979).

Other groups at the Beatson Institute working on these cell lines have found that in terms of several markers their status is identical to the original tumours. Mutations in *p53* are identical (Burns *et al*, 1993), the lack of RAS mutations in cell lines and tumours correlates perfectly (Clark *et al*, 1993), EGF receptor status is identical (Stanton *et al*, submitted for publication) and cyclin D1 status is identical (Nikolic *et al*, submitted for publication). This encourages us to believe the cell lines are truly representative of at least a large portion of the original tumours.

4.1.2 Oral squamous carcinoma: when do immortal variants arise?

Since this work started several other cell lines have been derived in the same manner by E.K.Parkinson. These will be included in the discussion in order to provide a context for the work described in this thesis. A summary of the relevant characteristics of all the cell cultures obtained is shown in table 6. Information for the DOK cell line, a gift from S.Chang, is also shown. DOK was derived from dysplastic oral epithelium adjacient to a carcinoma of the tongue, so represents a premalignant cell line.

Where an immortal cell line was established there was only one instance where a crisis was observed, that is in BICR 7. We have concluded that the cells giving rise to the lines were an immortal population present in the tumour and that this phenotype does not represent an adaptation to tissue culture. On this basis, immortality is a very common phenotype in oral SCC. With reference to table 6, it occurred in 2/4 early stage tumours, 10/12 late stage and recurrent tumours, and 2/3 lymph node metastases. Although the tumour numbers are not large, immortality appears to be increasingly common as tumours progress. The overall frequency of senescence in tumours disregarding staging is 54%, comparing well to Rheinwald and Beckett's finding of 47% for cultures in similar conditions (Rheinwald and Beckett, 1981).

The fact that it was possible to establish two cell lines from four early clinical stage (T2) tumours whereas all four erythroplakias senesced leads us to conclude that immortality most probably arises late in the transition from premalignancy to malignancy. This is a tentative conclusion since we were only able to obtain four erythroplakias, but it agrees with findings for melanoma, where indefinite lifespan is commonly seen in vertical growth phase tumours with potential for metastasis, but rarely in radial growth phase melanoma and never premalignant melanocytic nevus stages (Mancianti and Herlyn, 1989). In the colon system cell lines have been derived from premalignant adenomas of types

having both low and high malignant potential (Paraskeva *et al*, 1984). Cell lines from adenomas with low progression rate require specific passaging conditions. Although it would seem immortality is an earlier event in the colon, it is difficult to compare stages in different cancers. It is certainly clear however that at later stages of progression the immortal phenotype is very common in all three types of cancer.

Although DOK comes from a premalignant lesion and is immortal it does have a *p53* mutation, which none of the other premalignant lesions have. In addition, since it was isolated from a dysplasia adjacient to an SCC it may be further advanced than the erythroplakias (Chang *et al*, 1992).

It is clear that immortality is not crucial for SCC formation, since BICR 7, 59, and 37 are not immortal. BICR 37 is a metastasis so immortalisation was not required for migration and colonisation of a new site in the body. This could be explained by there being more than one pathway to tumour formation with one or some not involving immortality, or by immortality being a by-product and not an essential event in tumour formation. The answer to this question will be found when putative senescence genes have been isolated and analysed genetically and functionally in different cancers and in normal cells.

4.1.3 The tumorigenicity in nude mice of the cell lines

Most SCC cell lines previously studied form tumours when injected subcutaneously into immunosuppressed mice (Easty *et al*, 1981a, Easty *et al*, 1981b, Rheinwald and Beckett, 1981, Rupniak *et al*, 1985, Sacks et al, 1988). However the relationship of tumorigenicity to the state of progression of the original tumour has not been investigated. The tumorigenicity data for the erythroplakia and BICR cultures (table 4) shows that only keratinocytes from malignant lesions are consistently tumorigenic. Further, cells from early (T2) stage carcinomas are much less tumorigenic. Thus there appears to be a fairly good relationship between the extent of progression of the original tumour and the ability of the

resultant cell line to form experimental tumours. This may be a reflection of increasing genetic events in later tumours.

BICR 68 was derived from a late-stage tumour and is non-tumorigenic. This may be because it does not display as readily three phenotypes which are generally associated with transformation. These are multiplication in low serum, multiplication in agar, and feeder-independence (Edington *et al*, submitted for publication).

The two lines derived from metastases, BICR 18 and 22, were also not as strongly tumorigenic as one might expect. Both are capable of growing in low serum levels and 18 is feeder-independent but neither will multiply in agar.

The SV40 model of cellular immortalisation discussed in section 1.3.3 shows that there may be two mortality mechanisms. M1 is bypassed during extended lifespan and M2 is inactivated by rare genetic events at crisis (Wright *et al*, 1989). Since SV40 large T antigen can be replaced by HPV 16 or 18 E6 and E7 (Hawley-Nelson *et al*, 1989), and since all these transforming genes affect the tumour suppressors *p53* and RB-1 (Lane and Crawford, 1979, DeCaprio *et al*, 1988, Munger *et al*, 1989b, Werness *et al*, 1990), then *p53* and RB-1 could be part of M1. As part of the investigation of immortality in oral SCC, the BICR cell lines were first tested for the presence of HPV 16 and 18.

4.1.4 Human papillomaviruses in oral SCC

None of the cell lines tested contained DNA sequences from the E6 and E7 genes of HPV 16 or 18 as determined by PCR. The assay proved to be sensitive to one copy per cell of HPV 16 and five copies per cell of HPV 18. Thus these viruses are not contributing in any continuous fashion to the immortality of the cells. However other less transforming types of papillomavirus are not excluded, nor is a "hit and run" effect (Smith and Campo, 1988).

Other workers have reported HPV 16 and 18 in oral SCCs, some to the extent of 46% (Milde and Loning, 1986, Maitland *et al*, 1987, Syrjanen *et al*, 1988). In the latter case in all lesions except one the DNA was episomal, contrasting with the situation in cervical cancer where the DNA is frequently integrated (Boshart *et al*, 1984, Durst *et al*, 1985). Another study has found HPV 16 DNA in 50% of biopsies of oral mucosa and cultures derived from these (Maitland *et al*, 1989). Equally Brachman *et al* (1992) found a frequency of only 10%, so average levels are nearer 20-30%.

Using the same primers and very similar PCR conditions to those used in this work, Yeudall and Campo (1991) found 25% of oral cancers contained HPV 16 and 20% HPV 18 DNA. PCR in the study was followed by Southern blotting of the amplified DNA and hybridisation to internal oligonucleotides. This procedure increases the sensitivity of detection so would explain the higher frequency of viral presence. Only 3/39 cases contained one copy per cell or more of virus.

4.2 Genetic analysis

4.2.1 Clues from the karyotype of BICR 6

The karyotype shows several features typical of cell lines derived in a similar manner to ours and also primary untreated tumours. First of all it is hypotriploid and has many complex changes. SCCs range from diploid to highly aneuploid (Jin *et al*, 1990, Cowan *et al*, 1992, Patel *et al*, 1993, Worsham *et al*, 1993). They often have highly complex cytogenetic alterations with many rearrangements and breakpoints.

Abnormalities of chromosome 8p, 9p, 11, 13 and 14 are specific events which are consistently found in these lesions (Jin *et al*, 1990, Cowan *et al*, 1992, Patel *et al*, 1993, Worsham *et al*, 1993). These were also found in BICR 6. Some common changes which were not seen are breakpoints on both 1p and 1q, 3p deletions and abnormalities of chromosome 7. A lack of one copy of chromosome 3 may tie in with this, and extra copies

of chromosomes 1 and 7 may have microdeletions or insertions or point mutations which were not visible cytogenetically.

The translocation at 13q14 could be the event which leads to the LOH at RB in this cell line. The abnormalities of 9p additionally agree with LOH data. However lack of a copy of chromosome 6 would not necessarily lead to detection of LOH at 6q (none was found) since the two other copies could carry the relevant information.

It is difficult to draw any firm conclusions from cytogenetics data, especially since we only have data for one cell line. Loss of heterozygosity analysis provides additional, more specific information about the location of tumour suppressor genes.

4.2.2 *p53* mutation in oral SCC and its relationship to the immortal phenotype

The results of the LOH studies using D17S520 at 17p12 agree well with work describing *p53* mutations as discovered using PCR-direct sequencing (Burns *et al*, 1993). They also highlight the importance of using markers close to the target gene for accurate information, since a marker within the *p53* gene shows LOH in BICR 56, and this was not seen with D17S520.

The results agree with several other studies of *p53* in both esophageal tumours and cell lines. Loss of heterozygosity at *p53* has been detected to levels of approximately 50% as was found for the BICR lines (Meltzer *et al*, 1991, Huang *et al*, 1992). Mutations in the *p53* gene have also been demonstrated to between 30 and 90% in cell lines and primary tumours (Hollstein *et al*, 1990, Sakai and Tsuchida, 1992).

No one else to our knowledge has analysed *p53* in erythroplakias. Retention of heterozygosity in the erythroplakias agrees with normal immunohistochemical staining patterns and sequencing data.

Unlike other groups we have been able to correlate LOH and mutation in the same cell lines. Table 6 shows how *p53* staining and mutation (Burns *et al*, 1993), compare with

Tumour stage	Culture	Transformation	Blocked terminal differentiation	Proliferative capacity	p53 staining	p53 mutation
normal	HEK	-	-	senescent	normal	no
premalignant	E1	+	+	senescent	normal	no
premalignant	E2	+	+/-	senescent	normal	no
premalignant	E4	+	+	senescent	normal	no
premalignant	E5	+	+	senescent	normal	no
premalignant	DOK	n.d.	n.d.	immortal	high	yes
$T_2N_0M_0$	BICR 1	n.d.	n.d.	senescent	high	n.d.
$T_2N_0M_0$	BICR 66	+	+	senescent	normal	n.d.
$T_2N_{2B}M_0$	BICR 3	+	+	immortal	high	yes
$T_2N_{2B}M_0$	BICR 63	+	+	immortal	high	n.d.
N/A[a]	BICR 19	+/-	+/-	immortal	low	yes
$T_4N_0M_0$	BICR 68	+	+/-	immortal	high	n.d.
$T_4N_1M_0$	BICR 56	+	+/-	immortal	high	yes
$T_4N_1M_0$	BICR 6	+	+	immortal	low	yes
$T_4N_{2B}M_0$	BICR 7	n.d.	n.d.	crisis	high	yes
$T_4N_{2B}M_0$	BICR 31	+	+	immortal	high	yes
$T_4N_{2C}M_0$	BICR 36	n.d.	n.d.	senescent	normal	n.d.
$T_4N_1M_0$	BICR 78	n.d.	n.d.	immortal	high	n.d.
$T_4N_0M_0$	BICR 80	n.d.	n.d.	immortal	normal	n.d.

Table 6. Comparison of transformation, blocked terminal differentiation, lifespan, and p53 data in the BICR cultures. Taken from Burns *et al*, 1993, and Edington *et al*, submitted for publication. The transformation assay was as described in Rheinwald and Beckett, 1980. n.d.= not done.

Tumour stage	Culture	Transformation	Blocked terminal differentiation	Proliferative capacity	p53 staining	p53 mutation
N/A[b]	BICR 10	+	+	immortal	low	n.d.
N/A[b]	BICR 16	+	+	immortal	low	yes
N/A[b,c]	BICR 59	n.d.	n.d.	senescent	normal	n.d.
n.d.	BICR 82	n.d.	n.d.	immortal	low	n.d.
$T_4N_1M_0$	BICR 18	+	+	immortal	low	n.d.
$T_4N_3M_0$	BICR 22	+	+	immortal	low	yes
$T_4N_{2C}M_0$	BICR 37	n.d.	n.d.	senescent	normal	n.d.

a. Large, aggressive epidermal tumour: TNM staging not appliccable.
b. Recurrent tumours.
c. Patient did not complete course of radiotherapy.

Table 6, continued.

culture lifespan. Of the eight cultures which had normal p53 staining (most cells being negative with the occasional cell staining), seven were senescent. BICR 80 stains normally but is immortal, and could represent a subset of tumours which do not require *p53* mutation to escape senescence. Equally it could contain a mutation downstream of *p53* itself.

14/16 cell cultures which had abnormal staining (either high or low: some cultures showed no staining whatsoever) were immortal. Those which were not immortal were BICR 7, which enters crisis, and BICR 1 which senesces. BICR 7 may not have accumulated enough genetic events to escape crisis. Certainly it is clear that at least one other event is required. In a tumour such as this immortality may be a late by-product of carcinogenesis. There are several other late-stage tumours which are not immortal so this is not a precedent.

BICR 1 appears to have a dominant-negative type of mutation from its staining pattern and yet senesces. One conclusion from this is that *p53* mutation is not the only event required to escape M1, fitting with the idea that the transforming genes SV40 T antigen and HPV 16 or 18 E6 and E7 have several effects, not just on *p53*.

To date there have been no cultures with abnormal staining which have not proved to have p53 mutations by sequencing.

We can conclude from these data that *p53* mutation is important in escape from senescence, but is not the only event and may not be the only pathway to immortality. The erythroplakias could represent precursors of tumours with no *p53* mutation. They are definitely premalignant lesions as three out of four patients from which they came developed carcinomas within a year. On the other hand laryngeal papillomas, which have a much lower frequency of progression than erythroplakias (less than 1% versus 30-55%), contain the HPV 6 or 11 E6 transforming gene, which may inactivate to an extent the *p53* gene (Crook *et al*, 1991, Burns and Clark *et al*, submitted for publication). The erythroplakias do not contain HPV 6 or 11. This appears to be a paradox since *p53* mutation is so common in SCC. Still, *p53* mutation may not be an initiating event in SCC.

The gene product has a range of functions and it is not clear how its mutation influences development and progression. There is evidence for a role in transcription, permissivity of gene amplification and stability of the genome, terminal differentiation, and as a G1 checkpoint controlling cycling, repair of DNA damage and apoptosis (Kern *et al*, 1991, Yonish-Rouach *et al*, 1991, Aoyama *et al*, 1992, Kuerbitz *et al*, 1992, Lane, 1992, Livingston *et al*, 1992). Furthermore, in keratinocytes the different kinds of mutation of *p53* may have differing effects. Stabilisation of the protein may give keratinocytes a selective advantage at an early stage (Gusterson *et al*, 1991, Dolcetti *et al*, 1992, Ogden *et al*, 1992, Nees *et al*, 1993). These are likely to be dominant-negative mutations. But loss of function for example in null and heterozygote mice may be more important in progression from premalignancy to malignancy (Kemp *et al*, 1993).

BICR 19 is heterozygous but has a loss of function mutation, deletion of 107 base pairs such that codons 332 onwards are out of frame (Burns *et al*, 1993). Normal sequence was detected. It seems to have very few alterations in parameters measured to date. EGF receptor levels are normal (Stanton *et al*, submitted), and no loss of heterozygosity has been found apart from 9p. Since one normal *p53* allele can stabilise and prevent gene amplification (Livingston *et al*, 1992), this could explain the lack of abnormalities. Of course many more cases like this need to be investigated to support this hypothesis. BICR 66, an early carcinoma which senesces, also shows no LOH on 9p, 9q, or 3p (section 3.2.7 and O.Loughran, unpublished data). It has normal *p53*. This may simply reflect the fact that it is an early tumour so has not accumulated many changes or alternatively the lack of *p53* mutation may be the cause.

4.2.3 The retinoblastoma protein in head and neck SCC

Several groups have found loss of heterozygosity at the RB locus in esophageal SCC (Boynton *et al*, 1991, Huang *et al*, 1992, Huang *et al*, 1993). All were in the range 30-50% of informative tumours tested. Only one of the groups looked at the RB messenger

RNA (Huang *et al*, 1993). They found no alterations in seven SCCs, although there were alterations in adenocarcinomas.

From these data loss of heterozygosity at RB is frequent, but the the relationship to this of the state of the protein is unknown. We have described loss of heterozygosity in three of six informative SCC cell lines. In the same cell lines there is normal staining as regards protein quantity and subcellular location (E.K.Parkinson, unpublished data), and no change in protein size and phosphorylation state (A.Malliri, unpublished data). In a different panel of SCC cell lines (those derived by Rheinwald and Beckett, 1981) RB-1 mRNA is of normal size (J.Burns, unpublished data). The region D13S25, just distal to RB and deleted more frequently than RB in B-cell leukaemia, remains in at least one cell line with LOH at RB-1.

Cyclin D is part of the larger family of cyclins which function in the regulation of the cell cycle, probably by interacting with kinase catalytic subunits such as cdk 4 (Matsushime, 1992). The target proteins of cyclin D1 have not been identified but it can override inhibition of a human osteosarcoma line by pRb-1 without affecting the nuclear localisation of pRb-1 (Hinds *et al*, 1992). This effect was slightly less than that of cyclins A and E. However only D-type cyclins activate cdk4 in insect Sf9 cells such that pRb becomes phosphorylated at sites identical to those phosphorylated in human T cells (Kato *et al*, 1993). Cyclins D2 and D3 but not D1 bind pRb strongly in intact Sf9 cells, but hyperphosphorylation of pRb leads to their dissociation (Kato *et al*, 1993). Thus the D cyclins may both regulate cdk4 and target enzyme complexes to particular substrates.

The cyclin D1 gene is frequently amplified in squamous cell carcinoma of the head and neck (Jiang *et al*, 1992). Its expression has been detected along with cyclin D3 in human epidermal keratinocytes by Northern analysis (M.Nikolic, unpublished data). In the BICR lines cyclins D1 and D3 are expressed but again D2 was not detectable (M.Nikolic, unpublished data). Since cyclin D1 overexpression may inactivate the pRb-1 growth inhibitory pathway and does not affect RB protein localisation or mRNA we thought it was possible that in those BICR cell lines without RB-1 LOH cyclin D1 would be

overexpressed, and in those lines with RB-1 LOH it would not, either because of a reduction in pRb expression or an inactivating RB-1 mutation. A comparison of cyclin D1 protein expression (Nikolic *et al*, submitted for publication) and the status of the *Xba 1* RFLP of RB-1 is shown in table 7. It is clear from the limited numbers available that there is no simple quantitative correlation between the two parameters. In fact BICR 6, which has LOH at RB, shows relatively high overexpression of cyclin D1. Thus although these data are not simple they suggest that the RB-1 growth inhibitory pathway is disrupted in the majority of the BICR lines.

There remain two explanations for the results. Firstly there may be a mutation in RB which is not reflected in a change in message or protein size or protein phosphorylation. Sequencing the remaining allele or using single-stranded conformation polymorphism analysis would resolve this. Preliminary results show there may be generally higher protein levels in most BICR lines (A. Malliri, personal communication). Thus there may be a stabilising mutation in the remaining alleles in cases of LOH.

Secondly there could be another tumour suppressor proximal to RB (since D13S25 is distal) which is in fact the target of the LOH.

Two other groups have found that LOH at RB does not correlate with protein alterations in breast and bladder cancer (Ishikawa *et al*, 1991, Borg *et al*, 1992). Borg *et al* saw LOH in 26% of primary breast tumours which often expressed high levels of protein. They found low to absent protein levels in a further 15% which did not show LOH. They concluded that RB inactivation was important in breast cancer but the alteration was acquired by mechanisms other than the unmasking of a recessive mutation by allele loss, and that LOH at RB was a stochastic event reflecting the cells' genetic instability. They also suggested the presence of another tumour suppressor gene. Ishikawa *et al* reported similar findings, with inactivation of protein in a subset of bladder cancer lines and primary tumours, but in none of the tumours showing LOH. Neither group has as yet established the genetic events leading to such findings.

Cell line	3	19	6	10	18	22
RB-1 *Xba* 1 RFLP status	het	n/i	LOH	n/i	n/i	LOH
$p34^{cycD1}$ fold overexpression (relative to TFK 104)	1	13	10	1	9	3

Table 7. Comparison of loss of heterozygosity data at RB-1 and cyclin D1 expression in the BICR lines stated. TFK 104 are HPV 16 E6,E7 immortalised, non-tumorigenic primary keratinocytes. Het= heterozygous, n/i= non-informative, LOH= loss of heterozygosity. Cyclin D data taken from Nikolic *et al*, submitted for publication.

An interesting observation is that in each case of LOH in the BICR lines the allele containing the restriction site is the one which is lost (figures 16 and 17). Apart from being a coincidence in a small number of samples, this could represent abnormal DNA methylation in the cell lines. It is likely that the matched normal DNAs are genuinely heterozygous since abnormal imprinting in the germline would predispose to retinoblastoma, and none of the patients from which the BICR tumours came had retinoblastoma.

The preferential loss could also be due to an imprinting effect such that the retained allele is functionally inactive. However this seems unlikely since Rb expression appears to be normal in BICR 6 and 22. In addition it would mean the allele without the restriction site was always the imprinted copy. The data regarding RB and imprinting are confusing at the moment. In hereditary retinoblastoma the germline mutation is more likely to occur on the paternal allele, but there is no corresponding bias in sporadic tumours (Dryja *et al*, 1989, Zhu *et al*, 1989). Yet in sporadic osteosarcoma, where the predisposing mutation is also at RB, there is preferential retention of the paternal allele (Toguchida *et al*, 1989). One conclusion which can be drawn is that the RB alleles in bone differ in a way that they do not in retinal tissue (reviewed in Ponder, 1989). This allows that imprinting could also occur in epithelial cells.

It is still questionable that RB-1 inactivation is necessary for immortalisation of primary human keratinocytes: Jewers *et al* (1992) have found that mutations which abrogate Rb binding by HPV 16 E7 under the control of its homologous promoter in keratinocytes do not prevent immortalisation. On the other hand the integrity of the zinc-binding domain was essential for this function. It is possible that either E6/p53 binding plays a major role in immortalisation or that E7 affects other cellular processes.

4.2.4 Absence of loss of heterozygosity at 1q, 4p and 6q in the BICR cell lines

Cytogenetic data for oral SCC agrees with a lack of involvement of 4p and 6q in carcinogenesis in squamous epithelium. As has been described in the previous section abnormalities of these regions are rarely seen. The lack of LOH at 1q however is puzzling given the frequent breakpoints seen here (Jin *et al*, 1990, Patel *et al*, 1993). Cytogenetics though are relatively crude compared with LOH analysis so would not always identify important regions.

The demonstrated lack of LOH at these regions does not necessarily mean that they do not harbour tumour suppressor genes. Such genes may not be active in squamous epithelium and therefore their inactivation would not benefit the cells concerned in terms of growth potential. For example there are thought to be two regions on 1q where senescence genes might reside, 1q25 and 1q42 (J.C.Barrett, personal communication). However the work defining the regions was carried out in a breast cancer system and we do not know whether the tumours were immortal. The regions may also be tissue-specific and thus not applicable to SCC.

Conversely any tumour suppressor may be inactivated in ways which do not lead to LOH. Possible mechanisms are:

1) Loss of one active copy of the gene can confer an advantageous phenotype.

2) The second allele is inactivated by imprinting.

3) The two alleles could both be inactivated by point mutation (although this is rare relative to non-disjunction or deletion).

4) The gene could be rearranged such that the marker region is still intact but normal expression is prevented.

5) There could be a mutation in a cooperating gene product.

If the above five mechanisms are important to any great extent 1q, 4 and 6q must be studied by a different approach to learn whether any senescence genes (or other types of suppressor) are located there.

Finally there is a possibility that a senescence gene or tumour suppressor is part of a pathway of cooperating gene products. This would mean inactivation of the pathway could take place via inactivation of any one of the genes, which could be located throughout the genome and would explain low level LOH.

Due to the lack of informative microsatellites on 4q and 6p we cannot draw any firm conclusions for these regions. It will be necessary to analyse 4q in more detail as there is one case of LOH at D4S175 (4q21-25). It has been postulated however that a senescence gene lies on 4p and not 4q in keratinocytes (N.Fusenig, personal communication). At both 4p and 6q there could be small interstitial deletions which have not been detected by the markers used, but it is unlikely that in all the informative cell lines tested it would not have been seen.

The background of heterozygosity demonstrated by these results in cell lines and by K.Ah-See in primary tumours (in press) show that the mutation pattern in head and neck SCC tends towards fewer deletions than in colon cancer where almost every chromosome arm is involved (Fearon and Vogelstein, 1990).

4.2.5 83% of informative immortal oral SCC cultures show LOH at 9p.

With the markers used to date a candidate tumour suppressor gene can be tentatively assigned to the marker D9S168 at 9p22-23 or distal to this (figure 31). The marker with the highest frequency of loss (4/4 immortal cell lines) is D9S199 at 9p23 (figure 27). Loss of heterozygosity has also been found to a level of 25% *in vivo* in untreated tumours of head and neck using the marker D9S54 (K.Ah-See, personal communication). This lower frequency could be due to the fact that primary tumours are

likely to be contaminated with normal material, masking any LOH. It is also possible that the primary tumours used were of earlier stage on average than those from which the BICR lines were derived.

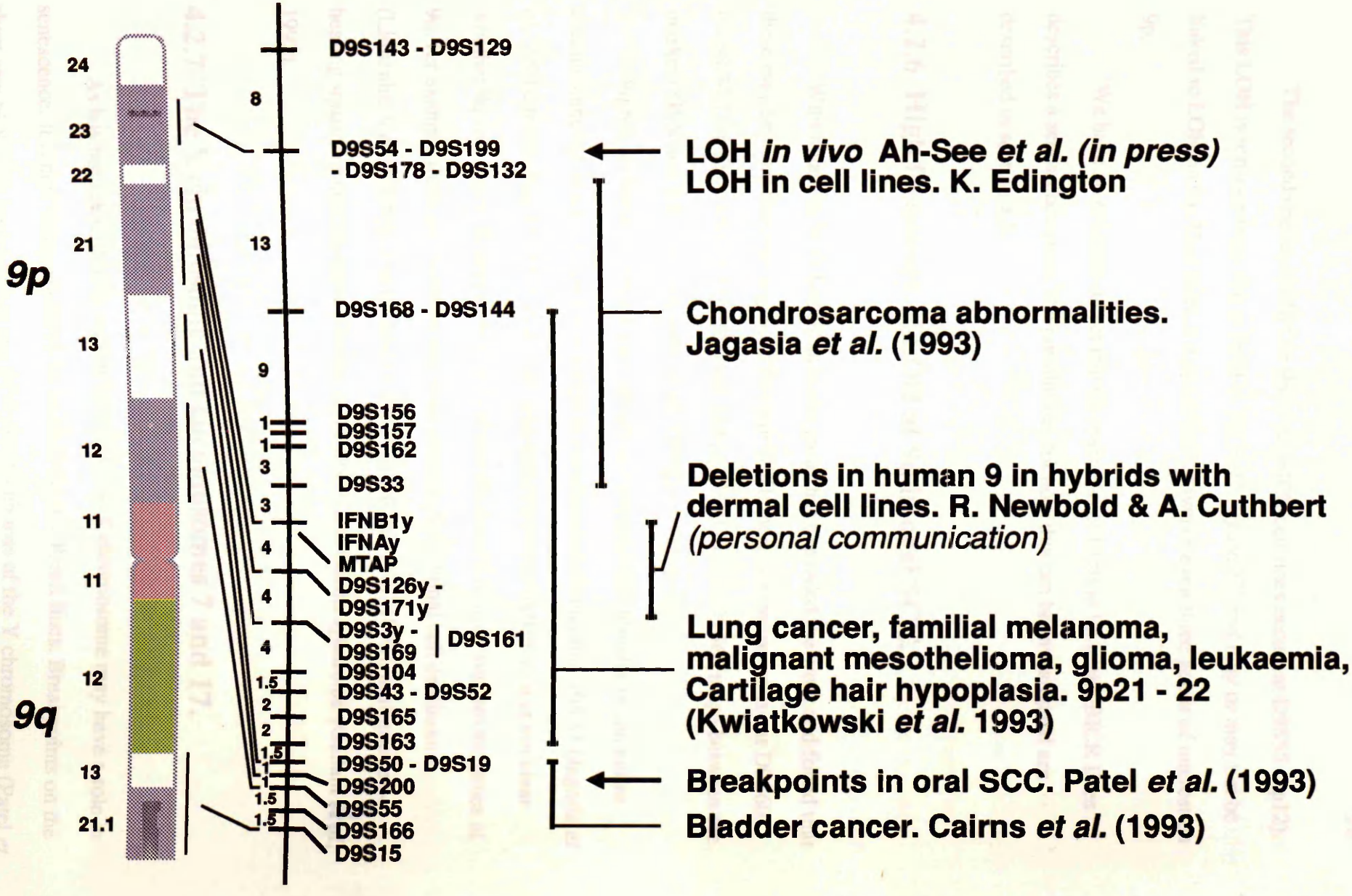
Figure 36 relates these losses to information on other candidate suppressor genes at 9p. For oral cancer apart from the data described above, Patel *et al* (1993) have found breakpoints at 9p12-13 in untreated oral SCC cultures.

There has been a lot of interest recently at the interferon locus at 9p21, where there are deletions in lung cancer (Center *et al*,1993, Olopade *et al*, 1993), familial melanoma (Fountain *et al*, 1992, Cannon-Albright *et al*, 1993, Coleman *et al*, 1993), malignant mesothelioma (Cheng *et al*, 1993), glioma (James *et al*, 1993) and acute lymphoblastic leukaemia (Olopade *et al*, 1992). All of these deletions map either at or very close to the interferon and MTAP genes. There are abnormalities at 9p22 in chondrosarcoma (Jagasia *et al*, 1993) and a bladder cancer gene maps between 9p12-13 and 9q22 (Cairns *et al*, 1993, Miyao *et al*, 1993).

Of more interest to this work is the report that when a normal chromosome 9 is transferred into mouse A9 cells by monochromosome transfer it is not tolerated without deletions in 9p (R.Newbold and A.Cuthbert, personal communication). This would imply that 9p suppresses the lifespan of the hybrids. The deletions have been mapped to the region between the markers IFN A and D9S171 (see figure 36), which includes the loci deleted in the cancers mentioned above. Upon transfer into dermal cell lines 9p also has deletions which from preliminary data appear to match those in A9 cells (A.Cuthbert, personal communication). There are additionally more complicated rearrangements of 9p in dermal cell lines.

The markers from IFN to D9S171 have not been used in the BICR lines so as yet we do not know whether the deletions found by Newbold and Cuthbert are also found in oral SCC cell lines and whether or not they correspond to the loss of heterozygosity described above. Chromosome 9 does reverse the immortal phenotype in several different lineages (see table 1) but this could be due to several different genes on the chromosome.

Figure 36. Information to date for tumour suppressor genes on chromosome 9p. Markers map to the positions indicated. LOH= loss of heterozygosity, SCC= squamous cell carcinoma.


9p
9q
24
23
22
21
13
12
11
11
12
13
21.1
D9S143 - D9S129
8
D9S54 - D9S199
- D9S178 - D9S132
13
D9S168 - D9S144
9
1
D9S156
D9S157
1
D9S162
3
D9S33
3
IFNB1y
4
IFNAy
MTAP
4
D9S126y -
D9S171y
4
D9S3y -
D9S169
D9S161
1.5
D9S104
D9S43 - D9S52
2
D9S165
2
D9S163
1.5
1
D9S50 - D9S19
1
D9S200
1.5
D9S55
1.5
D9S166
D9S15
LOH in vivo Ah-See et al. (in press)
LOH in cell lines. K. Edington
Chondrosarcoma abnormalities.
Jagasia et al. (1993)
Deletions in human 9 in hybrids with
dermal cell lines. R. Newbold & A. Cuthbert
(personal communication)
Lung cancer, familial melanoma,
malignant mesothelioma, glioma, leukaemia,
Cartilage hair hypoplasia. 9p21 - 22
(Kwiatkowski et al. 1993)
Breakpoints in oral SCC. Patel et al. (1993)
Bladder cancer. Cairns et al. (1993)

The second region of LOH on 9p in the BICR cell lines occurs at D9S55 (9p12). This LOH is separate from that at 9p23 in BICR 22 (figure 27) and may or may not be linked to LOH on 9q. Thus there is a possibility of two or even three genes of interest on 9p.

We have no evidence as yet that the region(s) of LOH at 9p in the BICR lines describes a senescence gene. The possibilities for how this can be investigated are described in section 4.3.

4.2.6 High frequency of LOH at 9q in oral SCC.

With regard to 9q O.Loughran in the group has expanded the work and found that there may be more than one region of frequent LOH: there is heterozygosity at D9S60 (9q33-34.1) in lines with LOH both distal and proximal to this. D9S60 maps between the markers GSN and ABL (Kwiatkowski *et al*, 1993).

9q has not been associated with senescence except when transfer of the entire chromosome led to a senescence phenotype in the chondrosarcoma line 105AJ (Jagasia *et al*, 1993). In this case MTAP was used as a natural selectable marker so it is not clear whether 9q was intact. However there is evidence for several tumour suppressor genes at 9q, for example there are deletions and breakpoints in transitional cell carcinoma (Linnenbach *et al*, 1993), non-Hodgkin's lymphoma (Offit *et al*, 1993), multiple self-healing squamous epitheliomata (Goudie *et al*, 1993), and Gorlin syndrome (Gailani *et al*, 1992).

4.2.7 The X chromosome and chromosomes 7 and 17.

As has been described in section 1.3.3.2.A, the X chromosome may have a role in senescence. It is now being examined for LOH in the BICR cell lines. Breakpoints on the short arm of X have been noted in oral SCC along with loss of the Y chromosome (Patel *et*

al, 1993), and breakpoints on the short arm of the inactive X were observed in an SCC of the vulva (Worsham *et al*, 1991). Loss of a Y chromosome was also a primary abnormality in an SCC of the vocal cord (Worsham *et al*, 1993). Initially however there seems to be no LOH at Xp22-pter (O.Loughran, unpublished data).

Recently chromosomes 7 and 17 have been transferred by microcell fusion into immortalised fibroblasts and MCF-7 breast cancer cells respectively. They caused symptoms of senescence (Casey *et al*, 1993, Ogata *et al*, 1993). The chromosome 17 effects were not due to the p53 tumour suppressor. Breakpoints at 7p and 17q have been noted before in SCC (Jin *et al*, 1990, Jin *et al*, 1993, Patel *et al*, 1993), but are not among the most common abnormalities. Ah-See *et al* (in press) have not found any LOH in either of these regions in primary tumours either so it is not yet clear what significance these chromosomes have for oral SCC.

4.2.8 What genetic abnormalities are important for the immortal phenotype in head and neck SCC?

From the work described in this thesis and from further investigation of the BICR cultures the main areas of alteration in oral SCC with significance for escape from senescence are the *p53* tumour suppressor gene and 9p. Additionally there are consistent losses of heterozygosity at 3p25-pter, 3p21, and 3p13-14 in the immortal lines (O.Loughran, unpublished data). Although preliminary results of microcell transfer of 3p13 into SCC lines do not show that hybrids senesce (R.Newbold, personal communication), this could mean either that 3p13 does not contain a senescence gene or that it requires expression of a cooperating region to have such an effect.

We also have some evidence for inactivation of growth control by RB-1 via deregulation of cyclin D1, and low level loss of material at 1q42.

Thus the immortal phenotype appears to be genetically complex and it is not yet clear how these data tie in with the complementation group theory described by Pereira-

Smith and Smith (1988). As discussed in the introduction to section 1.3, they were able to assign 20 cell lines to one of four complementation groups for limited lifespan. Chromosome 4 microcell transfer caused senescence in several lines of different lineages from complementation group B and not in lines from other groups (Ning *et al*, 1991). From this and other chromosome transfer experiments (table 1) we can conclude that single chromosomes can in some cases reverse the immortal phenotype. However the number of chromosomes identified as carrying putative senescence genes now exceeds the number of complementation groups, although it is likely that the effects of some of these will be tissue specific.

What is clear is that immortal cells can be complemented by fusion with normal cells (Bunn and Tarrant, 1980, Muggleton-Harris and DeSimone, 1980, Pereira-Smith and Smith, 1983, Berry *et al*, submitted for publication). It may be that cells which have achieved immortality have accumulated more alterations in many instances than can be successfully complemented by other immortal cells. This hypothesis is indirectly supported by work showing that some cell lines cannot be assigned to only one complementation group using the indicator lines of Pereira-Smith and Smith (Duncan *et al*, 1993, Berry *et al*, submitted for publication). Also seven different chromosomes have induced senescence when transferred singly into various cell lines (Yamada *et al*, 1990, Ning *et al*, 1991, Klein *et al*, 1991, Porterfield *et al*, 1992, Casey *et al*, 1993, Jagasia *et al*, 1993, Ogata *et al*, 1993), but although deletions in chromosome 3p have been consistently found in immortal SCC cultures (O.Loughran, unpublished data), transfer of chromosome 3 alone to cell lines does not cause senescence. One might also expect from the SV40 model of immortalisation that expression of both wild type *p53* and RB-1 in immortal cultures would induce senescence via mortality mechanism 1. In fact antisense *p53* and RB oligomers cooperate to extend lifespan in human diploid fibroblasts (Hara *et al*, 1991). Finally the work described in this thesis shows many changes have occurred in immortal variants in head and neck tumours.

It is tempting to speculate as to the functions of senescence genes in the light of recent developments in the p53 and pRb growth regulatory pathways and in control of telomere length. The WAF1 protein provides a molecular link between p53 and cell cycle arrest since its transcription is induced by wild type p53 (El-Deiry *et al*, 1993) and it binds cyclin-dependent kinases and inhibits their action including phosphorylation of pRb-1 (Harper *et al*, 1993, Xiong *et al*, 1993). p16 and p20 are also proteins which are inhibitory to cdk action (Gu *et al*, 1993, Serrano *et al*, 1993). It could be that these proteins which act to inhibit cell cycle progression are senescence genes acting in concert, given the evidence for a role for p53 and pRb-1 in senescence.

Several yeast mutants have been dicovered which affect telomere length; the genes involved are RAP1 (Conrad *et al*, 1990), RIF1 (Hardy *et al*, 1992), EST1 (Lundblad and Szostak, 1989), TEL1 & TEL2 (Lustig and Petes, 1986), and PIF1 (Schulz and Zakian, 1994). Particularly since the EST1 mutation leads to a senescence phenotype, and given that telomere shortening stops at crisis in immortal variants (Counter *et al*, 1992) some of these genes may also turn out to be senescence genes which are inactivated in immortal cells.

4.3 Future prospects

A collaboration is underway to transfer normal chromosome 9 back into the BICR lines with LOH at 9p to test for the function of this gene so that senescence can be distinguished from suppression of tumorigenicity. It will be important to separate the actions of 9p and 9q. The markers used to define the 9p deletions in the A9 and dermal cell hybrids will also be examined in the BICR lines in order to define the region of LOH at 9p23 more closely. The region at the IFN A locus may or may not be lost in oral SCC, and could be separate or linked to LOH at 9p23.

Another method of distinguishing senescence and more general suppression would be to use 9p microsatellite markers to find if senescent cultures of both carcinoma and

metastatic oral SCCs contain this deletion or not. It will be of interest whether BICR 7, a culture which enters crisis, has 9p deletions as well. If it has the chromosome 9p gene may be grouped with *p53* and RB-1 as being involved in mortality mechanism 1. In this case it could have a function similar to the cell cycle control proteins described in the previous section. If BICR 7 has not lost 9p this gene could be the first of the mortality mechanism 2 genes to be described apart from th example discussed in section 1.3.3.2.A on chromosome 6q.

The BICR lines can then be used as a target for functional assay of transferred chromosomes or fragments of chromosomes, whether they carry specifically senescence or other tumour suppressor genes. This could also be achieved now that the entire human genome exists in yeast artificial chromosomes (YACs) and there are techniques enabling the fusion of YACs with selectable markers and their transfer into mammalian cells (Traver *et al*, 1989, Markie *et al*, 1993). Hence it may be possible to clone any suppressors which are defined by this process.

Finally we should analyse chromosomes 7 and the rest of 17 for LOH in the system.

This work will go some way towards defining whether or not there are genes whose products cooperate to bring about senescence in mammalian cells, and will enhance our understanding as to whether their loss of function contributes to tumour development *in vivo*.

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