Investigation of the Role of Chromosome 7 in Human Cell Immortalisation and Cancer

Mary Berrington, BA

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CRC Beatson Laboratories

Beatson Institute for Cancer Research

Glasgow

&

Faculty of Medicine

University of Glasgow

Contents

List of Tables and Figures

Chapter 4

Chapter 5

Appendix

Declaration

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

Abbreviations

ALT	alternative lengthening of telomeres
AT	ataxia telangiectasia
BAC	B1 artificial chromosome
BICR	Beatson Institute for Cancer Research
c-	cellular
CDK	cyclin dependent kinase
CHLC	Cooperative Human Linkage Centre
CRC	Cancer Research Campaign
DDS	Denys-Drash syndrome
ER	oestrogen receptor
EST	expressed sequence tag
FAP	familial adenomatous polyposis
FISH	fluorescent in situ hybridisation
GAP	GTPase activating protein
GFP	green fluorescent protein
GSE	genome suppressor element
HNPCC	hereditary non-polyposis colon cancer
HPV	human papilloma virus
HSP	heat shock protein
ICRF	Imperial Cancer Research Fund
LOH	loss of heterozygosity
LTR	long-terminal repeat
M1	mortality stage 1
M2	mortality stage 2
MEF	mouse embryonic fibroblast
MEN	multiple endocrine neoplasia
MMCT	microcell mediated monochromosome transfer
MMR	mismatch repair
NER	nucleotide excision repair
p	protein
PAC	P1 artificial chromosome

PCR	polymerase chain reaction
RDA	representational difference analysis
RFLP	restriction fragment length polymorphism
SAGE	serial analysis of gene expression
SNP	single nucleotide polymorphism
SSCP	single strand conformation polymorphism
SSR	simple sequence repeat
TRF	terminal repeat fragment
TUNEL	terminal uracil nucleotide end labelling
UV	ultra violet
v-	viral
VHL	von Hippel-Lindau
WAGR	Wilm's tumour, aniridia, genito-urinary abnormalities and mental retardation
YAC	yeast artificial chromosome

Reagents

APS	ammonium persulphate
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
DAB	diaminobenzidine
dH_2O	de-ionised water
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	3′ deoxyribonucleoside 5′-triphosphate
EDTA	ethylenediaminetetra-acetic acid
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
IgG	immunoglobulin G
LB	Luria broth
MES	2-(N-morpholino) ethansulfonic acid
mRNA	messenger ribonucleic acid

PBS	phosphate buffered saline
PEG	polyethylene glycol
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
TAE	tris, acetic acid, ethylenediaminetetra-acetic acid
TBE	tris, boric acid, ethylenediaminetetra-acetic acid
TE	tris, ethylenediaminetetra-acetic acid
TEMED	tetramethylenediamine
Tris	2-amino-2-(hydroxymethyl)propane-1, 3-diol

Units

bp	base pair
Ci	Curie
cm	centimetre
Da	dalton
g	gram
g	gravity
hr	hour
k	kilo
l	litre
M	mega
μ	micro
m	milli
m	metre
M	molar
min	min
n	nano
°C	degree Celsius
PD	population doublings
rpm	revolutions per minute
RT	room temperature
s	second
U	unit

v/v	volume for volume
w/v	weight for volume

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Abstract

High frequencies of loss of heterozygosity have been observed at the q31 region of chromosome 7 in a variety of tumour types, suggesting that a multi-tissue tumour suppressor gene maps to this region. Introduction of an intact copy of human chromosome 7 into non-tumourigenic cell lines that have lost 7q31 markers and belong to immortality complementation group D causes a proportion of hybrids to senesce. This indicates that the group D senescence gene maps to chromosome 7. Moreover, this gene may be the target of losses observed in tumours.

To further investigate these effects I have used the microcell mediated monochromosome transfer technique to introduce chromosome 7 into the ovarian tumour cell line OVCAR5 and the breast tumour cell line MDA-MB-231. I had previously shown that both these cell lines are hemizygous for chromosome 7q and belong to senescence complementation group D as determined by mortalin staining and cell-cell fusion analysis. Following microcell transfer approximately 30% of hybrids of each cell type underwent a rapid growth arrest. Growth-arrested hybrids stained positive for senescence-associated endogenous β-galactosidase activity and showed reversion of the mortality marker mortalin to its mortal distribution. In total 79 immortal hybrids, which have presumably deleted or otherwise inactivated the senescence gene on the introduced chromosome, were also generated. PCR-based microsatellite deletion analysis demonstrated that more than half of these had lost markers from the introduced chromosome, but no single region of loss was identified.

To investigate whether the 7q senescence and tumour suppressor gene are one and the same, I compared the growth in soft agar and the tumourigenicity in nude mice of three immortal OVCAR5/Chromosome 7 hybrids—specifically hybrids for which no microsatellite losses had been found—to that of the parent cell line. A reduced tumourigenicity of these hybrids would suggest that the senescence-inducing and tumour suppressive effects of the chromosome are separable. Although two of the hybrids did indeed exhibit reduced colony formation in soft agar and delayed tumour formation in nude mice, interpretation of these results was clouded by the high level of clonal variation found within the cell line as a whole.

To circumvent this problem I investigated the soft agar growth and tumourigenicity of a panel of OVCAR5/Chromosome 7 hybrids that I had derived from the same single OVCAR5 cell clone. Five of the six hybrids generated showed a

marked reduction in colony formation in soft agar compared to the parent cell clone. Three of these hybrids were then tested for tumourigenicity in nude mice: two displayed a significant delay in tumour formation. These results suggest that there is a gene on chromosome 7, independent of the senescence gene, that can suppress the tumourigenicity of the OVCAR5 cell line. The challenge now is to identify these genes and to assess their relative contributions to tumour suppression and tumourigenicity.

CHAPTER 1

INTRODUCTION

1. Introduction

1.1 Cancer as a genetic disease: oncogenes and tumour suppressor genes

Cancer is a genetic disease, arising from the progressive accumulation of mutations that promote the clonal selection of cells with increasingly deregulated growth. Most of these alterations are somatically acquired, but some are inherited. During the last two decades many of the genetic targets of the mutations have been identified. However, except for one or two notable examples little is known about the precise combination of genetic alterations required to produce a particular type of tumour and of what determines the tissue specificity of these changes. Moreover, the cancer phenotype is a result of complex gene interactions, not only involving the known cancer gene products but also a multitude of other proteins. A great many 'modifier' and low penetrance cancer susceptibility genes are still to be discovered—the individual impact of any one of these genes is likely to be small compared to that of the known cancer genes, but collectively their variant alleles may hugely influence an individual's cancer risk. Additionally, all these interactions are subject to modification by poorly understood environmental factors. The disentangling of all such interrelations clearly poses a tough challenge to cancer biologists.

Cancer genes fall into two broad classes: proto-oncogenes, which are positive regulators of cell proliferation, survival, invasion and metastasis, and tumour suppressor genes, which are negative regulators. The coordinated regulation of these diametrically opposed gene classes is responsible for maintaining tissue homeostasis; cancer reflects a fundamental breakdown in this control. The types of alteration that occur in proto-oncogenes and tumour suppressor genes display intrinsic differences. Point mutations in proto-oncogenes are usually missense and tend to activate—or give new functions to—the gene product. Alternatively proto-oncogenes can be subject to amplification or to translocations that put them under the control of active promoter sequences or that generate a fusion product with new or constitutive function. Tumour suppressor genes, on the other hand, are subject to loss of function alterations, most commonly through deletions or through point mutations that truncate the protein product or alter a crucial functional domain. These differences partially account for the dominant transforming ability of oncogenes and the recessiveness (at the cellular level) of tumour suppressors.

1.1.1 Oncogenes

The identification of oncogenes began with studies of acutely transforming retroviruses. Although not oncogenic in humans, these can rapidly induce tumours in animals and transform cells *in vitro*. The discovery that *v-src*, the transforming gene of the Rous sarcoma virus, has a counterpart in the cellular gene *c-src* was groundbreaking in this field (Stehelin et al., 1976). Since then, it has transpired that nearly all retroviral oncogenes are transduced cellular proto-oncogenes whose expression and activity is increased as a consequence of the transduction process. Many DNA viruses of the adenovirus, herpesvirus, poxvirus and papovavirus families also encode oncoproteins; however, the transforming genes rarely have proto-oncogene homologues within the normal genome. Chronic transforming retroviruses (such as mouse mammary tumour virus and avian leukosis virus) also possess no cellular sequences. These viruses induce neoplastic transformation after a latency period of months or years, via the integration of proviral DNA in such a way as to activate specific host target genes: cellular genes are either brought under the control of the viral LTR or are fused to viral sequences with the consequent production of novel proteins (Hayward et al., 1981). This knowledge led to the prospective use of insertional mutagenesis as a means of identifying novel proto-oncogenes that are consistent targets for proviral insertion (Nusse and Varmus, 1982). It was suggested that *spontaneous* alteration of any of these genes might account for tumours—including most human tumours—whose initiation is virus-independent; the finding that around one in five tumours possesses genomic sequences with intrinsic transforming ability gave weight to this (Shih et al., 1981). Indeed, tumour gene transfer experiments identified oncogenes already known to be the cellular counterparts of various retroviral oncogenes, in addition to several novel genes (many of which belonged to the *src* and *ras* superfamilies). The presence of activating alterations in many of these cellular genes was subsequently demonstrated (Santos et al., 1982; Tabin et al., 1982; Taparowsky et al., 1982). Other oncogenes were identified directly through their association with translocations, which caused activation either by the generation of new fusion products or by bringing the proto-oncogene under the transcriptional control of nearby enhancer elements (Adams et al., 1983; Gale and Canaani 1984). Probes and primers derived from the oncogenes identified in these various ways were then used to isolate further, homologous, genes by cross-hybridisation or PCR. Table 1.1 summarises some of the oncogenes identified by these various methods.

The precise location of oncogenic mutations often gave the first indication of the important functional domains of the protein (McKay et al., 1986). Genetic, biochemical and cellular studies in both humans and model organisms have further illuminated the functions of these genes. The majority of proto-oncogenes are now known to be components of signal transduction pathways, which are responsible for translating extracellular signals into nuclear changes in gene expression. Activation of proto-oncogenes (and inactivation of tumour suppressor genes) results in the deregulation of these pathways, such that aspects of cellular behaviour—for example proliferation and survival—become autonomous. Proto-oncogenes have been discovered at most stages of the signal transduction pathways, acting as (among others) ligands, receptors, membrane-associated tyrosine kinases, lipid kinases, cytoplasmic regulators, G proteins, kinases, transcription factors and transcriptional co-activators—Table 1.2 classifies oncoproteins according to these biochemical roles in signal transduction. A small subset of proto-oncogenes are more directly involved in cellular proliferation and survival, and function as components of the cell cycle or anti-apoptotic machinery. *CCND1* and *CDK4*, encoding cyclin D1 and cyclin dependent kinase 4 (CDK4) respectively, have both been found to be activated in tumours (Hall and Peters, 1996). The anti-apoptotic gene *Bcl-2* is another target for activation, and was first identified at the breakpoint of translocations in follicular lymphomas (Bakhshi et al., 1985; Tsujimoto et al., 1985). An important consequence of oncogenic activation, therefore, is to uncouple cell proliferation and survival from regulation by extrinsic factors; this can be achieved either by constitutive activation of mitogenic signalling pathways or through direct stimulation of cell cycle progression or cellular survival.

Certain oncogenes are important in the advanced stages of tumourigenesis. A variety of proto-oncogenes—often also components of signal transduction pathways—are involved in the regulation of the actin cytoskeleton, of cell adhesion, and of the extracellular matrix. Deregulation of these facets via oncogene activation promotes the cell migration and substrate independence required for tumour cell invasion and metastasis. Other oncogenes are thought to have a role in the process of angiogenesis, the induction of new blood vessel growth from existing vessels; this process is probably essential if solid tumours are to enlarge beyond around one millimetre square.

Proto-oncogene activation probably contributes to the development of all sporadic cancers. Activating *germline* mutations in proto-oncogenes are likely to be lethal, but mutations in a limited number of proto-oncogenes are heritable and give rise

to familial cancer syndromes. Germ-line mutations in *MET*, *RET* and *CDK4*, for example, are respectively responsible for hereditary papillary renal cancer, multiple endocrine neoplasia type 2 (MEN2), and some cases of familial melanoma (Mulligan et al., 1993; Zuo et al., 1996; Schmidt et al., 1997). However, the majority of cancer syndromes are caused by germline mutations in recessive-acting tumour suppressor genes, where loss of the remaining wild-type allele is the initiating transforming event (see Section 1.1.2.1). Even here, progression to full tumourigenicity requires further mutations in additional, probably multiple, proto-oncogenes and tumour suppressors.

1.1.2 Tumour suppressor genes

1.1.2.1 Origins of the tumour suppressor gene concept: the study of hereditary cancer syndromes

That cancer might arise from the effects of negative as well as positive regulators of cell growth was first suggested early this century (Boveri, 1914). The modern day concept of tumour suppressor genes, however, stems from two more recent lines of enquiry. First, somatic cell fusion studies demonstrated that hybrid cells formed by fusing tumourigenic with non-tumourigenic cells *could* give rise to tumours in suitable hosts, but only following the ejection of particular chromosomes (Reviewed in Harris, 1988). This provided the first real evidence that tumourigenicity is suppressed by specific genetic sequences, and that neoplastic transformation results (at least partly) from the loss of function of normal alleles. Analysis of the chromosome loss incurred in tumourigenic variants of suppressed hybrids allowed putative tumour suppressor genes to be mapped to specific chromosomes (Stoler & Bouck, 1985).

Studies of hereditary cancer syndromes further ensconced the concept of genes that negatively regulate tumour development, and led to the first cloning of a tumour suppressor gene. Although cancer that is unmistakably hereditary accounts for only 1% of all cancer cases, the genes that are altered are frequently mutated in sporadic tumours too. The childhood ocular tumour retinoblastoma is unusual in that around 40% of cases are inherited. Like most hereditary cancer syndromes, the inheritance pattern is autosomal dominant. The ground-breaking achievement of Alfred Knudson was to account for this inheritance in terms of recessive, loss-of-function mutations in a putative tumour suppressor gene (Knudson, 1971). He suggested that members of families exhibiting a predisposition to retinoblastoma transmit an initial mutation in their germline; the acquisition of a further, somatic mutation that inactivates the

remaining allele constitutes the initiating, rate-limiting step in development of the tumour. In cases of retinoblastoma where there is no family history (that typically occur in slightly older children), Knudson postulated that two independent somatic mutations needed to occur in a single retinoblast. This 'two-hit' hypothesis was based purely on epidemiological data. However, the finding that a proportion of individuals who inherit retinoblastoma possess a deletion in all their cells at chromosome 13q14 provided clear experimental evidence of the inherited alteration—the 'first hit' (Yunis & Ramsay, 1978). Subsequently, the demonstration of 'loss of heterozygosity' (LOH) of polymorphic markers at 13q14 in both familial and sporadic tumours gave evidence for the second hit, arising via chromosome loss, deletion, mitotic recombination or gene conversion (Cavenee et al., 1983; Knudson, 1978). The fact that this second hit occurs in almost all carriers of the germline mutation accounts for retinoblastoma's apparent dominant inheritance. A particular probe mapping to chromosome 13q14 identified a homozygous deletion in two tumours (Friend et al., 1986). This sequence was used in a 'chromosome walk' to identify sequences that were expressed in normal retinoblasts but not in retinoblastomas, and thus paved the way for isolation of the *RB1* gene.

Following the demonstration of internal deletions within the *RB1* gene and of the ability of normal *RB1* cDNA to revert the tumourigenic properties of mutant tumour cells in culture, *RB1* was universally acknowledged as a true tumour suppressor gene (Friend et al., 1986; Huang et al., 1988). Several different types of genetic lesion have been shown to inactivate the *RB1* locus, including large and small scale deletions, splicing mutations and point mutations (Bookstein et al., 1990a; Horowitz et al., 1989; Kaye et al., 1990). *RB1* is now known to encode a ubiquitously expressed, 105kDa nuclear phosphoprotein called pRB (Lee et al., 1987), which appears to function as a negative regulator of cell cycle progression (see Section 1.1.2.3). The importance of this role is reflected in the fact that *RB1* mutations have also been found in a wide variety of sporadic tumours. These include sarcomas, small cell carcinomas of the lung, bladder carcinomas and breast carcinomas (Horowitz et al., 1990; Lee et al., 1988). Interestingly, the proteins p107 and p130 are structurally and functionally similar to pRB (Reviewed in Mulligan & Jacks, 1998). The involvement of these proteins in tumour suppression, however, remains uncertain.

More than 20 different hereditary cancer syndromes have now been attributed to germline mutations in specific tumour suppressor genes (Table 1.3). These tumour suppressors usually follow the 'two hit' paradigm, with inactivation of the second copy

of the gene being the initiating, rate-limiting event in tumourigenesis. The acquisition of additional mutations in oncogenes and tumour suppressors is then required for conversion to full malignancy. Like *RB1*, many of the tumour suppressor genes unearthed through the study of hereditary cancer syndromes are also now known to be inactivated in sporadic tumours. Again in line with Knudson's prediction, this usually requires the acquisition of two somatic mutations, the second mutation arising in a descendant of the cell that incurred the first. Such a sequence of mutational events is likely to occur only rarely, thus accounting for the fact that sporadic tumours tend to occur singly and relatively late in life. Patients with an inherited cancer syndrome, on the other hand, often present with multiple tumours at a young age.

1.1.2.2 Departure of tumour suppressor genes from the classic retinoblastoma model

Tumour suppressor genes do not always follow the classic Knudson paradigm, and can differ from the retinoblastoma model in several ways. In some cases, inactivation of one allele only appears sufficient for tumour initiation. The *WT1* gene has been implicated in the growth abnormality syndromes WAGR and DDS, of which the paediatric nephroblastoma Wilm's tumour is one manifestation, as well as in isolated inherited, and some sporadic, Wilm's tumour cases (Little & Wells, 1997; Pelletier et al., 1991). WAGR syndrome patients usually display large, constitutional deletions spanning the *WT1* gene and the nearby *PAX6* gene. Not all tumours from these patients, however, show a somatic mutation in the remaining *WT1* allele (Little & Wells, 1997). In addition, the non-tumour manifestations of the growth abnormality syndromes mentioned above are associated with germline *WT1* mutation (or deletion) only. The *TP53* gene may also be haploinsufficient for tumour suppression in some instances, studies of murine *p53* heterozygotes indicating that tumourigenesis does not always require loss of the second allele—again, gene dosage appears important (Venkatachalam et al., 1998). Mice heterozyous for the murine tumour suppressor gene $p27^{Kip1}$ are also predisposed to cancer, the wild type allele being neither mutated nor silenced in tumours (Fero et al., 1998). As a CDK inhibitor $p27^{Kip1}$ is a candidate human tumour suppressor (Coats et al., 1996); indeed, many tumours display low levels of the protein (Porter et al., 1997; Yasui et al., 1999; Yatabe et al., 1998), and hemizygous alterations of the chromosomal region to which it maps have been observed (Spirin et al., 1996; Stegmaier et al., 1996; Takeuchi et al., 1996).

Heritable retinoblastoma is a phenotypically simple disease. Some heritable cancer syndromes, however, are characterised by extensive phenotypic variation in terms of penetrance, severity of symptoms, tumour type and age of onset. von Hippel-Lindau (VHL) disease, which predisposes to kidney, brain and eye neoplasms, and familial adenomatous polyposis (FAP), which predisposes to colon cancer, both display such variation. These syndromes are associated with germline mutations in the genes *VHL* and *APC* respectively. In each of these genes, certain mutations are known to give rise to particular phenotypes (Reviewed in Kaelin & Maher, 1998; Kinzler & Vogelstein, 1996); this 'allelic heterogeneity' accounts for some—but not all—of the variability in symptoms (although clear molecular explanations for the genotype-phenotype relationships are lacking). Extensive phenotypic variation also exists within the multiple endocrine neoplasia syndrome MEN1. However, in this case there are no clear correlations between specific mutations within the *MEN1* gene (the functions of which little is known) and the clinical manifestations of the disorder (Reviewed in Thakker, 1998). This phenotypic variation—and much of that of the VHL and FAP syndromes—is presumably attributable to variant alleles of modifier genes and to environmental factors. Such variables are likely to be equally important in determining susceptibility to (and clinical outcome of) sporadic tumours.

The correlation of particular mutations with variations in phenotype implies that alterations of tumour suppressor genes more subtle than simple, complete loss of function mutations can contribute to cancer susceptibility. As further evidence of this, polymorphisms within the *TP53* coding sequence may confer differential susceptibility to oncogenesis (Buller et al., 1997; Wang-Gohrke et al., 1998). Moreover, a germline 'mutation' of *APC* found in Ashkenazi Jews does not alter the function of the encoded protein, but generates a hypermutable tract in the coding sequence such that somatic mutations near it arise at increased frequency (Laken et al., 1997). These subtle variations in known cancer genes may account for some familial cancer clusterings that do not show simple Mendelian inheritance. A variety of undiscovered low penetrance susceptibility and modifier genes are also likely to contribute to such aggregations.

RB1 appears to be inactivated in almost all retinoblastomas, be they sporadic or familial (Horowitz et al., 1990). Many tumour suppressor genes isolated through study of a hereditary cancer syndrome, however, are inactivated in only a subset of sporadic tumours of the same type. Only around 10% of sporadic Wilm's tumours, for example, show mutation or deletion of *WT1* (Little & Wells, 1997). Indeed, a second *WT* locus

has been identified on chromosome 11p15 that is implicated in some sporadic Wilm's tumours and in the growth abnormality syndrome BWS, and five other loci are also thought to harbour genes involved in the biology of this tumour (Reviewed in Coppes & Egeler, 1999). Germline mutations in *BRCA1* and *BRCA2* predispose to female breast and ovarian cancer and to male & female breast cancer respectively, but mutations in these genes are very rarely observed in sporadic breast and ovarian tumours (Futreal et al., 1994; Lancaster et al., 1996). It is in fact now widely accepted that inherited and sporadic breast tumours develop through very different mutational pathways (see Section 1.1.2.3).

Perhaps more fundamentally, the dogma that tumour suppressors act to suppress proliferation or promote apoptosis can also be questioned in some instances. For example, while some studies support the hypothesis that WT1 plays a tumour suppressing, anti-proliferative role, other analyses suggest that the wild-type protein can promote proliferation and suppress apoptosis. (Yamagami et al., 1998). Moreover, the presence of wild-type WT1 in acute myeloid leukaemia has been proposed by some as an indicator of poor prognosis and drug resistance (Bergmann et al., 1997; King-Underwood & Pritchard-Jones, 1998). The prototypical tumour suppressor pRB has also been demonstrated to suppress apoptosis in some circumstances (Morgenbesser et al., 1994; Whitaker & Hansen, 1997). Interestingly, there is evidence to suggest that some *TP53* mutations in tumours are in fact gain-of-function mutations, that endow the protein with new properties. One such activating mutation may promote genetic instability by disrupting spindle checkpoint control, thus promoting—rather than inhibiting—tumour progression (Gualberto et al., 1998). The existence of such mutations might partly account for the fact that different classes of *TP53* mutations have different effects on resistance of tumour cells to chemotherapy (Blandino, Levine & Oren, 1999).

The Knudson paradigm has provided a useful framework upon which much of our current understanding of tumour suppressor genes rests. Not surprisingly, however, it has proved too simplistic in some instances. As alluded to in Section 1.1.2.4, novel approaches to the isolation of cancer genes will almost certainly result in the identification of an increasing number of genes whose inactivation does not fulfil classical tumour suppressor gene criteria.

1.1.2.3 Gatekeepers and Caretakers

Some rare syndromes of which an increased cancer risk is one manifestation differ from the retinoblastoma model in that they have a recessive mode of inheritance. These syndromes include ataxia telangiectasia (susceptibility to lymphoma and breast cancer), Bloom's syndrome (susceptibility to many solid tumours), xeroderma pigmentosum (susceptibility to skin cancer caused by heightened sensitivity to UV-irradiation) and Werner's syndrome (susceptibility to multiple tumour types). Although these syndromes predispose to different types of tumour, they all result from germ-line mutations in genes responsible for maintaining genomic integrity. Xeroderma pigmentosum sufferers inherit a mutation in one of the nucleotide excision repair (NER) genes (Hoeijmakers, 1993), Bloom's and Werner's sufferers in the DNA helicases BLM and WRN respectively (Ellis et al., 1995; Yu et al., 1996), and ataxia telangiectasia sufferers in the DNA damage response gene ATM (Savitsky et al., 1995). These defects, although not directly responsible for tumour development, increase the chances of mutations arising in those genes that are.

In view of these findings, Kinzler and Vogelstein proposed that tumour suppressor genes should be classified as either 'gatekeepers' or 'caretakers' (Kinzler & Vogelstein, 1997). Gatekeepers directly inhibit cell proliferation or promote cell death, and their inactivation provides cells with a direct growth advantage. Each cell type appears to have only one or a few gatekeepers, with inactivation of a particular gatekeeper leading to very specific types of tumour. Inactivation of *RB1*, *APC* and *NF1*, for example, constitutes the initiating, rate limiting step in development of retinoblastomas, colon tumours and Schwann tumours respectively. Although mutations in other cancer genes clearly play a role in the progression of these tumours, such mutations do not appear to be able to efficiently *initiate* the neoplastic process (Garber et al., 1991; Jen et al., 1994). Caretaker genes are involved in maintaining genetic stability and preventing mutation. Unlike gatekeepers, inactivation of a caretaker gene does not directly promote tumour initiation. Instead, their disruption gives rise to a 'mutator phenotype', where the observed mutation frequency of all genes—including cancer gatekeepers—is increased. The fact that tumour initiation depends upon the acquisition of further mutations is thought to partly account for the non-dominant pattern of inheritance of the cancer syndromes referred to above.

Undisputed gatekeepers include the prototypical tumour suppressor pRB, which acts to inhibit progression through the cell cycle. pRB is hypophosphorylated during G0

and early G1, but from mid to late G1 becomes increasingly phosphorylated by cyclin dependent kinases (CDKs) (Lundberg & Weinberg, 1998). Hypophosphorylated pRB binds to the transcription factor E2F, forming a complex that actively represses the transcription of genes required for S phase entry; recruitment of either a histone deacetylase or a CtIP/CtBP co-repressor complex appears to mediate this repression (Luo, Postigo & Dean, 1998; Meloni, Smith & Nevins, 1999). Hyperphosphorylation of pRB disrupts the pRB/E2F repression complex, allowing transcription of S phase genes and G1 to S phase progression (Harbour et al., 1999). Thus pRB plays a central role in cell-cycle regulation. In view of this, it is perplexing that germline *RB1* mutations predispose carriers to only retinoblastoma and osteosarcoma; the reasons for the tissue-specificity of pRB's gatekeeping functions have yet to be elucidated, but redundancy of function in non-affected tissues may offer a partial explanation. The significance in cancer development of the pRB/E2F cell cycle control pathway is further emphasised by the frequent inactivation in tumours of the tumour suppressor $p16^{INK4a}$ (Reviewed in Pollock, Pearson & Hayward, 1996). The $p16^{INK4a}$ protein binds to and inhibits the cyclin D dependent kinases CDK4 and CDK6, thus negatively regulating pRB phosphorylation (Sherr & Roberts, 1995). In addition to its involvement in sporadic tumours, germline mutations in $p16^{INK4A}$ are associated with familial melanoma and familial pancreatic adenocarcinoma (Hussussian et al., 1994; Whelan, Bartsch & Goodfellow, 1995).

Abnormalities of the p53 tumour suppressor constitute one of the most common cancer defects. Germline mutation of *TP53* is associated with Li Fraumeni syndrome, an autosomal dominant predisposition to cancers of the breast, brain, bone, adrenal cortex, soft tissues and haematopoietic system (Malkin et al., 1990). Mutation or deletion of *TP53* is also displayed by a huge array of sporadic tumours (Hainaut et al., 1998). The p53 protein is a modular transcription factor that integrates signals arising from a wide array of cellular stresses (such as DNA damage, hypoxia, hyperoxia and heat shock) and elicits an appropriate response via activation or repression of specific downstream effector genes (Reviewed in Prives & Hall, 1999). The p53-mediated response to stress is usually one of apoptosis, G1 or G2 arrest or induction of differentiation, the precise outcome being highly dependent upon poorly understood factors specific to the cell or tissue type. p53 activity is subject to many levels of regulation, mediated by protein-protein interactions, post-translational modification and (probably) subcellular localisation (Reviewed in Prives & Hall, 1999). The best

characterised p53-activating cellular stress is DNA damage, and in the absence of a p53-mediated response genetic lesions induced by DNA damaging agents more readily lead to neoplastic transformation (Kemp, Wheldon & Balmain, 1994). Many of the diverse array of cellular functions that p53 has been implicated in thus concern the maintenance of genomic integrity; the fact that loss of p53 leads to increased genomic instability argues in favour of a caretaker role for this protein. However, overexpression of p53 suppresses transformation (Finlay, Hinds & Levine, 1989), and its loss can clearly endow cells with a *direct* proliferative and survival advantage. These features indicate that the inactivation of p53 could well constitute the initiating step of tumourigenesis. Two mammalian homologues of p53, p73 and p63 (originally named KET) have been identified in recent years which also appear to function as transcription factors (Kaghad et al., 1997; Schmale & Bamberger, 1997). There is currently no conclusive evidence that either homologue functions as a tumour suppressor (Kovalev et al., 1998), and it is unclear whether the three gene products activate the same, overlapping, or different target genes. Two further, as yet largely uncharacterised, proteins also show strong similarity to p53 (Bian & Sun, 1997; Zeng, Levine & Lu, 1998).

p53 has also been implicated in oncogene-induced apoptosis and cell cycle arrest (see Section 1.2.5), responses that may serve as a tumour suppressive adaptation to inappropriate proliferative signals. It is unclear whether loss of this function or of the DNA damage response pathway is of most importance to tumourigenesis. The p53 response to oncogene expression is mediated by $p14^{ARF}$ (Sections 1.2.1 and 1.2.5), which may also represent a bona fide tumour suppressor gene. A role of $p14^{ARF}$ in tumour suppression has been hard to demonstrate conclusively, since its coding sequence overlaps that of $p16^{INK4a}$ (Mao et al., 1995); the deletions of the locus that are very commonly found in cancer affect both genes. The fact that few point mutations within the locus appear to specifically affect $p14^{ARF}$, while many have been shown to specifically affect $p16^{INK4a}$, argues against a tumour suppressor function for $p14^{ARF}$ (Munro et al., 1999; Sharpless & DePinho, 1999). However, mice null for $p19^{ARF}$ (the murine homologue of $p14^{ARF}$) that retain functional $p16^{INK4a}$ are highly tumour prone (Kamijo et al., 1997). This, together with the very high frequency of homozygous deletion of the *INK4A/ARF* locus in sporadic tumours, suggests that $p14^{ARF}$ loss may yet be considered to play an important role in tumour progression in humans.

Another presumed gatekeeper is the product of the *APC* gene. In addition to the association of germline *APC* mutations with FAP (sufferers of which develop hundreds

of intestinal polyps, a very small number of which will progress to malignancy), somatic *APC* mutations are present in the majority of sporadic intestinal adenomas and carcinomas (Powell et al., 1992). The APC protein functions as a negative regulator of β-catenin, which was originally identified as a cadherin binding protein. APC targets free β-catenin (that which is not sequestered by E-cadherin) for degradation, via complex-formation with glycogen synthase kinase 3 (GSK3, a component of the WNT signalling pathway) and Axin; free β-catenin is phosphorylated by GSK3 and subsequently degraded by the ubiquitin-proteasome pathway (Munemitsu et al., 1995; Nakamura et al., 1998; Rubinfeld et al., 1996). β-catenin is stabilised in response to either WNT signalling (via inhibition of GSK3) or APC inactivation; in addition, activating mutations of β-catenin have been observed in a subset of tumours that lack *APC* mutations (Kolligs et al., 1999; Morin et al., 1997). Accumulation of β-catenin allows it to complex with the Tcf-4 transcription factor and induce the expression of Tcf-4 regulated genes. APC thus regulates cellular levels of β-catenin and consequently the formation of active β-catenin/Tcf-4 transcription complexes. The identity of the β-catenin/Tcf-4 target genes is an area of intense investigation. Recent studies have demonstrated Tcf-4/β-catenin-mediated upregulation of the proto-oncogene c-MYC following loss of APC function, thus providing a functional connection between APC inactivation and the hyperproliferation of colon endothelial cells (He et al., 1998; Pignatelli, 1999). It is also possible that the Tcf-4/β-catenin complex represses expression of E-cadherin (Huber, Bierkamp & Kemler, 1996), whose cell-cell adhesion function is known to be lost in most epithelial cancers (Birchmeier & Behrens, 1994). (Loss of sequestration of β-catenin by E-cadherin may lead in turn to accumulation of free β-catenin and Tcf-4 activation (Fagotto et al., 1996).) Significantly, loss of cell-cell adhesion characterises the progression of benign lesions to invasive, metastatic tumours. Deletion or mutation of the E-cadherin gene has been observed in several tumours (Guilford et al., 1998; Huber et al., 1996); it may therefore itself represent a bone fide tumour suppressor gene. Negative regulation of the β-catenin/Tcf-4 pathway is unlikely to be the only means by which APC exerts its tumour suppressive effects. In particular, the observation that *APC* germline mutations are frequent in FAP patients while β-catenin germline mutations are rare suggests that β-catenin mutation cannot substitute for *APC* mutation in the initiation of FAP (Cao et al., 1999). APC is known to upregulate the differentiation-inducing *CDX2* gene in a manner that is not directly

mediated by β-catenin/Tcf-4; in rare cases of colorectal cancer where the APC/β-catenin pathway is intact, mutation of *CDX2* or of other APC regulated genes may constitute the initiating event (da Costa et al., 1999). An APC homologue known as APC2 has recently been identified; APC2 can also regulate the formation of active β-catenin/Tcf complexes (Nakagawa et al., 1998; van Es et al., 1999). Any role of APC2 in cancer, however, has yet to be elucidated.

Other tumour suppressor gene products also appear to be involved in specific signalling pathways. Germline mutations in the *NF1* gene are associated with the neurofibromatosis type 1 syndrome (Cawthon et al., 1990; Viskochil et al., 1990). Neurofibromin, the *NF1* gene product, contains a GTPase-activating protein (GAP)-related domain and is a major negative regulator of the Ras signal transduction pathway; its loss leads to increased levels of activated Ras and constitutive downstream mitogenic signalling (Feldkamp, Gutmann & Guha, 1998). The tumour suppressor PTEN is another negative regulator of signal transduction. The *PTEN* gene is deleted or mutated in a wide variety of sporadic tumours (Steck et al., 1997), and has also been implicated in the Cowden tumour susceptibility syndrome (Liaw et al., 1997). PTEN functions as a phosphatase, modulating signal transduction pathways that involve lipid second messengers. Specifically, PTEN is thought to dephosphorylate insulin-induced phosphatidylinositol (3, 4, 5) trisphosphate (Maehama & Dixon, 1999), and may thereby inhibit activation of the AKT protein kinase and the subsequent generation of AKT-induced cell survival signals (Coffer, Jin & Woodgett, 1998). The PTCH tumour suppressor also modulates a signal transduction pathway, but is unusual in that it encodes a transmembrane protein. PTCH is known to suppress the sonic hedgehog signalling pathway, but the target genes activated in its absence in tumours are mainly unknown (Ingham, 1998). Germline mutation of *PTCH* is associated with Gorlin's syndrome, which predisposes to basal cell carcinomas, medullablastomas or meningiomas, while somatic *PTCH* mutations are found in some sporadic basal cell carcinomas (Gailani et al., 1996; Johnson et al., 1996). Rather than negatively regulating a mitogenic or anti-apoptotic signalling pathway, the DPC4/Smad4 tumour suppressor (a homologue of the Drosophila mothers against dpp (MAD) protein) is a positive mediator of anti-proliferative TGFβ signalling; it probably effects the TGFβ response by inducing the transcription of TGFβ responsive genes (Duff & Clarke, 1998). Smad4 has also been linked to the SAPK/JNK cascade, thus implicating Smad4 in the control of both cell cycle arrest and apoptosis (Atfi et al., 1997). Loss of Smad4

is strongly associated with both pancreatic and colorectal malignancy (Hahn et al., 1996; Takagi et al., 1996). Interestingly, the *SMAD2* gene, which maps adjacent to *SMAD4* at chromosome 18q21, has also been implicated in tumour suppression and may be involved in the control of cellular invasion (Prunier et al., 1999).

The Wilm's tumour suppressor gene *WT1* encodes four protein isoforms that appear to act as zinc finger-containing transcription factors involved in both transactivation and repression (Haber et al., 1991; Madden et al., 1991). Many transcriptional targets have been proposed, but none have been conclusively verified in vivo (Reviewed in Little, Holmes & Walsh, 1999). Recent data suggest that WT1 may also regulate gene expression through interactions with RNA, probably via a role in gene splicing (Davies et al., 1998). In addition, WT1 binds to a variety of proteins, including p53. Again, the physiological relevance of many of these interactions remains unclear (Little et al., 1999).

The VHL tumour suppressor appears to have multiple, tissue-specific functions, with different germline mutations predisposing to different tumour types. One of the roles of the cytoplasmic protein involves inhibition of the vascular endothelial growth factor VEGF (Iliopoulos et al., 1996). VHL loss leads to inappropriate accumulation of VEGF mRNA even under normoxic conditions, possibly via constitutive activation of the oxygen-regulated transcription factor HIF1 (Maxwell et al., 1999); inactivation of VHL could thus be critical to the process of angiogenesis. The protein forms a complex with elongins B and C and the cullin protein family member Cul2 (Duan et al., 1995; Pause et al., 1997). The homology of elongin C and Cul2 with the yeast proteins Skp1 and Cdc53 suggests that the pVHL/elongins/Cul2 complex could also be involved in targeting cell cycle control proteins for ubiquitination and degradation (Pause et al., 1999).

Caretaker genes include the seven NER xeroderma pigmentosum complementation group genes *XPA-XPG*. Inactivation of the NER gene products impairs repair of UV-induced cyclobutane pyrimidine dimers and pyrimidine-pyrimidone (6-4) photoproducts, thus increasing the likelihood of mutation at the next DNA replication cycle. XPA and XPE are responsible for recognising DNA lesions, XPB and XPD are DNA helicases required for unwinding the DNA duplex at regions of damage, XPF and XPG are endonucleases responsible for excising the lesions, while XPC is a single-stranded DNA binding protein.

The BLM and WRN helicases, implicated in Bloom's syndrome and Werner's syndrome respectively, also function as caretakers. Again, genomic instability is a feature of these disorders, manifested in Bloom's syndrome as an increased frequency of chromosome breakage and chromatid exchange between homologous chromosomes and sister chromatids. In Werner's syndrome, chromosomal instability is manifested by inversion, translocation, and deletion. The rate of spontaneous mutation is increased in both syndromes; this is despite apparent normal functioning of DNA repair pathways (Warren et al., 1981; Fukuchi et al., 1989).

The product of the *ATM* gene, implicated in ataxia telangiectasia (AT), is probably another caretaker. Cells from AT individuals display an elevated level of spontaneous chromosomal breaks, are extremely sensitive to ionizing radiation and appear defective in a number of DNA-damage cell cycle checkpoints (Friedberg et al., 1995). These features of AT imply a role for ATM in the repair or processing of DNA double-strand breaks. Certain human and mouse cells with *ATM* defects fail to upregulate p53 on induction of DNA damage, suggesting that ATM lies upstream of p53 in the p53 DNA damage-recognition pathway (Kastan et al., 1992; Xu et al., 1996; Westphal et al., 1997). Indeed, it is now know that ATM functions as a kinase and is capable of phosphorylating p53 in response to DNA damage (Banin et al., 1998; Canman et al., 1998). ATM belongs to the same family as DNA-dependent protein kinase DNA-PK, which is implicated in non-homologous end-joining of double-strand DNA breaks and which also acts upstream of p53 in the DNA damage response pathway (Woo et al., 1998). ATM is also related to the MEC1 and TEL1 proteins of *S. cerevisiae*, Rad3 of *S. pombe*, and MEI-41 of *D. melanogaster*; these are all involved in DNA-damage sensitive checkpoint controls and cause repair deficient phenotypes when mutated.

Mismatch repair (MMR) genes also fall under the caretaker category. Germline mutations in the MMR genes *MLH1*, *PMS1*, *PMS2* and, particularly, *MSH2*, are associated with the dominant inherited syndrome of nonpolyposis colorectal cancer (HNPCC); in contrast to FAP, HNPCC sufferers typically develop only one or two intestinal polyps, each of which rapidly progresses to malignancy. Not surprisingly, HNPCC tumour cells have an increased spontaneous mutation rate and show microsatellite length heterogeneity. The latter is also observed in some *sporadic* colorectal tumours and in certain other sporadic tumour types; such tumours are often associated with *somatic* mutation of *MSH2* (Bubb et al., 1996; Risinger et al., 1995).

The gatekeeper/caretaker classification of other tumour suppressor genes is less clear-cut, partly because the functions of many tumour suppressor genes are not yet well defined, and partly because the same gene may be able to act as both gatekeeper and caretaker depending on the circumstances. BRCA1 and BRCA2, for example, both possess a transactivation domain that is compromised by mutations found in *BRCA1* and *BRCA2* families (Chapman & Verma, 1996; Milner et al., 1997). BRCA1, moreover, can inhibit cell proliferation via induction of $p21^{WAF1}$ (Somasundaram et al., 1997), and expression of both BRCA1 and BRCA2 correlates with cell cycle progression (Ruffner & Verma, 1997; Vaughn et al., 1996). These are features that would be expected of a gatekeeper. However, BRCA1 and BRCA2 can both bind (directly or indirectly) to the RecA homologue RAD51, which is involved in homologous recombination and DNA damage repair (Scully et al., 1997; Sharan et al., 1997). Furthermore, mice embryos that are null for Brca1 or Brca2 display increased sensitivity to radiation (Sharan et al., 1997; Shen et al., 1998). These findings suggest that both BRCA1 and BRCA2 are involved in RAD51-mediated repair and recombination and may thus function as caretakers. Indeed, the generation of a conditional *Brca1* knockout in mouse mammary epithelial cells has convincingly demonstrated that *Brca1* disruption causes genetic instability and triggers further genomic changes (Xu et al., 1999). Tumours arose only after a long latency period and displayed frequent chromosomal alterations, many of which involved the *TP53* gene. Significantly, introduction of a loss-of-function *TP53* allele into the *Brca1* conditional mutant was found to accelerate tumour formation. Loss of the p53 checkpoint function may thus represent one genetic change required for Brca1-mediated tumourigenesis. Given that accumulation of p53 has been found to activate a cell cycle checkpoint and inhibit the malignancy of *Brca2* defective tumours, the same may also be true of Brca2-mediated tumourigenesis (Connor et al., 1997).

A caretaker function for these genes could explain a puzzling finding, namely the lack of somatic *BRCA1* and *BRCA2* mutations found in sporadic breast cancers. Kinzler and Vogelstein (1997) argue that mutations in caretaker genes would not necessarily be expected to occur in sporadic cancers, since tumour initiation would require the accumulation of four independent mutations—inactivation of two caretaker alleles and two gatekeeper alleles—in a single cell. Progression to full malignancy would require even more mutational events. In all likelihood, a cell would undergo terminal differentiation, replicative senescence or apoptosis before the necessary number of mutations had been acquired. If this scenario holds true then familial and sporadic

breast tumours must arise through distinct mutational pathways, and inherent differences should exist between the aetiology of the two tumour types. A number of observations indicate that the genetic makeup of familial and sporadic breast tumours does indeed differ. Most fundamentally, breast tumours of patients with a germline *BRCA1* or *BRCA2* mutation display a greater number of genetic defects than sporadic breast tumours (Tirkkonen et al., 1997). As in the mouse Brca1 model, these defects include an increase in *TP53* alterations (Crook et al., 1997; Eiriksdottir et al., 1998). In summary, these findings are fully consistent with a caretaker role of BRCA1 and BRCA2.

Tumour suppressor genes thus constitute critical points in a variety of complex cellular pathways. These pathways control proliferation, differentiation, apoptosis, response to DNA damage or mutation, angiogenesis and cell-cell adhesion. As discussed later tumour suppressor genes are also involved in replicative senescence, the process that limits the proliferative lifespan of normal somatic cells but which is abrogated in most malignancies. Another cellular property that is altered in tumours is that of gap junctional intercellular communication; genes with a role in this aspect of cell behaviour, most notably the connexins, may also function as tumour suppressors (Reviewed in Yamasaki et al., 1999). Many tumour suppressor genes, for example *SMAD4*, *WT1* and *BRCA1*, encode proteins that have essential roles in development. Indeed, it is becoming increasingly apparent that these developmental roles may account for the involvement of such genes in neoplasia.

The role of tumour suppressor genes in protecting against cancer is underscored by the finding that several DNA tumour viruses, including the oncogenic human papillomaviruses, encode oncoproteins that bind to and inhibit the normal activity of tumour suppressor gene products. Adenovirus E1A proteins, polyoma T antigen and HPV E7 can all bind to and inhibit the action of pRB, while adenovirus E1B and HPV E6 inhibit p53. The SV40 large T antigen can inhibit both pRB and p53 via distinct binding sites. Many other cellular proteins are detected in complexes with these and other DNA viral antigens; it is probable that these proteins include currently unknown tumour suppressors.

1.1.2.4 The identification of tumour suppressor genes

The isolation of recessive-acting tumour suppressor genes—whose existence becomes apparent only after their inactivation—has proved much more difficult than that of

dominant-transforming oncogenes. Functional screens greatly facilitated the isolation of novel oncogenes. Screening sequences for suppression of the transformed phenotype, however, has proved more difficult, not least because tumour cells usually possess multiple genetic defects and are extremely well adapted to evading growth inhibition. Moreover, tumour suppressor genes that function as caretakers and give rise to a mutator phenotype would not even be predicted to inhibit tumourigenicity: the genetic defects that directly confer enhanced growth and survival would have already occurred, and would not be reversed by subsequent restoration of genomic stability.

As discussed below, functional screens *can* prove useful in the isolation of novel genes that have direct growth inhibitory functions. However, most tumour suppressors have been identified by 'reverse genetics', or positional cloning. The list of cancer genes isolated by this approach includes *APC*, *ATM*, *BLM*, *BRCA1*, *BRCA2*, *DCC*, *DPC4*, *FHIT*, *LKB1*, *NF1*, *NF2*, *MEN1*, *MMAC1*, *RB1*, *TSC1*, *TSC2*, *VHL*, *WRN* and *WT1*. In the mapping of genes associated with hereditary cancer syndromes, linkage analysis frequently identified the relevant chromosomal region. In future, the resolution of linkage analyses may be greatly improved by the advent of DNA chip technology, which can be used to identify and monitor thousands of genome-wide single nucleotide polymorphisms (SNPs) (Reviewed in Gerhold, Rushmore & Caskey, 1999). Cytogenetic clues can then help refine a region identified by linkage studies (and in fortuitous cases such as the mapping of *RB1* to chromosome 13q14 can define a region independently of linkage); gross chromosomal deletions in normal cells are rare, but if detected in just one family can be of great assistance. Candidate genes can then be identified from within the area of interest. Any known tumour suppressor genes residing within the region are obvious subjects for germline-mutation screening; this approach linked *TP53* to Li Fraumeni syndrome and *CDKN2A* to familial melanoma. Completion of the human genome project and of a genome-wide transcript map will greatly facilitate the identification of novel candidates (Schuler et al., 1996).

However, the identification of genes that confer inherited susceptibility to cancer is likely to remain a formidable task. This is partly because of a lack of families suitable for genetic studies. Linkage studies have the greatest chance of success in families containing multiple affected and unaffected individuals in two or more generations, but such families are hard to find. This is partly because of difficulties in distinguishing a real familial cancer syndrome from chance familial aggregations of sporadic tumours, particularly when a syndrome predisposes to common forms of the disease. Incomplete

penetrance can pose a further complication, as can the development of sporadic cancers in family members who do not carry the mutant allele. Moreover, germline mutations in several different inherited cancer genes (usually those whose products lie on the same functional pathway) can give rise to indistinguishable clinical symptoms. Even leaving these difficulties aside, traditional linkage analyses are unlikely to remain the principal means of mapping inherited cancer genes. This type of study *is* very effective at mapping rare alleles that cause Mendelian, highly penetrant syndromes. However, only 1% of cancers are associated with such syndromes, although more than 15% of all cancer may have a major inherited component. Indeed, the isolation of highly penetrant cancer susceptibility genes has now largely been completed, and attention is shifting towards the identification of low penetrance susceptibility genes. New methods of epidemiological analysis will be needed to detect non-Mendelian familial clusterings, and alternative mapping strategies—such as sib-pair gene sequence segregation analysis—will doubtless be required to locate the genes responsible.

The study of chromosomal abnormalities is central to the mapping of tumour suppressor genes involved in both sporadic and familial tumours. Consistent with Knudson's 'two-hit' hypothesis, chromosomal regions at which high frequencies of LOH are found in tumours often harbour a tumour suppressor gene (Ponder, 1988). LOH at several chromosomal locations has now been described for most tumour types. Once a region of LOH has been defined and cloned, tumour-specific alterations and candidate genes within it can be identified. LOH mapping has thus helped isolate genes involved in both sporadic and familial tumours including *TP53*, *DCC*, *NF2* and *MEN1*. The utility of LOH mapping, however, is limited by its insensitivity, with losses often spanning very large areas. The presence in tumours of homozygous deletions can be much more informative. Moreover, subtractive hybridisation techniques such as representational difference analysis (RDA) can be used to hunt for these deletions even when no positional information is available (Lisitsyn & Wigler, 1995). This technology helped in the cloning of *BRCA2* at 13q12 and of *PTEN* at 10q22-23 (Li et al., 1997; Wooster et al., 1995). RDA and LOH mapping would be expected to identify not only genes whose inactivation constitutes the initial, rate-limiting step of tumourigenesis, but also genes involved in tumour progression.

Studies of genomic loss have thus been the foundation of most efforts to isolate tumour suppressor genes. However, it is becoming increasingly apparent that alteration to DNA sequence is not the only means by which tumour suppressor genes can be

inactivated. Epigenetics—the mitotic (or meiotic) inheritance of information on the basis of gene expression levels rather than gene sequence—almost certainly also plays a part in tumour development. The methylation of cytosine residues in promoter CpG islands is a powerful mechanism for suppression of gene activity, resulting from an inhibition of transcription initiation caused by reduced binding of sequence-specific transcription factors and (possibly) recruitment of methylation-dependent transcriptional repressors (Asiedu et al., 1994; Clark, Harrison & Molloy, 1997; Prendergast & Ziff, 1991). The promoters of a number of known tumour suppressor genes contain CpG islands and show evidence of methylation silencing. The best documented of these is *p16*^{INK4A}, but *RB1*, *VHL* and *APC* are additional examples (Hiltunen et al., 1997; Myohanen, Baylin & Herman, 1998; Ohtani-Fujita et al., 1997; Prowse et al., 1997). Moreover, there is now good evidence from tumour studies that methylation of these promoters can constitute one—and sometimes both—of the Knudson hits (Reviewed in Jones & Laird, 1999). Over 80% of hits to the mismatch repair gene *MLH1* appear to be achieved by promoter methylation; significantly, treatment of cells derived from tumours that display a methylated *MLH1* promoter with the DNA methyltransferase inhibitor 5-aza-deoxycytosine leads to re-accumulation of the MLH1 protein and restoration of mismatch repair activity (Herman et al., 1998). Many tumour suppressors are preferentially inactivated by a particular pathway: for example, point mutation is the most common means of p53 inactivation while homozygous gene deletion is the main means of p16^{INK4A} inactivation. Jones and Laird (1999) argue that a group of tumour suppressor genes may exist unrecognised whose primary means of inactivation is through DNA hypermethylation.

A number of new techniques have been developed to scan the genome for changes in methylation, and are beginning to isolate a range of new genes relevant to cancer. The methods include restriction landmark genome scanning (Hatada, Sugama & Mukai, 1993), methylation-sensitive arbitrarily primed PCR (Gonzalgo et al., 1997) and methylated CpG islands amplification (Toyota et al., 1999); RDA can also be used to detect methylation differences (Ushijima et al., 1997). Other new methodologies have greatly improved the accuracy of measuring methylation levels at known CpG islands (Reviewed in Jones & Laird, 1999), and may thus confirm the involvement in cancer of suspected genes in which no tumour-specific mutations or deletions have been found.

Other strategies for isolating tumour suppressors involved in sporadic cancers aim not to identify genetic or epigenetic genomic alterations, but to detect differences in

expression directly—thus exploiting the fact that the expression of tumour suppressor genes is usually decreased in tumours. Differential hybridisation in its simplest form has been in use for several years, and helped isolate the metastasis suppressor Nm23 (Rosengard et al., 1989). Now, complex, fluorescently labelled cDNA populations derived from tumour and normal cells can be hybridised to DNA chips that bear tens of thousands of gene tags, and differentially expressed genes identified with fairly high sensitivity (Reviewed in Gerhold et al., 1999). Completion of the human genome project will give rise to gene expression arrays that represent the whole genome. Subtractive hybridisation, differential display and, more recently, the technique of serial analysis of gene expression (SAGE) are also identifying many new potential tumour suppressors. This technology, of course, will identify many sequences whose downregulation is not the disease triggering event but is rather a consequence of previous oncogene or tumour suppressor gene mutation. Increased knowledge of signal transduction pathways should help us make sense of the huge amounts of data generated by these methods, as well as exposing new candidate tumour suppressors based on biochemical information alone.

Functional approaches are unearthing further candidate tumour suppressors. These approaches may prove particularly useful in isolating genes whose inactivation is important to the later stages of tumourigenesis (those involved in invasion, metastasis, and angiogenesis) about which little is currently known. Large fragments of genomic material can be introduced into cells and the effects on the transformed phenotype analysed; microcell mediated monochromosome transfer, for example, is being extensively used to introduce entire human chromosomes into tumour cells. Simple *in vitro* and *in vivo* assays can then be used to determine any effect of the chromosome on invasive or metastatic ability (Nihei et al., 1999). Effects on additional transformed phenotypes, such as unlimited replicative capacity, anchorage independent growth in soft agar or primary tumour formation in nude mice can also be analysed (Gustafson et al., 1996; Ogata et al., 1995). The subsequent identification of the gene's sub-chromosomal location is not always easy, but deletion analysis of the donor chromosome in revertant hybrids can prove informative (England et al., 1996). Once a region has been defined, increasingly smaller fragments of the chromosome can be introduced using radiation hybrids, BACs, PACs, P1s and cosmids.

Novel functional techniques will increasingly be used to isolate candidate tumour suppressor genes for which no positional information is available. One such

approach relies upon the production of genomic suppressor elements (GSEs), gene fragments that give rise to antisense molecules or dominant negative peptides which can inhibit the action of the gene from which they are derived (Holzmayer, Pestov & Roninson, 1992). PCR-amplified cDNA fragments derived from normal cells are inserted into viral vectors such that they can be expressed in sense or antisense orientiation, and are used to infect untransformed cells via a virus-packaging cell line. Any insert expressed in the antisense orientation that, for example, extends replicative lifespan, confers resistance to apoptosis or permits anchorage independent growth in soft agar, can be PCR-cloned using primers specific to the vector sequences. The candidate tumour suppressor gene $p33^{ING1}$ was isolated by this approach; in this instance the GSE library used was the product of a subtractive hybridisation between non-tumourigenic and tumourigenic breast cell line cDNA, and was thus presumably already enriched for tumour suppressor sequences (Garkavtsev et al., 1996).

Animal models are another useful tool in the isolation of tumour suppressor genes, exploiting the fact that cancer genes have been widely conserved through evolution. The amenability of *Drosophila melanogaster* for use in comprehensive mutagenesis screens makes the fruit fly a particularly valuable resource. Recent studies demonstrate that the same gene can indeed function as a tumour suppressor in both vertebrates and invertebrates. The *Lats* gene was identified in a *Drosophila* mutagenic screen, its inactivation causing aberrant proliferation and altered differentiation (Xu et al., 1995). The human homologue *LATS1* has now been cloned, and the product shown to bind CDC2 in a cell cycle dependent manner (Tao et al., 1999). Moreover, mice deficient in Lats1 are prone to tumours (St. John et al., 1999). Whether or not *LATS1* is declared a true human tumour suppressor, it is clear that the use of non-mammalian model organisms is a valid approach in the hunt for novel human cancer genes. Murine models of inherited cancer are likely to prove particularly useful in the identification of low penetrance susceptibility genes and modifier genes; for example, allelic variants of the phospolipase A2-encoding *Mom-1* gene have been shown to account for variation in the number of intestinal polyps that arise in inbred mice strains carrying identical germline *APC* mutations (Dietrich et al., 1993).

Collectively these approaches are already providing a huge pool of candidate tumour suppressor genes, increasing the need for clear definition of the essential characteristics of a tumour suppressor. Haber and Harlow (1997) argue that merely being down-regulated in cancer is not enough: to qualify as a bona fide tumour

suppressor, a gene must sustain loss of function mutations during cancer development. They say this still allows for epigenetic mechanisms of inactivation but does not discriminate on the basis of function, thus including, for example, mismatch repair genes whose re-introduction into cancer cells has no direct effect on cellular proliferation. The advent of SNP DNA chips may greatly facilitate the screening of candidate tumour suppressor genes for loss of function mutations in tumours.

As highlighted by the lack of understanding of the workings of many known tumour suppressors, elucidating the function of a given tumour suppressor gene—particularly when not cloned via a functional assay—can be extremely difficult. Re-introducing the gene into tumour cells does not always give much of a hint as to even its broad mechanism of action. Ectopic expression of *RB1*, for example, suppresses both growth rate and tumourigenicity in some cell lines but tumourigenicity only in others (Bookstein et al., 1990b; Huang et al., 1988). Moreover many tumour suppressors, such as VHL, BRCA1 and MEN1, have little homology with known proteins. Some tumour suppressor genes encode proteins that have several functional domains and a few, such as *WT1*, have many splice variants. Analysis of mutational hotspots can prove useful in indicating the relative importance of functional domains, but does not always. Much of our information about the function of novel tumour suppressors, therefore, has been gained through use of animal models. The generation of knockout mice via introduction of specific mutations into chosen genes by gene targeting has proved especially useful, particularly given the extreme rarity of human homozygote nulls. Mouse knockouts of most known tumour suppressor genes die in utero, indicating that the genes have essential developmental, as well as tumour suppressive, functions. Analysis of the abnormal embryos can indicate what these functions are, as demonstrated by the *WT1* knockout mouse whose renal and gonadal development is completely obliterated (Kreidberg et al., 1993). Chimeras can be generated that allow survival of null cells to a later developmental stage and, as in the *Brca1* study discussed earlier, conditional targeting can be used to introduce a chosen mutation into a particular tissue and at a particular stage of development. The use of heterozygotes, on the other hand, allows study of cancer susceptibility.

The identification of novel tumour suppressor genes and the elucidation of their function will doubtless provide new therapeutic targets. Some therapies will aim to restore specific genes; in this respect the possibility of reversing the epigenetic inactivation of tumour suppressor genes whose promoter has been methylated is

particularly encouraging. Our increasing understanding of the biochemical pathways in which tumour suppressor genes function will illuminate further molecular targets for therapeutic intervention.

1.1.2.5 Evidence for a tumour suppressor gene on human chromosome 7

A wealth of cytogenetic and molecular data point to the existence of a multi-tissue tumour suppressor gene on chromosome 7. Karyotypic alterations of chromosome 7 are common in many tumour types (Reviewed in Zenklusen & Conti, 1996). Monosomy 7, for example, is often observed in lymphoid, myeloid and other non-epithelial disorders, while trisomy 7—cases of which often also contain interstitial deletions of the chromosome—is frequently found in epithelial tumours. Significantly, the chromosome loss associated with monosomy 7 appears to occur at an early developmental stage. Specific deletion of the long arm of chromosome 7 is common in squamous cell carcinomas of the head and neck (where the deletion appears to be the primary event) and has also been observed in prostate cancers and malignant melanomas. Interstitial losses of chromosome 7 give an indication of the subchromosomal location of the gene; deletion of the 7q31 to q32 region is common in malignant andrological neoplasias and has also been observed in breast and ovarian carcinomas and myeloid disorders.

High frequency LOH of a particular genomic marker is similarly indicative of the existence in that region of a tumour suppressor gene. Since the introduction of $(C\text{-}A)_n$ microsatellite repeat analysis as a means of investigating LOH, several studies have demonstrated a high incidence of allelic imbalance on 7q. LOH at this region is found in a wide range of tumour types, including carcinomas of the breast (Bieche et al., 1997; Zenklusen et al., 1994a), colon (Zenklusen et al., 1995a), kidney (Shridhar et al., 1997), ovary (Kerr et al., 1996; Koike et al., 1997; Zenklusen et al., 1995b), pancreas (Achille et al., 1996), prostate (Zenklusen et al., 1994c), stomach (Kuniyasu et al., 1994), mouth (Wang et al., 1998b) and head & neck (Loughran et al., 1996; Zenklusen et al., 1995a). The highest reported incidence of LOH—in a panel of breast tumours at the 7q31.1 marker D7S522—was 83% (Zenklusen et al., 1994a). Collectively the allele loss centred on 7q31.1 to q31.2, thus implicating this region as harbouring a tumour suppressor gene whose inactivation is important to the development of several tumour types. Studies in our laboratory support this conclusion, Adam Hurlstone finding 40% LOH—again centring on 7q31—in a series of breast carcinomas (unpublished data). Collectively, the studies indicate that 7q31 alteration is an early rather than late event in

the development of many tumours. Significantly, LOH at this region in breast tumours has been associated with a higher risk of relapse and decreased survival rates (Bieche et al., 1992). High frequencies of LOH have also been found on the syntenic region of mouse chromosome 6 (band 6A1) in chemically induced skin carcinomas and hepatomas (Kemp, Fee & Balmain, 1993; Zenklusen et al., 1996). Again, LOH appeared to be important in the initiation rather than progression of these tumours. These studies indicate that the chromosome 7 gene—like all tumour suppressors cloned to date—is evolutionarily conserved.

Functional evidence for the existence of a tumour suppressor gene on chromosome 7 has also been obtained. When microcell mediated monochromosome transfer was used to introduce human chromosome 7 into a murine squamous cell carcinoma cell line, five of seven hybrids generated were significantly suppressed for tumour formation in nude mice (Zenklusen et al., 1994b). The suppressed hybrids only regained their tumourigenicity after expelling the donor chromosome. Significantly, one of the two clones whose tumourigenicity was not suppressed contained a deletion on the donor chromosome spanning 7q31.1 to q31.3. The suppression/non-suppression of tumourigenicity did not correlate with variations in *in vitro* surface growth rates, indicating that the gene does not function by slowing progression through the cell cycle. Chromosome 7 has also been found to suppress the tumourigenicity of a human choriocarcinoma cell line (Miyamoto et al., 1991). However, introduction of human chromosome 7 into a rat prostate cell line was found to cause suppression of metastatic ability rather than of tumourigenicity (Nihei et al., 1999). Microsatellite analysis of the hybrids generated indicated that the gene responsible was not located at 7q31.1; this, together with the fact that LOH at 7q is usually an early event in tumour progression, suggests that the metastasis suppressor is not the same as the gene defined by the studies described above.

Much of the evidence for a chromosome 7 tumour suppressor gene therefore implicates the 7q31.1 to 31.2 region. However, it is probable that other regions of chromosome 7 also contain tumour suppressor genes of relevance to certain neoplasias. For example, breakpoints of the q22 and q31-32 regions of chromosome 7 are both common in myeloid disorders (Tosi et al., 1999), while LOH studies indicate that the q22 region is also a common target of loss in uterine leiomyomas and breast carcinomas (van der Heijden et al., 1998; Zeng et al., 1999). Advanced ovarian carcinomas, conversely, display high frequencies of LOH at 7q31.3 (Edelson et al., 1997). Despite

the probability of the existence of multiple tumour suppressor genes on chromosome 7, the q31.1 to 31.2 region stands out as being of particular relevance to a very wide range of tumour types.

1.2 Replicative Senescence

Replicative senescence—the phenomenon that limits the replicative lifespan of most normal cells—may act *in vivo* to curb the proliferation of cells carrying growth-promoting mutations. It has therefore been proposed as one mechanism of tumour suppression (see Section 1.2.6). Indeed, many malignant tumours contain immortal cells that have apparently overcome the proliferative barrier that senescence imposes. Cessation of cell division at senescence is accompanied by alterations in cellular physiology, morphology and gene expression—changes that may endow replicative senescence with an additional role in human ageing (Reviewed in Faragher & Kipling, 1998).

The limited proliferative lifespan of normal cells was first described for human fibroblasts in culture. Although they remained viable, the cells were observed to enter a non-dividing state after between 50 and 70 population doublings (Hayflick, 1965). Senescence—also known as M1—has since been observed in a wide variety of primary cell cultures of both mesenchymal and epithelial origin, always occurring after a specific, cell type-dependent, number of cell divisions. An accumulating body of evidence indicates that replicative senescence also occurs *in vivo*. The replicative lifespan of fibroblasts in culture, for example, is inversely correlated to the age of the donor (Martin, Sprague & Epstein, 1970), a finding that has since been extended to keratinocytes (Gilchrest, 1979), lens epithelial cells (Lipman & Taylor, 1987) and T cells (Perillo et al., 1989). As further correlative evidence, fibroblasts isolated from sufferers of premature ageing conditions such as Werner's syndrome undergo senescence earlier than do their normal counterparts (Brown, 1990). The most convincing evidence for *in vivo* senescence, however, comes from a study of skin: analysis of the senescence-associated endogenous β-galactosidase marker has identified an age-dependent increase in its expression in dermal fibroblasts and epidermal keratinocytes (Dimri et al., 1995).

Because senescence is related to the number of elapsed population doublings rather than chronological time, cells must possess a means to count the number of divisions they have undergone. One such 'mitotic clock' is the progressive erosion of telomeres that occurs with each cell cycle (Olovnikov, 1973). The shortening of telomeres to a critical length may trigger senescence via a DNA damage response pathway. Ultimately, immortalisation requires the activation of mechanisms that halt

telomere erosion. Sections 1.2.3 and 1.2.5 give a more in depth discussion of these topics.

1.2.1 The effector molecules of senescence (M1)

Fusion of normal and immortal (tumour-derived) cells usually produces hybrids that have a limited lifespan (Pereira-Smith & Smith, 1983). This indicates that the mortal phenotype is dominant and that immortality is the result of recessive defects in senescence-inducing genes. One such gene is the *TP53* tumour suppressor. Inactivating *TP53* mutations or the use of antibodies against the protein product extend the proliferative lifespan of fibroblasts (Bond, Wyllie & Wynford-Thomas, 1994; Gire & Wynford-Thomas, 1998). Fibroblasts isolated from sufferers of Li Fraumeni syndrome, which is caused by a germline mutation in *TP53*, also display an extended lifespan after spontaneous loss of the remaining allele (Bischoff et al., 1990). Similarly, abrogation of p53 function by expression of the HPV16 protein E6 has been shown to increase the lifespan of breast epithelial cells (Shay et al., 1993). Consistent with these findings, the transactivating ability of p53 increases during senescence (Bond et al., 1996) despite no simultaneous increase in protein levels (Afshari et al., 1993). This activation is believed to occur as a response to a DNA damage signal induced by telomeric erosion (Section 1.2.5). The direct cause of p53 activation is still uncertain, but upregulation of transcriptional cofactors such as $p33^{ING1}$ may be partly responsible (Garkavtsev et al., 1998; Garkavtsev & Riabowol, 1997).

Other senescence effector molecules are upregulated at the protein level. The CDK inhibitor $p21^{WAF1}$, for example, is upregulated in senescent fibroblasts and keratinocytes (Noda et al., 1994; Sayama et al., 1999). $p21^{WAF1}$-mediated inhibition of cyclin E/CDK2 helps retain pRB in its hypophosphorylated state and thus prevent expression of genes required for entry into S phase (Dimri, Hara & Campisi, 1994; Dulic et al., 1993). In early senescence, $p21^{WAF1}$ may additionally play a role in the inactivation of the DNA replication factor PCNA (Stein et al., 1999). Disruption of $p21^{WAF1}$ allows fibroblasts to temporarily bypass senescence, confirming that $p21^{WAF1}$ is an essential component of the senescence machinery (Brown, Wei & Sedivy, 1997). Moreover, its ectopic expression induces senescence in bladder carcinoma cells (Fang et al., 1999). It is now known that $p21^{WAF1}$ is a downstream effector of p53, inactivation of which abolishes $p21^{WAF1}$ expression and induces escape from senescence (Gire & Wynford-Thomas, 1998). However, $p21^{WAF1}$ is not the only downstream senescence

effector of p53, as a partial loss of p53 expression (insufficient to turn off $p21^{WAF1}$) still prevents the onset of senescence (Bond et al., 1995; Tahara et al., 1995). Moreover, it is likely that $p21^{WAF1}$ can be induced during senescence in a p53-independent manner (Tahara et al., 1995).

Another CDK inhibitor that is upregulated in senescence and that maintains pRB in its growth-suppressive, hypophosphorylated state is the tumour suppressor $p16^{INK4a}$ (Alcorta et al., 1996; Hara et al., 1996). In contrast to $p21^{WAF1}$, whose expression in fibroblasts increases sharply just before senescence but then declines, $p16^{INK4a}$ accumulates gradually to a sustained, greatly elevated level in the later stages of senescence. While $p21^{WAF1}$ may be responsible for the induction of senescence, $p16^{INK4a}$ may be essential for its maintenance (Stein et al., 1999). The upstream effector of $p16^{INK4a}$ is still unknown; its expression is clearly p53 independent. However, it has recently been shown that treating fibroblasts with agents that induce double strand breaks results in both $p16^{INK4a}$ enrichment and premature senescence (Robles & Adami, 1998). It is plausible, therefore, that $p16^{INK4a}$ is also induced in response to a DNA damage signal from eroded telomeres. Another recent study indicates that $p16^{INK4a}$ (and $p14^{ARF}$) is under the transcriptional control of the Polycomb-group protein Bmi1, which represses transcription by packaging DNA into higher order chromatin (Jacobs et al., 1999). Fibroblasts from mice that lack *bmi1* express very high levels of $p16^{INK4a}$ and undergo premature senescence, suggesting that downregulation of this gene could play a crucial role in the induction of $p16^{INK4a}$ in normal senescence. It has been difficult to demonstrate conclusively that $p16^{INK4a}$ is essential for senescence. As discussed in Section 1.1.2.3 this is partly because deletion of the *INK4a* locus affects the overlapping gene encoding $p14^{ARF}$, which has also been implicated in cell cycle arrest (Agarwal et al., 1995; Liggett et al., 1996). However, $p16^{INK4a}$ expression is clearly very frequently lost by mutation, deletion or promoter silencing in both immortal tumour cell lines and *in vitro* immortalised, non-tumourigenic cell lines (Okamoto et al., 1994; Vogt et al., 1998). Furthermore, loss of the protein by methylation of its promoter is associated with the increased lifespan of a subset of normal human breast epithelial cells (Brenner, Stampfer & Aldaz, 1998). Treatment of these cells with the methylation inhibitor 5-aza-2-deoxycytidine restores p16 expression and induces premature senescence. Re-expression of $p16^{INK4a}$ in this manner can also induce senescence in an oral squamous cell carcinoma cell line and in immortalised human fibroblasts (Timmermann, Hinds & Munger, 1998; Vogt et al., 1998). Further evidence is now emerging for the specific

involvement in human replicative senescence of $p16^{INK4a}$ rather than $p14^{ARF}$. In a recent study of keratinocytes, no $p14^{ARF}$ protein was detected in senescent cells and no $p16^{INK4a}$ -independent deletions or mutations of *p14ARF* observed in neoplastic cells (Munro et al., 1999). $p14^{ARF}$ exerts its growth inhibitory effects through the inhibition of mdm2-induced degradation of p53 (Stott et al., 1998). The fact, then, that p53 levels do not increase during senescence also argues against a role of $p14^{ARF}$ in replicative senescence in humans, and suggests that $p16^{INK4a}$ is indeed the target of losses at the INK4a locus in immortal cell lines. (However, as discussed in Section 1.2.5 $p14^{ARF}$ probably *is* involved in the premature senescence induced by oncogenes.) The role of $p16^{INK4a}$ in pRB-mediated replicative senescence is further emphasised by the fact that loss of function of $p16^{INK4a}$ or pRB, but not of both together, occurs in most immortal cell lines (Whitaker et al., 1995). Inactivation of pRB, as predicted, also extends the replicative capacity of fibroblasts (Shay et al., 1993); furthermore, its re-expression in tumour cells induces senescence (Xu et al., 1997).

$p21^{WAF1}$ and $p16^{INK4a}$-mediated inhibition of CDKs thus results in retention of the pRB/E2F transcriptional repressor complex, lack of expression of genes required for entry into S phase, and G1 arrest (Figure 1.1). Senescent cells are characterised by their insensitivity to mitogen stimulation. While this may be explained in part by the direct inhibition of CDKs and pRB/E2F-mediated transcriptional repression, it is apparent that key S phase entry genes regulated by other means also fail to be induced by the signal transduction machinery. Such genes include that encoding the transcription factor c-fos (Seshadri & Campisi, 1990), whose activity is essential for DNA replication (Riabowol, Schiff & Gilman, 1992), and the 'immediate-early' response gene *Egr-1* (Meyyappan, Wheaton & Riabowol, 1999). Suppression of these genes appears to result from alterations to—but not a complete breakdown of—the early signal transduction pathway, specifically from a decrease in activity of the serum response factor that regulates their transcription (Meyyappan et al., 1999). The block to cell cycle progression in senescence is thus a fairly complex phenomenon, that involves the coordinated upregulation of growth inhibitory factors together with the repression of key growth-activating genes.

1.2.2 Intermediate lifespan barriers and the regulation of senescence: differences between cell types

The escape from senescence induced by inactivation of any one of the components of the p53/p21 or p16/pRB pathway is only temporary. For example, expression of a mutant *p53* or of HPV16 *E6* confers on fibroblasts a lifespan extension of around 20 population doublings, after which the cells enter a senescence-like growth arrest known as M1b (Bond et al., 1999). Fibroblasts cultured from Li-Fraumeni syndrome patients enter a similar state of arrest after the lifespan extension conferred by spontaneous loss of the wild-type p53 allele (Rogan et al., 1995). During this growth arrest $p16^{INK4a}$ accumulates to a level greater than that observed in M1, suggesting that cells compensate for loss of the p53 pathway through upregulation of the pRB pathway. The targeting of pRB with antisense oligomers or HPV16 E7 also demonstrates the existence of a second barrier to proliferation, cells undergoing growth arrest after approximately the same number of additional population doublings as conferred by loss of p53 (Bond et al., 1999; Hara et al., 1991).

Accumulating evidence indicates that there are major cell type-specific differences in the regulation of senescence. Most fundamentally the Hayflick limit, the number of divisions that cells undergo before they senesce, varies widely. Differences in the functional significance of the p53 and p16/pRB effector pathways also exist. The lifespan of the majority of normal breast epithelial cells, for example, can be extended by expression of *E7* but not of *E6*. This indicates that these cells senesce through a p53-independent pathway (Foster & Galloway, 1996). This is in contrast to fibroblasts in which the p53 and p16/pRB pathways are both essential components of the M1 machinery. Interestingly, breast epithelial cells that have bypassed senescence through expression of *E7* do eventually enter a senescence-like state that is now p53-dependent (Foster et al., 1998). The delay in p53-mediated arrest is such, however, that it may not represent a barrier to cancer progression. Other examples of p53-independent senescence have been documented in subsets of keratinocytes and uroepithelial cells (Kang, Guo & Park, 1998; Puthenveettil, Frederickson & Reznikoff, 1996). In contrast, the lifespan of a population of long-lived normal breast epithelial cells can be further extended by the expression of *E6* but not of *E7* (Shay et al., 1993). It is now known that this apparent p53-dependent senescence is actually a consequence of previous inactivation of the p16/pRB pathway (Foster et al., 1998). These cells appear to consistently suppress this 'back-up' pathway early in their life history, and may exist *in*

vivo as an oestrogen receptor negative stem cell pool that form a sub-set of particularly aggressive breast tumours (Wynford-Thomas, 1999). Thyroid epithelial cells also differ from the fibroblast model of senescence, and can apparently undergo a senescence-like growth arrest in the absence of both p53 and pRB (Bond et al., 1996).

Not surprisingly, species-dependent variations in the regulation of senescence also exist. Mouse and human cells, the cell types in which most senescence research has been carried out, appear to differ in both the way that senescence is triggered and in the actual effector pathways. While the primary 'mitotic clock' in human cells is believed to be progressive erosion of telomeres, the telomere dynamics of rodent cells are such that this clock can be of little functional significance (see Section 1.2.5). As discussed above, there is little direct evidence in human cells for the involvement in replicative senescence of $p14^{ARF}$, despite its role in the inhibition of mdm2-induced degradation of p53 and the fact that its ectopic expression induces growth arrest. However, this is not the case for the murine homologue $p19^{ARF}$, which accumulates greatly as rodent fibroblasts approach senescence (Zindy et al., 1998). A mouse knockout of *p19*^{ARF} in which the coding sequence of $p16^{INK4a}$ is unaffected has now been generated (Kamijo et al., 1997). Significantly, embryonic fibroblasts derived from these mice do not undergo senescence. This is reflected by the fact that establishment of mouse embryonic fibroblast cultures is usually accompanied by loss of function of just $p19^{ARF}$ or p53. These differences in the trigger of senescence and in the effector mechanisms may be functionally related: while in humans senescence may be largely triggered by telomeric attrition and subsequent, $p14^{ARF}$-independent activation of p53, a different trigger in mice could result in the $p19^{ARF}$-mediated p53 response. Consistent with this, there is no evidence for the involvement of $p19^{ARF}$ in DNA damage signalling to the cell cycle machinery (Stott et al., 1998). Species-dependent differences in the effector pathways of senescence also account for variations in the stringency of senescence. While inactivation of the p19/p53 pathway is enough for murine cells to escape senescence, human cells invariably need to disrupt—at a minimum (see Section 1.2.4)—both the p53/p21 and p16/pRB pathways. Perhaps in consequence, human cells very rarely spontaneously immortalise in culture whereas rodent cells do so much more easily. The existence of 'back-up' senescence pathways in human cells is probably a reflection of the importance of cancer avoidance mechanisms in long-lived animals.

1.2.3 Crisis (M2) and immortalisation

Evasion of the proliferative barriers imposed by both M1 and M1b is still not sufficient for cellular immortality. Abrogation of both p53 and pRB function by expression of the SV40 large T antigen (or of HPV16 *E6* and *E7*) extends the lifespan of human fibroblasts beyond M1b; however, the cells eventually hit a block known as crisis (M2) (Wright, Pereira-Smith & Shay, 1989). This barrier is characterised by extreme genomic instability, and is manifested by a marked increase in cell death rather than a decrease in proliferation *per se*. During the period of extended lifespan conferred by large T antigen, the telomeres continue to shorten (Counter et al., 1992). Indeed, crisis probably represents the state at which the extent of telomeric erosion is incompatible with cell viability, following the loss of telomere binding proteins such as TRF2 that protect chromosomes from end-fusion and non-dysjunction (van Steensel, Smogorzewska & de Lange, 1998). Very rare clones emerge from crisis that are capable of indefinite division (Wright et al., 1989). Significantly, this gain of immortality is invariably correlated with stabilisation of telomeres. Telomere stabilisation most commonly occurs through upregulation of the multimeric ribonucleoprotein telomerase, which adds new telomeric repeats to chromosome ends (Counter et al., 1992). The suggestion that tumour cells too may become immortal through re-activation of telomerase was supported by the discovery that nearly 85% of malignant human tumours express telomerase (while most normal somatic cells contain very low levels or none at all) (Shay & Bacchetti, 1997). The cloning of the catalytic subunit of telomerase (hTERT) allowed the causal link between telomerase expression and immortality to be tested directly. Indeed, overexpression of the subunit in normal human fibroblasts and retinal pigment epithelial cells fully reconstituted telomerase activity and resulted in telomere elongation and an apparent indefinite expansion of proliferative lifespan (Bodnar et al., 1998); the pRB and p53 pathways in these cells appear to be intact (Jiang et al., 1999; Morales et al., 1999). Moreover, inhibition of telomerase by antisense oligomers had previously been shown to cause telomere shortening and the restoration of a finite lifespan (Feng et al., 1995). However, in at least some cell types expression of telomerase is not sufficient for immortality. Introduction of certain chromosomes by microcell transfer can induce senescence in target tumour cell lines despite the continued expression of telomerase components (Tanaka et al., 1999). In addition, immortalisation of keratinocytes and breast epithelial cells appears to require inactivation of the p16/pRB pathway in addition to hTERT expression (Kiyono et al.,

1998); however, it is possible that this requirement was simply a consequence of inadequate *in vitro* growth conditions. The mechanisms by which tumour cells upregulate telomerase have not been fully elucidated. However, ectopic expression of *c-myc* and of the gene encoding the helix-loop-helix protein Id-1 have both been shown to increase hTERT expression and extend lifespan (Alani et al., 1999; Wang et al., 1998a). Repression or inactivation of telomerase inhibitors is also likely to play a major role; indeed, microcell transfer experiments have indicated the existence of a repressor of hTERT on a region of chromosome 3 that is commonly altered in tumours and immortal cell lines (Horikawa, Oshimura & Barrett, 1998). Regulation of the RNA component of telomerase may also be functionally significant: the gene encoding it is over-represented and amplified in many tumours and cell lines (Soder et al., 1997). The high frequency with which telomerase is expressed in tumour cells makes it an extremely attractive therapeutic target. Recent studies demonstrate that chemical inhibitors of telomerase and dominant-negative mutants of hTERT induce telomere shortening and cell death in human tumour cell lines (Naasani et al., 1999; Hahn et al., 1999; Zhang et al., 1999). However, some caution is required—in certain genetic backgrounds and at certain stages of tumour development the inhibition of telomerase might promote, rather than inhibit, tumour growth (Chin et al., 1999).

Not all immortal cells stabilise their telomeres through expression of telomerase: a quarter of *in vitro* immortalised cell lines and a small number of tumours appear to use instead a poorly defined mechanism known as alternative lengthening of telomeres (ALT) (Bryan et al., 1997; Bryan et al., 1995). This mechanism is probably recombination-based. Cells that use ALT contain novel promyelocytic leukaemia (PML) bodies which, in addition to telomeric DNA and telomere binding proteins, contain the DNA recombination factors RAD51 and RAD52 (Yeager et al., 1999). Activation of the ALT pathway appears to involve the loss of ALT repressors that are present in normal cells (Perrem et al., 1999). Interestingly, cells of mesenchymal origin seem to utilise ALT more commonly than epithelial cells. This may reflect inherent differences in the ability of these cell types to upregulate telomerase. Figure 1.2 illustrates the two-stage (M1/M2) model of cellular immortalisation.

1.2.4 Multiple pathways to senescence

As discussed above, inactivation of both the p53/p21 and p16/pRB pathways precedes the immortalisation of many cell types. Suppression of systems that repress the

maintenance of telomeres is also essential for immortality. However, there is evidence that many cell types employ additional senescence pathways, and that these must also be inactivated in order to become immortal.

Microcell mediated monochromosome transfer experiments have demonstrated that at least ten human chromosomes can restore the senescence programme in particular immortal cell types (Reviewed in Oshimura & Barrett, 1997). Furthermore, somatic cell fusion experiments have identified four distinct immortality complementation groups, A to D (Pereira-Smith & Smith, 1988). Over thirty immortal cell lines have been inter-fused and assigned to one of these groups on the basis of the mortality or immortality of their hybrids. Cell lines that formed immortal hybrids were presumed to have inactivated the same senescence gene (and were hence assigned to the same complementation group), while those that formed mortal hybrids had inactivated different genes (and were assigned to different complementation groups). The cell lines that complemented each other, namely whose hybrids senesced, were frequently dysfunctional for p53 and pRB; this indicated that at least four more genes were targets for inactivation during immortalisation (Whitaker et al., 1995). A variety of immortal fibroblast, endothelial and tumour cell lines were included in the original study, the results indicating that complementation group assignment did not correlate to cell type, tumour type, embryonal layer of origin, or expression of particular activated oncogenes. However, the majority of cell lines assigned to group A had been immortalised by the SV40 virus, indicating a subsequent mutational bias in these cells. Moreover, a subsequent study assigned each of seven lymphoid cell lines to complementation group D, indicating that T and B cells undergo a common mechanism of immortalisation (Goletz, Robetorye & Pereira-Smith, 1994).

Human chromosomes 4, 1 and 7 have been found to induce senescence specifically in cell lines belonging to immortality complementation groups B, C and D respectively, thus mapping three of the complementation group genes to specific chromosomes (Hensler et al., 1994; Ning et al., 1991; Ogata et al., 1995). The chromosome of the complementation group A gene is not yet known. Significantly, the chromosomal regions that the B and D group genes appear to map to are frequent targets of alteration in tumours or tumour cell lines (Loughran et al., 1997; Zenklusen & Conti, 1996). In one study, immortal keratinocytes were found to display LOH at either 4q32-34 (the putative group B locus) or 7q31 (the putative group D locus) but not at 1q25 (the putative group C locus) (Loughran et al., 1997). The mutually exclusive nature of this

LOH suggests that the complementation group B and D genes encode proteins on the same pathway to senescence. None of the complementation group genes have yet been cloned and little is known about their precise role in senescence. The observation that the group D gene is lost in some telomerase negative cell lines suggests that the complementation group pathway does not function by suppressing telomerase (Loughran et al., 1997). However, there's evidence nevertheless that the group D gene *is* involved in the suppression of telomere maintenance, presumably via repression of the ALT mechanism (Nakabayashi et al., 1997)—the group D gene may thus be involved in crisis, M2, rather than the true senescence program of M1. The fact that p53 and pRB are inactivated in many cell lines that belong to a specific complementation group also argues that the complementation group gene products do not reside on the p53 or pRB senescence pathway (alternatively, of course, inactivation of p53 or pRB may confer selective advantages in addition to the extension of replicative lifespan). Significantly, the chromosomes carrying the complementation group genes can induce senescence in the absence of p53 or pRB (Ning et al., 1991). It is intriguing that the distribution of the heat shock protein mortalin correlates with complementation group assignment (see Chapter 3). Mortalin has itself been implicated in the processes of replicative senescence and immortalisation but, at least in immortal keratinocyte cell lines, does not appear to be a common target for inactivation during immortalisation (Loughran et al., 1997).

Other chromosomes capable of inducing senescence do not correlate with complementation groups A-D. Indeed, the fact that there are more chromosomes with senescence-inducing capability than there are immortality complementation groups is somewhat paradoxical; there may be other complementation groups that are yet to be defined (Moy et al., 1997). Some chromosomes appear to contain more than one senescence gene. Human chromosome 6 appears to harbour at least 3 senescence genes: of these, one seems to be inactivated in immortalised human fibroblasts, a second in ovarian tumour cell lines and a third in head and neck squamous cell carcinoma cell lines (Sanhu et al., 1994; Sandhu et al., 1996; Sara Fitzsimmons, BICR, personal communication). Similarly, two senescence loci exist on chromosome 1, one of which represents the complementation group B gene (Vojta et al., 1996). In some cell lines senescence can be induced by the introduction of at least two different chromosomes (Sasaki et al., 1994), implying that the cell line in question has inactivated both the relevant genes and hence that the senescence genes on these chromosomes are involved

in different senescence pathways (each of which must be inactivated if immortality is to occur). It is therefore likely that the senescence genes mapped by MMCT have different functions. It is probable that some of these genes will participate in the actual mechanisms of growth arrest while others will be involved in the regulation of senescence. Introduction of chromosome 3 into a particular renal carcinoma cell line causes loss of telomerase activity, telomere shortening and restoration of senescence (Horikawa et al., 1998); this indicates that at least one of the non-complementation group senescence genes mapped to a particular chromosome by MMCT functions by suppressing telomerase activity, and is therefore more correctly defined as a crisis gene rather than a senescence gene. However, mortal hybrids generated by fusing together complementing immortal cell lines sometimes remain telomerase positive (Bryan et al., 1995), demonstrating that the putative gene products do not all function as telomerase suppressors. It is likely that each of the senescence pathways involves multiple genes; for example, chromosome 3 cannot induce cellular senescence in *all* telomerase positive cell lines, implying that not all tumour cells are defective for the same gene controlling telomerase activity. Cloning and characterisation of the genes involved should greatly enhance our understanding of the mechanisms of these pathways.

Chromosome transfer experiments and studies with SV40 large T antigen have thus demonstrated in many cell types the existence of multiple, independent pathways of cellular senescence, each of which must be disrupted in order to attain immortality. The genetic defects leading to keratinocyte immortality have been particularly well characterised, with studies suggesting that at least four pathways—those of p53/p21, p16/pRB, telomerase repression, and the complementation group B/D gene products—must be inactivated (Loughran et al., 1997). Inactivation of individual pathways, although unable to cause immortalisation, is likely to confer an extension in proliferative lifespan such as that seen after inactivation of the p53 or pRB pathway. This in turn would provide the opportunity for cells to acquire the mutations in additional pathways that are required for full immortality. Such a 'multiple pathways' scenario is entirely consistent with the multi-step nature of chemically induced immortalisation and *in vivo* tumourigenesis (Bols, Naaktgeboren & Simons, 1991).

1.2.5 The triggers of senescence

The progressive erosion of telomeres that occurs with each round of cell division is thought to be the primary mechanism by which human cells 'count' the number of

doublings they have been through (Olovnikov, 1973). Telomere length is usually estimated from the length of the terminal restriction fragment (TRF), which regardless of the initial length usually declines to approximately 6 Kb at senescence (Allsopp et al., 1992). TRF lengths vary widely within a cell, so it is possible that senescence is triggered when one or more of the telomeres reaches a critical length (Allsopp & Harley, 1995). Indeed, experimental amputation of a yeast telomere has been shown to induce proliferative arrest (Sandell & Zakian, 1993). The intrinsic telomere clock is likely to be prone to environmental modulation. Hyperoxia, for example, is known to accelerate telomeric erosion and to reduce the replicative lifespan of fibroblasts (von Zglinicki et al., 1995).

Precisely how critically short telomeres signal to and activate the senescence machinery has yet to be fully elucidated. One hypothesis proposes that telomeric proteins sequester transcription factors. As the telomeres erode, these factors would be released and available to repress growth promoting genes (or activate senescence genes). There is no evidence in mammalian cells to support such a hypothesis. However, the yeast telomere-associated protein Rap1 has been shown to sequester the silencing factors SIR3 and SIR4 which are able to act at other sites when not sequestered (Marcand et al., 1996). Moreover, mutations of SIR4 can inhibit yeast senescence (Kennedy et al., 1995). An alternative hypothesis suggests that the heterochromatic structure of sub-telomeric DNA may repress genes involved in senescence. As telomeres shorten the extent of the heterochromatin domain may diminish and lead to activation of genes formerly residing within it (Wright & Shay, 1992). Again, yeast cells provide a precedent for such a scenario, silencing of loci near their telomeres having been reproducibly demonstrated (Laurenson & Rine, 1992). However, the extent to which senescence of individual human cells resembles that of yeast is unclear.

The hypothesis currently most in favour is that telomeres bind proteins that prevent the chromosome end from being recognised as a DNA double strand break; when the telomeres reach a critical length these proteins may be released, resulting in activation of a DNA damage response pathway. Since normal cells possess no mechanism to repair shortened telomeres the growth arrest induced would be maintained for as long as the mechanisms that respond to the damage remain intact. In support of this proposal, both p53 and its target $p21^{WAF1}$ are known to be activated by DNA double strand breaks (Atadja et al., 1995; Noda et al., 1994). Recent data suggest that the same is also true of $p16^{INK4a}$ (Robles & Adami, 1998). Indeed, the sequence of

events induced in mortal fibroblasts by agents that cause DNA damage—transient induction of p53 and $p21^{WAF1}$ followed by delayed, sustained activation of $p16^{INK4a}$—is identical to that occurring in fibroblast senescence. Moreover, these agents induce a state of growth arrest that has many morphological and biochemical markers of senescence. Certain telomere binding proteins have already been identified. The TRF2 protein, for example, probably plays a key role in protecting chromosome ends from DNA damage checkpoints by maintaining the correct 'duplex loop' structure of telomeric termini (Griffith et al., 1999). Loss of TRF2 as a consequence of telomeric erosion may be responsible for induction of the M1 DNA damage response pathway as well as the formation of end-end chromosome fusions in crisis. In support of this, expression of mutant forms of TRF2 induces a senescence-like growth arrest (Griffith et al., 1999). Several known genes encode proteins that recognise DNA damage and directly or indirectly activate p53. ATM, DNA-PK (see Section 1.1.2.3) and poly(ADP-ribose) polymerase (PARP) are three such proteins. Of these PARP may play a particularly important role in senescence. Activation of PARP by hyperoxia induces premature senescence, and its inactivation by chemical inhibitors extends cellular lifespan (Vaziri et al., 1997). PARP physically associates with p53 and may thus be directly responsible for the post-translational modification of p53 that appears to occur in senescent cells (Vaziri et al., 1997).

A large body of evidence supports the hypotheses that telomeric erosion can trigger senescence and that evasion of telomeric shortening is necessary for immortalisation (Section 1.2.3). However, telomere erosion is probably not the primary trigger of senescence in all cell types. Syrian hamster embryo cells, for example, express telomerase and senesce with long telomeres (although in such cases it cannot be ruled out that at least one telomere in each cell shortens to the extent that it can trigger senescence) (Carman, Afshari & Barrett, 1998). Mice possess extremely long telomeres, and the effects on the germline and proliferative tissues of telomerase loss become apparent only after several generations (Rudolph et al., 1999). It is therefore unlikely that telomere length provides any barrier to tumourigenesis in rodents. Telomeric erosion is also unlikely to be the senescence clock in some human cell types, for example the sub-populations of keratinocytes and uroepithelial cells that senesce in a p53-independent manner. These cells have been shown to be constitutively telomerase positive and to senesce without detectable telomere shortening (Belair et al., 1997; Kang et al., 1998). Indeed, a growing number of normal human cell subpopulations are now

believed to express telomerase constitutively. These subpopulations include stem cells, for example basal keratinocytes in epidermis, and cells, for example lymphocytes, that are required to undergo frequent clonal expansion (Harle-Bachor et al., 1996; Counter et al., 1995). More controversially, cell types such as terminal ductal breast epithelium may also express telomerase. Other cell types that appear telomerase negative may in fact express telomerase when subjected to the appropriate proliferative stimuli (Belair et al., 1997).

Given the suggestion that over 90% of cancers arise from cell types that now appear to be telomerase positive (Wynford-Thomas, 1999), do tumour cells *need* to escape senescence, and, if they do, could telomere shortening provide the selective pressure for senescence evasion? Studies of telomere length in tumours indicate that the answer to both questions is yes: the finding that most human cancers have much shorter telomeres than the corresponding normal tissue indicates that in most tumours telomere shortening *has* occurred and was followed by telomerase or ALT activation (Hastie et al., 1990). It is probable that the activity of telomerase in many cell types is simply not great enough to prevent telomeric erosion. This, together with the evidence that many cancer cells appear to lose genes encoding repressors of telomerase (Horikawa et al., 1998), suggest that telomere erosion *does* usually provoke selection pressure for telomerase activation and hence for immortalisation during human tumourigenesis.

Other senescence clocks, however, are probably important in some human cell types. One possible clock is the decrease in the level of methylation of cytosine bases that occurs with cell division (Matsumura, Malik & Holliday, 1989). Like telomeric shortening, the process of demethylation is halted in immortal cells (Wilson & Jones, 1983). DNA methylation is associated with repression of transcription, and the lack of methylation in aged cells may activate certain senescence-inducing genes. This might at least partly explain the induction in senescence of $p16^{INK4a}$, whose promoter is known to be silence-able by methylation. Another possible 'clock' is the gradual accumulation of random macromolecular damage that occurs with time. Indeed, it is known that a variety of environmental stimuli can induce a premature senescence-like state. Some such stimuli, for example oxidation, may induce senescence by accelerating telomeric erosion (von Zglinicki et al., 1995); other stimuli, however, appear to have no connection with telomeres. Reddel (1998) proposes that there are multiple mitotic clocks, each characterised by an accumulation of a particular intracellular change. Any of these pathways may cause senescence when a certain

threshold (a critically short telomere, a critically low level of methylation or a critically high level of macromolecular damage) is reached. One of these clocks may predominate in a particular cell type because it usually reaches its threshold before the others. The existence of multiple clocks may reflect the existence of multiple senescence effector pathways, with each pathway being activated by a particular trigger.

Another stimulus that can trigger senescence (at least *in vitro*) is the expression of oncogenes. It has been known for several years that adenovirus 5 *E1A* and *myc* expression can lead to stabilisation of p53 and apoptosis (Hermeking & Eick, 1994; Lowe & Ruley, 1993). More recently, it has been demonstrated that ectopic expression of oncogenic *ras* or *raf,* or constitutive activation of MEK, can induce senescence in primary cells (Lin et al., 1998; Serrano et al., 1997; Zhu et al., 1998). These cellular responses to oncogene expression are partly mediated by upregulation of $p19^{ARF}$ and (probably) the human homologue $p14^{ARF}$ (de Stanchina et al., 1998; Palmero, Pantoja & Serrano, 1998; Zindy et al., 1998), whose inhibition of mdm2-induced degradation of p53 causes upregulation of the CDK inhibitor $p21^{WAF1}$ (Kamijo et al., 1998; Stott et al., 1998). Inactivation of either p53 or $p19^{ARF}$ is sufficient for murine, but not human, fibroblasts to escape ras-induced arrest (Palmero et al., 1998; Serrano et al., 1997). It has recently been shown that $p14^{ARF}$ is transcriptionally activated by unsequestered E2F, thus providing the missing molecular link between hyper-proliferative signalling and ARF induction and revealing further inter-relations between the p53 and pRB pathways (Bates et al., 1998). However, $p16^{INK4a}$ is also induced during ras and raf-induced senescence, and in human cells may be the main senescence effector (Serrano et al., 1997; Zhu et al., 1998). Again, inactivation of $p16^{INK4a}$ is sufficient for evasion of senescence in murine cells but not in human cells (Serrano et al., 1997).

The induction of senescence or apoptosis by oncogenes may represent an *in vivo* safeguard against inappropriate proliferative signals, and thus represent a mechanism of tumour suppression. However, the precise relevance of oncogene-induced senescence to tumourigenesis remains uncertain. Oncogene expression (rather than telomeric shortening) may in some tumours provide the selective pressure for events such as *TP53* or $p16^{INKa}$ mutation that disable the senescence and apoptotic pathways. Consistent with this view, *ras* mutation does sometimes precede *TP53* or *INK4A* mutation during tumourigenesis (Fearon & Vogelstein, 1990). In model systems of skin carcinogenesis, however, ras activation is an early event while immortalisation occurs much later (Balmain et al., 1984). It is also possible that the cellular response to the artificially

high levels of ras and raf induced by ectopic expression using heterologous promoters has little *in vivo* relevance.

Whatever the selective pressure driving evasion of M1, telomere length will ultimately become the limiting factor. It is thus probable that *all* tumour cells must activate a mechanism of telomere maintenance in order to escape the chronic genomic instability and loss of cell viability characteristic of crisis.

1.2.6 Senescence as a mechanism of tumour suppression

There is a significant body of evidence to suggest that tumour cells do indeed face selection pressure to overcome the proliferative lifespan barrier imposed by senescence. However, this view has been opposed on the basis that a lifespan of 50 population doublings (the Hayflick limit of fibroblasts in culture) would still be enough to generate an extremely large tumour mass. Aside from ignoring the possibility of premature senescence induced by oncogenes, this argument fails to take into account ongoing cell death and differentiation (both of which may greatly decrease the number of cells generated by a particular number of divisions), and the fact that most tumours are clonal in origin. In addition, it is probable that the majority of tumours originate from cell types whose Hayflick limit is considerably less than that of fibroblasts. Despite this it is unlikely that replicative senescence (as opposed to oncogene-induced senescence) poses a barrier in the early growth stages of many tumours, particularly those that develop from cells close to the beginning of their proliferative lifespan. However, escape from senescence may well be essential for advanced tumour growth, metastasis, recurrence and hence lethality. While immortalisation is not a prerequisite for tumourigenicity, escape from senescence would clearly permit the extensive cell division required for acquisition of the several mutations required for malignant growth. Indeed, the time required to accumulate the minimum number of defects needed for malignancy is likely to be considerable, and may well bring cells close to (or beyond) the limit of their normal proliferative lifespan. The notion that immortality may facilitate tumour progression is supported by observations that immortal cells are more susceptible than normal cells to spontaneous, oncogene or carcinogen-induced transformation.

There is evidence that senescence, and escape from senescence, do occur in tumours. Most fundamentally, not all tumours are capable of unlimited expansion. The growth of many benign, early stage tumours is limited, and senescence is a likely candidate for the cause of this growth limitation (Wynford-Thomas, 1997). The

majority of tumours, however, appear to contain cells that are immortal. Furthermore, studies of head and neck squamous cell carcinomas indicate that late stage and recurrent tumours contain immortal cells more frequently than early stage tumours (Edington et al., 1995). This implies that there *is* selection pressure during tumourigenesis for cells with increased replicative potential, and that this pressure arises when tumour cells reach the end of their proliferative lifespan. Cells extracted from intermediate-stage tumours have lost some but not all of their senescence regulatory controls, and may have an extended rather than unlimited lifespan (Loughran et al., 1996).

Genetic evidence also indicates that senescence is a mechanism of tumour suppression, with many immortal cell lines exhibiting losses of multiple tumour suppressor genes. The existence of multiple mutations may reflect evasion of the multiple lifespan barriers that exist in many cell types (Sections 1.2.2, 1.2.3 and 1.2.4). Indeed, the extent of genetic defects correlates well with lifespan in culture: in the study of head and neck cancers referred to above, immortality correlated with p53 dysfunction and high frequencies of allele loss at other loci; allele loss in senescent neoplastic cultures, however, was rare (Edington et al., 1995). Wynford Thomas (1998) has described further evidence that proliferative lifespan barriers impose selection pressure for tumour suppressor gene mutation. He suggests that the pattern of mutations observed in different tumour types reflect differential selection pressure for the inactivation of particular senescence pathways. Only around 30% of invasive ductal breast carcinomas, for example, exhibit *TP53* mutation, and this correlates with lack of ER expression and poor prognosis. These features suggest that these tumours are derived from the long-lived breast cell sub-population referred to in Section 1.2.2. These cells (because of previous silencing of $p16^{INK4a}$) exhibit strictly p53-dependent regulation of senescence. Most breast tumours, however, retain wild type p53 and have a phenotype that correlates with that of the shorter-lived subpopulation of breast cells (Section 1.2.2). These shorter-lived cells initially show p53-independent senescence, with a p53-dependent barrier only kicking in after a large number of doublings; it is possible that a clinically significant tumour can be generated before this barrier is reached. The presence or absence of *TP53* mutation in a breast tumour could thus be an inevitable reflection of the senescence controls that normally operate in the cell of origin.

The proliferative barrier of M1 may thus have evolved as a mechanism of tumour suppression. However, the same is not necessarily true of M2. Crisis might

simply be the inevitable consequence of unimpeded proliferation in the absence of both functional telomeres and p53 (Chin et al., 1999).

1.2.7 Evidence for a senescence gene on human chromosome 7

Microcell mediated monochromosome transfer experiments have mapped the complementation group D senescence gene (Section 1.2.4) to chromosome 7. The immortal, non-tumourigenic cell lines SUSM1 and KMST6, established from normal human diploid fibroblasts by *in vitro* mutagenesis, have been mapped to immortality complementation group D by cell-cell fusion analysis of the immortal phenotype (Pereira-Smith & Smith, 1988). Both cell lines have defects at 7q: KMST6 displays LOH at 7q31-qter while SUSM1 has a homozygous deletion at 7q31-32. Introduction of chromosome 7 into these cell lines induced proliferative arrest within 10 to 30 population doublings (Ogata et al., 1993). Because the cell lines were fibroblast-derived and non-tumourigenic, it was unlikely that the growth arrest induced by the chromosome was a consequence of an effect unrelated to senescence such as tumour suppression or terminal differentiation. Indeed, the growth arrested hybrid cells were enlarged and flattened—a morphology characteristic of senescent cells—and stained positive for expression of senescence-associated endogenous β-galactosidase. Furthermore, the mortality marker mortalin reverted to the pancytosolic distribution associated with cells of limited proliferative potential (Nakabayashi et al., 1997). Introduction of chromosomes 1 and 11, which induce a delayed growth arrest in other immortal cell lines (Hensler et al., 1994; Koi et al., 1989), had no effect on the replicative potential of these cell lines. Similarly, chromosome 7 was found to have no effect on the tumour cell lines HT1080, HeLa and TE85, representative of immortality complementation groups A, B and C respectively. It has subsequently been demonstrated that chromosome 7 also induces senescence in the complementation group D hepatoma cell line HepG2 (Ogata et al., 1995), indicating that the senescence-inducing effect of the chromosome is irrespective of cell type or tumourigenicity. Significantly, the chromosome 7-induced growth arrest of SUSM1 is accompanied by telomeric shortening (Nakabayashi et al., 1997). SUSM1 does not express telomerase but has unusually long telomeres. These findings suggest that the chromosome 7 senescence gene may function by suppressing the ALT telomere maintenance mechanism.

1.3 Aims

There is evidence, therefore, for the existence on chromosome 7 of both a senescence gene and a multi-tissue tumour suppressor gene. The aim of my project was to functionally analyse these genes, using microcell mediated monochromosome transfer to introduce the entire chromosome into human tumour cell lines. My initial objective was to investigate whether chromosome 7 could induce senescence in breast and ovarian tumour cell lines that belong to immortality complementation group D. If it could, I hoped to narrow down the region within which the senescence gene is located by carrying out PCR-based microsatellite deletion analysis of the donor chromosome of immortal hybrids.

We also chose to examine experimentally the suggestion that the chromosome 7 senescence and multi-tissue tumour suppressor gene are one and the same. I thus planned to investigate whether any *immortal* microcell hybrids generated in the above experiments—which had presumably deleted or otherwise inactivated the senescence gene on the donor chromosome—showed evidence nevertheless of being suppressed for tumourigenicity. Were I to find no evidence for the existence of a separate tumour suppressor gene on chromosome 7, the argument that the senescence gene is the target of the frequent 7q31 losses found in tumours would be strengthened.

Table 1.1

Table 1.1
The identification of oncogenes

See Hesketh, 1997 for the majority of these entries.

The identification of oncogenes

A) Oncogenes first identified in acute transforming retroviruses

ABL, *AKT*, *CBL*, *CRK*, *ERB*-A, *ERB*-B, *ETS*, *FES/FPS*, *FGR*, *FMS*, *FOS*, *JUN*, *KIT*, *MIL/RAF*, *MOS*, *MYB*, *MYC*, H-*RAS*, K-*RAS*, *REL*, *ROS*, *SEA*, *SIS*, *SKI*, *SRC*, *YES*

B) Oncogenes activated by retroviral integration

AHI1, *BMI1*, *DSI1*, *EVI1*, *FIM1*, *FIS1*, *FLI1*, *FLVI1*, *GIN1*, *INT1/ WNT1*, *INT2*, *INT3*, *INT4/ WNT3*, *LCK*, *MIS1*, *MIS2*, *MIS3*, *MIS4*, *MLVI2*, *MLVI3*, *PIM1*, *SPI*, *TIAM1*, *TPL2*, *VIN1*

C) Oncogenes identified by gene transfer

activated in the tumours from which they were derived:
N-*RAS*, *NEU*, *MET*, *TRK*

activated during gene transfer:
DBL, *FGF5*, *HST1*, *LBC*, *MAS*, B-*RAF*, *RET*, *TRE*, *VAV*

D) Oncogenes identified by their association with chromosomal aberrations

translocation:
ALL1/MLL/HRX, *BCL1/PRAD1/CCND1*, *BCL2*, *BCL3*, *BCR*, *TAL1*, *TAL2*, *TAN1*

amplification:
L-*MYC*, N-*MYC*, *GLI1*, *AIB1/SCR1*

E) Oncogenes identified by cross hybridisation or by PCR with degenerate oligonucleotide primers

CCND2, *CCND3*, *CDK4*, *ELK1*, *ELK2*, *EPH*, *ERB-B2*, *ERB-B3*, *ERB-B4*, *ERG*, *FOS*B, *FRA1*, *FRA2*, *HCK*, *HST2*, *JUNB*, *JUND*, *LYN*, *MAX*

Table 1.1

Table 1.2

Table 1.2
The functions of oncoproteins

Classification of oncoproteins according to their biochemical role in signal transduction. This table is not intended to be comprehensive. See Hesketh, 1995 and 1997 for the majority of entries.

The functions of oncoproteins

A) Ligands

AIGF, HST1, INT2, NOV, SHH, SIS/PDGFB, WNT1, WNT2, WNT3

B) Receptor tyrosine kinases

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

C) Non-catalytic receptors

INT3/NOTCH4, MAS, TAN1/NOTCH1

D) Non-receptor tyrosine kinases

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

cytoplasmic:
ABL, FPS/FES

E) Lipid kinases

PI3K

F) Cytoplasmic regulators of protein activity

SH2/SH3 containing adaptors:
CRK, NCK, SHC

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

other:
CBL

G) Membrane-associated G proteins

HRAS, KRAS2, NRAS, GSP, GIP2

H) Cytoplasmic protein serine/threonine kinases

AKT, BCR, MEK1/2, MOS, PIM1, RAF/MIL, TPL2

I) DNA-binding nuclear proteins (transcription factors)

ALL1/MLL, ERB-A/THRA, ETS1, ETS2, FOS, JUN, MYB, MYC, REL, TAL1, SKI

J) Transcription co-activator (acetyl transferase)

AIB1/SCR1

Table 1.2

Table 1.3

Table 1.3
Genes involved in hereditary predisposition to cancer

BCNS: basal cell nevus syndrome. BZS: Bannayan-Zonana syndrome. CD: Cowden disease. HNPCC: hereditary non-polyposis colon cancer. HPRC: hereditary papillary renal carcinoma. JPS: juvenile polyposis syndrome. LDD: Lhermitte-Duclos disease. MEN: multiple endocrine neoplasia. PJS: Peutz-Jeghers syndrome. WAGR: Wilms' tumour, aniridia, genito-urinary malformation and mental retardation.

Genes involved in hereditary predisposition to cancer

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

Table 1.3

Figure 1.1

Figure 1.1
Mortality stage 1: Possible telomere to cell cycle signalling mechanisms

Recognition of critically shortened telomeres results in activation of p53 and upregulation of $p16^{INK4a}$. In turn, p53 induces expression of $p21^{WAF1}$. $p16^{INK4a}$- and $p21^{WAF1}$-mediated inhibition of CDKs prevents the phosphorylation of pRB. The pRB/E2F repressor complex is maintained, preventing expression of genes required for S phase entry. Arrows represent a positive effect, bars represent inhibition.

Mortality stage 1:

Possible telomere to cell cycle signalling mechanisms

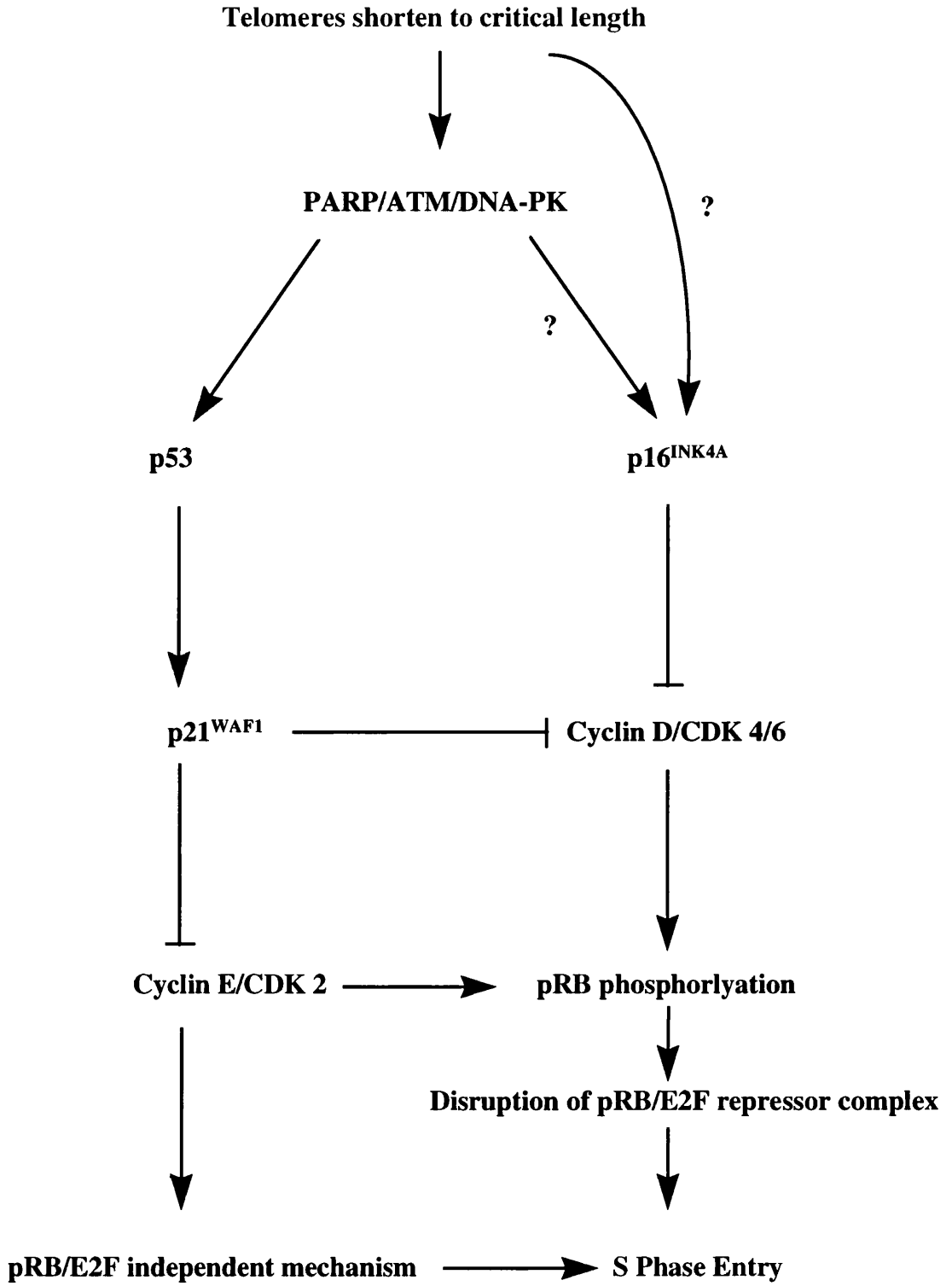


Figure 1.1

Figure 1.2

Figure 1.2
The two-stage model of cellular immortalisation

A) Mortality stage 1 (M1) involves loss of mitogen responsiveness and arrest in the G1 phase of the cell cycle. M1 is the process commonly viewed as *in vitro* replicative senescence. B) Bypass of M1 through, for example, expression of SV40 large T antigen extends the proliferative lifespan until cells reach a second stationary growth phase, mortality stage 2 (M2), corresponding to the classical description of postsenescent crisis. Inactivation of the M2 mechanism constitutes the final immortalising event; this inactivation is a rare event, suggesting a mutational origin, and correlates with the emergence of a telomere maintenance system. C) Bypass of M1 through, for example, expression of HPV E6 *or* E7 confers on fibroblasts a smaller extension of proliferative lifespan than does expression of SV40 large T antigen. Cells reach an intermediate lifespan barrier known as M1b.

The two-stage model of cellular immortalisation

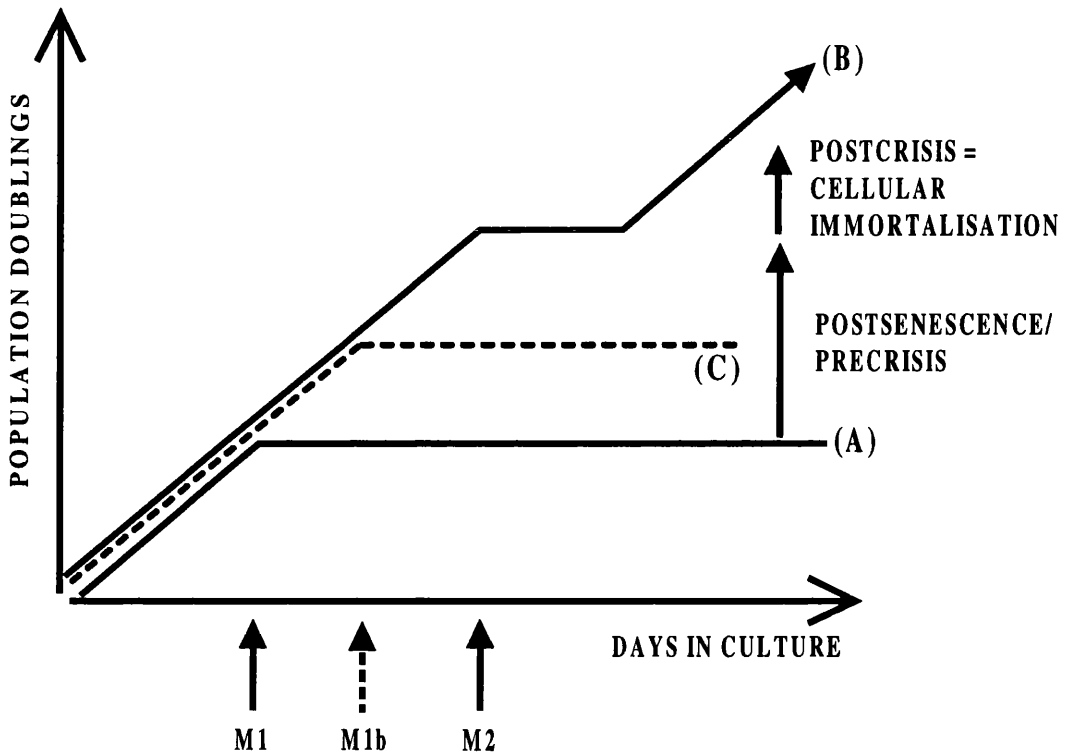


Figure 1.2

CHAPTER 2

MATERIALS & METHODS

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

Solutions and buffers were prepared using dH_20 from a Millipore MilliRO 15 System. Unless otherwise stated below, all chemicals were obtained from Fisher Scientific, Loughborough, UK.

Supplier	Chemical
Amersham International Little Chalfont, UK	Redivue[α^{32}P]dCTP~3000Ci/mmol
Bioline London, UK	X-Gal
Boehringer Mannheim UK Lewes, UK	Caesium chloride; Hepes
Fluka Chemika-Biochemika AG Buchs, Switzerland	DMF
Gibco BRL Life Technologies Paisley, UK	Tris
James Burrough Witham, UK	Ethanol
Promega Corporation Southhampton, UK	Deoxyribonucleotides
Sigma Chemical Company Poole, UK	Ethidium bromide; MES; TEMED; Tween 80 & 20; Triton X-100; EDTA; Potassium ferricyanide; Potassium ferrocyanide; Hydrogen peroxide;

2.1.2 Enzymes

Supplier	Enzyme
Bioline London, UK	Taq DNA polymerase
Boehringer Mannheim UK Lewes, UK	Proteinase K

Life Technologies Paisley, UK	*Msp*1 and React 1 buffer

2.1.3 General plasticware

Supplier	Material
Becton Dickinson Labware Plymouth, UK	Falcon tubes
Bibby-Sterilin Stone, UK	5 ml Bijous; 20ml Universals
Elkay Galway, Eire	Microcentrifuge tubes; Pipette tips
Greiner Labortechnik Stonehouse, UK	Filter pipette tips

2.1.4 Electrophoresis gels

Supplier	Material
Gibco BRL Life Technologies Paisley, UK	Agarose, electrophoresis grade; *Hae* III digested ϕX174 DNA
National Diagnostics Hull, UK	SequaGel, 19:1 acrylamide:bisacrylamide

2.1.5 Paper and X-ray film

Supplier	Material
Eastman Kodak Company Rochester, New York, USA	X-ray film (X-OMAT-AR)
Whatman International Maidstone, UK	3MM filter paper

2.1.6 Immunocytochemistry antibodies and materials

Supplier	Material
Affiniti Research Products Ltd Exeter, UK	Anti-Caveolin-1 antibody, [C13630] (rabbit polyclonal)

Autogen Bioclear UK Calne, UK	Anti-Mortalin antibody, [GRP 75 (C-19)] (goat polyclonal)
Sigma Chemical Company Poole, UK	FITC-labelled rabbit anti-goat IgG; DAB tablets (10 mg); Goat serum; Bovine serum albumin
Vector Laboratories Peterborough, UK	Vectashield mounting medium; Vectastain ABC kit, peroxidase rabbit

2.1.7 Microbial host, media and supplies

Supplier	Material
Difco, Becton Dickinson Plymouth, UK	Tryptone; Yeast extract
Bibby-Sterilin Stone, UK	Petri dishes
Gibco BRL Life Technologies Paisley, UK	Competent *E. coli* DH5α cells
Sigma Chemical Company Poole, UK	Ampicillin

2.1.8 Drug resistance plasmids

pBabe Hygro and psp72 containing a corrected PMC1 neo polA cassette were both obtained from Dr E K Parkinson, BICR.

2.1.9 General cell culture media and supplies

Supplier	Material
A/S Nunc Botolph Claydon, UK	Cryotubes
Becton Dickinson Labware Plymouth, UK	Falcon tissue culture dishes
Bioclear UK Devizes, UK	FBS
Calbiochem-Novabiochem UK Nottingham, UK	Hygromycin B

Fisher Scientific UK Loughborough, UK	DMSO
Gibco BRL Life Technologies Paisley, UK	L-glutamine; RPMI; Neomycin (G-418)
Sigma Chemical Company Poole, UK	DMEM; Penicillin; Sodium bicarbonate; Sodium pyruvate; Streptomycin;
Worthington Biochemical Corporation Reading, UK	Trypsin

2.1.10 Supplies for specialised cell culture techniques

Supplier	Material
Difco, Becton Dickinson Plymouth, UK	Noble agar
Costar Bucks, UK	5 μm filter membranes
Gibco BRL Life Technologies Paisley, UK	Lipofectamine
A/S Nunc Botolph Claydon, UK	Chamber slides; 25 cm^2 flasks
Sigma Chemial Company Poole, UK	Phytohemagglutinin PHA-P; Cytochalasin B; Demecolcine; PEG-1000

2.1.11 Cell lines

The ovarian carcinoma cell lines listed below were kindly provided by Dr R Brown, CRC Beatson Laboratories, Medical Oncology, Glasgow, UK, and the pancreatic carcinoma cell lines by Dr N Lemoine, ICRF, London, UK. All other cell lines were obtained from Dr E K Parkinson, BICR.

Cell line	Tumour of origin
MCF7	breast
MDA-MB-231	

MDA453	
ZRF75	
HT1080	fibrosarcoma
143BTK	osteosarcoma
OVIP	ovarian
OVCAR3	
OVCAR4	
OVCAR5	
CH1	
COLO357	pancreatic
SUIT2	
TMSG	
HeLa	cervix
KMST6	fibroblast*
SUSM1	

*both are non-tumourigenic, immortalised fibroblast cell lines

The donor cell lines for use in MMCT, A92/*Hytk*7 and A92/*Hytk*15, were generated in Dr R F Newbold's laboratory, Brunel University, Middlesex, UK.

2.1.12 Websites

Centre d'Etude du Polymorphisme Humain (CEPH)

http://www.cephb.fr/

Cooperative Human Linkage Centre (CHLC)

http://lpg.nci.nih.gov/CHLC

National Center for Biotechnology Information (NCBI)

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

UK Human Genome Mapping Project Resource Centre

http://www.hgmp.mrc.ac.uk

Whitehead Institute/MIT Center for Genome Research

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

2.2 Methods

2.2.1 Cell culture techniques

2.2.1.1 Culture of cell lines

Except when stated otherwise, all cell lines and clones were cultured in DMEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 0.3% sodium bicarbonate and 1 mM sodium pyruvate. The cell lines MCF7, OVCAR3, OVCAR4 and OVCAR5 were cultured in RPMI supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 0.2% sodium bicarbonate and 1 mM sodium pyruvate. A92/*Hytk*7 and A92/*Hytk*15 donor cells were cultured in DMEM supplemented as above and containing 800 units/ml hygromycin B. All cell lines were maintained in a humid 37 °C/5% CO_2 incubator.

Cryopreserved cell stocks were prepared from early-passage cell lines and clones as follows. Cells were maintained in culture until they reached approximately 75% confluence. They were then trysinised, neutralised with normal growth medium and pelleted. The cell pellet was resuspended in freezing medium (10% DMSO in normal growth medium) to a density of 1 x 10^6 cells/ml, and the suspension transferred into cryotubes (1 ml per vial). The cryotubes were wrapped in cotton wool and placed into a -70 °C freezer overnight, and the following day were immersed in liquid nitrogen for long-term storage. Cryotubes were recovered from liquid nitrogen by rapid immersion in a large volume of water at 37 °C. The cell suspension was then made up to a volume of 10 ml with normal growth medium and the cells pelleted. Cells were resuspended and plated at a density of 0.5-1.0 x 10^6 cells per 10 cm dish.

2.2.1.2 Transfection

Cells were cultured in 10 cm plates until approximately 80% confluent. 10 μg of plasmid DNA was diluted in 400 μl of serum-free medium, and added to a tube containing 50 μl of Lipofectamine reagent and 350 μl of serum-free medium. This mixture was incubated at room temperature (RT) for 20 min to allow DNA-liposome complexes to form; meanwhile, the cells were rinsed once with serum-free medium. Following the incubation, 6.4 ml of serum-free medium was added to the complexes and the whole mixture gently pipetted onto the cells. A control plate of cells was treated with Lipofectamine reagent/serum-free medium only. The plates were incubated overnight (approximately 16 hr) at 37 °C before the transfection mixture was removed and replaced with complete growth medium. The cells were incubated for a further 24

hr, and then passaged and plated at a density of 3 x 10^5 cells per 10 cm dish. Once the cells had adhered, hygromycin B or neomycin selection was added as appropriate (Table 2.1). Selection medium was changed weekly. After approximately four weeks, individual transfectant colonies were ring-cloned and expanded as required.

Cell Line	Hygromycin B concentration (U/ml)	Neomycin concentration (mg/ml)
OVCAR5	500	0.4
MDA-MB-231	500	1.4
HeLa	200	0.8
SUSM1	100	0.6

Table 2.1. The minimum concentration of hygromycin B or neomycin required to kill all non-transfected cells plated at a density of 3 x 10^5 cells per 10 cm dish. Selection medium is changed weekly. Cell death is complete within 2 weeks.

2.2.1.3 Microcell mediated monochromosome transfer

Donor cells (murine A92 cells containing an *Hytk*-tagged human chromosome) were plated into six Nunc 25 cm^2 flasks at a density of 1.25 x 10^6 cells per flask. After 24 hr demecolcine was added to a final concentration of 0.075 μg/ml, and the flasks were incubated at 37 °C for a further 48 hr. The medium was then replaced with 30 ml of 10 μg/ml cytochalasin B in serum-free medium. After a 20 min incubation (37 °C) the flasks were placed in a GSA rotor and spun in a pre-heated Sorvall RC 28S centrifuge, programmed with the following parameters: 7600 rpm/9500g; 75 min; slow start 15 min; slow stop 10 min; 37 °C; brake off. Following the run, the pellets (consisting of microcells and cell debris) were resuspended in a small amount of supernatent and transferred into a single 15 ml sterile Falcon tube. The suspension was centrifuged (3470 rpm, RT, 5 min), and the pellet resuspended in 10 ml of serum-free medium. The microcell suspension was then passed through three sterile 5 μm filters, and the final suspension centrifuged as before. Meanwhile the recipient cells, approximately 80% confluent on 10 cm plates, were rinsed twice with serum-free medium. The microcell pellet was resuspended in 3 ml of 20 μg/ml phytohemagglutinin PHA-P in serum-free medium, and the whole suspension added to a plate of recipient cells. A control plate was treated with PHA-P/serum-free medium only. The cells were then incubated at

37 °C for 1 hr. Following this incubation, the medium was removed and 3 ml of 45% PEG-1000 in serum-free medium added gently to the cells. After a 60 s incubation at RT, the cells were rinsed five times with serum-free medium (1 x 30 s, 1 x 45 s and 3 x 60 s rinses), and incubated at 37 °C overnight. The cells were then trypsinised and re-plated at 3 x 10^5 cells per 10 cm dish. Hygromycin B selection (500 U/ml for MDA-MB-231 and OVCAR5, 200 U/ml for HeLa and SUIT2) was added after 24 hr. The selection medium was changed weekly and colony formation monitored. Proliferating colonies were ring-cloned and expanded as required, and their DNA extracted. Figure 2.1 shows a schematic of this procedure.

2.2.1.4 Assay for endogenous β-galactosidase activity

Adhered cells were rinsed twice with PBS, and fixed by incubating in 3% (v/v) formaldehyde in PBS for 5 min at RT. The cells were then twice rinsed again with PBS. 5 ml of X-gal staining solution (47 mM MES, 140 mM sodium chloride, 1.9 mM magnesium chloride, 4.8 mM potassium ferrocyanide, 4.8 mM potassium ferricyanide and 0.95 mg/ml X-Gal) at pH 6 was added to each 10 cm plate. As a control, proliferating cells of the same cell type were also treated. The dishes were placed in a humid box, and incubated for 16 hr at 37 °C. Following this incubation the cells were rinsed with PBS and microscopically examined for blue staining.

2.2.1.5 Cell-cell fusion

Each parent cell population consisted of a pool of eight or more clones transfected with a plasmid conferring resistance to either hygromycin B or neomycin. In each experiment, a hygromycin B-resistant parent cell population was fused to a neomycin-resistant parent cell population.

1 x 10^5 cells of each parent cell population were seeded into the same 3.5 cm plate and incubated for 24 hr. The cells were rinsed twice with serum-free medium and fused during a 60 s incubation at RT in 45% PEG-1000 in serum-free medium. As a control, each parent cell population alone was also subjected to PEG-1000 treatment. Following this incubation the cells were rinsed four times with serum-free medium (1 x 30 s, 1 x 45 s and 2 x 60 s rinses). Normal growth medium was then added to the cells. After a 24 hr incubation, the cells were trypsinised and plated into 10 cm dishes at a density of 3 x 10^5 cells per 10 cm dish, or 1 x 10^5 per 6 cm dish. The parental control cells were mixed together at this stage, by seeding 1.5 x 10^5 cells of each parent

population into a 10 cm plate. After 24 hr cells were placed under dual hygromycin B and neomycin selection (Table 2.2). The selection medium was changed weekly. Hybrid colonies were ring-cloned and expanded as appropriate and passaged for 50 population doublings or until senescence.

Name of hybrid	Medium	Hygromycin B selection (U/ml)	Neomycin selection (mg/ml)
HeLa/HeLa	DMEM	200	0.8
HeLa /MDA-MB-231	DMEM	500	1.4
HeLa /OVCAR5	RPMI	500	0.4
MDA-MB-231/MDA-MB-231	DMEM	500	1.4
MDA-MB-231/OVCAR5	RPMI	500	1.4
MDA-MB-231/SUSM1	DMEM	500	1.4
OVCAR5/OVCAR5	RPMI	500	0.4
OVCAR5/SUSM1	RPMI	500	0.6
SUSM1/SUSM1	DMEM	100	0.6

Table 2.2. Minimum concentrations of hygromycin B and neomycin required to kill all non-hybrid cells plated at a density of 3 x 10^5 cells per 10 cm dish. Selection medium is changed weekly. Cell death is complete within 2 weeks.

2.2.1.6 Determination of population doublings

The number of population doublings (PD) that cell-cell hybrid clones had achieved was calculated at each passage according to the formula

$$PD = (\log N - \log N_o) \times 3.32$$

where N_o = initial cell number
N = final cell number

(Paul et al., 1975)

2.2.1.7 Assay for growth in soft agar

Base agar plates were poured by pipetting 4 ml of base agar medium (0.6% (w/v) noble agar in RPMI supplemented with 20% (v/v) FBS, 2 mM L-glutamine and 0.2% (w/v) sodium bicarbonate, 37 °C) into 6 cm Sterilin plates. The plates were chilled at 4 °C to allow the agar to set. Sub-confluent cells that had been cultured for seven days in the

absence of drug selection were trypsinised, pelleted, resuspended in RPMI (supplemented with 20% (v/v) FBS, 2 mM L-glutamine and 0.2% (w/v) sodium bicarbonate) to a density of 5000 cells/ml, and warmed to 37 °C. 4 ml of base agar medium at 37 °C was then mixed thoroughly with 4 ml of the cell suspension. 2 ml of the final suspension was added to a pre-warmed base plate; at least three replica plates were prepared for each cell line. The plates were chilled at 4 °C for 20 min, and then incubated at 37 °C in a 10% CO_2 humid incubator. Colonies with a diameter of greater than 0.1 mm were counted after between two and four weeks with the aid of a dissection microscope.

When required, colonies were extracted from the agar using a 200 µl Gilson and long pipette tip, again with the aid of a dissection microscope. The colony was resuspended in normal growth medium and placed into a well of a 24-well plate. The cells were then expanded and their DNA extracted (Section 2.2.4.1).

2.2.2 Mouse tumour studies

2.2.2.1 Preparation and injection of cells

Sub-confluent cells were trypsinised, pelleted and rinsed with PBS. The suspension was then re-pelleted and the cells resuspended in ice-cold PBS at a concentration of 3.333×10^6 cells/ml. 150 µl of the cell suspension was injected subcutaneously into athymic nude mice; four or five mice were used for each cell line or clone. The mice were monitored weekly for signs of tumour formation, and the diameter of any tumours measured using calipers.

2.2.2.2 Extraction of tumour and tail DNA and expansion of tumour cells

The mice were sacrificed and the tumours excised when the diameter of the tumours reached approximately 10 mm. Half of each tumour was flash frozen in liquid nitrogen and stored at -70 °C.

The remaining half was transferred into an eppendorf containing 1 ml of DNA lysis buffer (100 mM Tris.HCl pH 8.5, 5 mM EDTA, 0.2% (w/v) SDS, 200 mM NaCl and 100 µg/ml Proteinase K). The eppendorfs were incubated overnight at 55 °C with constant agitation and then vortexed. The DNA was then precipitated by addition of an equal volume of isopropanol, with gentle mixing until all viscosity was gone. The aggregated precipitate was removed by lifting from the solution with a sterile plastic

inoculation loop. Excess liquid was dabbed off and the DNA rinsed in 70% (v/v) ethanol. After being briefly allowed to air-dry, the DNA was dispersed into at least 0.5 ml of TE (10 mM Tris.HCl and 1 mM EDTA pH 8.0) and maintained at 37 °C with agitation until completely dissolved. DNA was prepared from 0.5 cm tail biopsies in a similar manner, except that after vortexing the tubes were centrifuged at 12 000 rpm for approximately 5 min. The supernatent was then used for isopropanol precipitation as before.

When required, cells from the newly excised tumours were expanded in culture. A tumour chunk was divided with a scalpel into very small pieces (approximately 1 mm square) under sterile conditions. The pieces were spread out on the base of a 10 cm tissue culture plate, and gently covered with FBS. Most of the FBS was immediately removed, and the dish transferred to a dry 37 °C area for 30 min to allow the tumour pieces to adhere to the plate. 4 ml of normal growth medium was then added to the cells. After 5 days of incubation at 37 °C in a humid incubator the medium was removed and replaced with 10 ml of normal growth medium. The dishes were then monitored periodically for signs of cell outgrowth from the tumour pieces. The cells were passaged after 3-4 weeks, and maintained in culture until only human cells were visible (while the murine cells eventually senesced, the immortal human cells continued to proliferate).

2.2.3 Immunocytochemistry

2.2.3.1 Immunofluorescence

Cells were plated into 8-well chamber slides at a density of 2 x 10^4 cells per well and incubated for 24 hr. The cells were then rinsed twice with ice-cold PBS, and fixed by incubating for 10 min at RT in 3.7% formalin in KRH buffer (120 mM NaCl, 6 mM KCl, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$ and 25 mM Hepes, pH 7.4). After fixing, the cells were rinsed three times with KRH buffer. The cell membranes were then permeablised, by incubating in 0.5% triton X-100 in KRH buffer at RT for 5 min. The cells were rinsed three times with KRH buffer containing 0.05% (v/v) Tween 80, and were then blocked by incubating in blocking buffer (10% FBS in KRH buffer/Tween) for 1 hr. After blocking, the cells were incubated for 1 hr at RT in the primary antibody diluted in blocking buffer (Table 2.3). No primary antibody was added to control wells. The cells were then rinsed in KRH buffer/Tween for three periods of 10 min. Subsequently, the

cells were incubated in the fluorochrome-conjugated secondary antibody diluted in blocking buffer (Table 2.3) for 1 hr at RT in a darkened humid box. The cells were again rinsed in KRH buffer/Tween for three periods of 10 min. The plastic chambers and sealing gasket were removed from the slides and a drop of Vectashield mounting medium applied to each area of cells. A glass coverslip was then placed over the cells and sealed in place using clear nail varnish.

2.2.3.2 Visualisation by confocal microscopy

Slides were imaged using a confocal microscope equipped with a krypton/argon ion laser (model MRC 600, BioRad, Hercules, CA). 488/568nm line excitation and dual channel 522 and 585nm emission filters were used. Image analysis was performed using BioRad software.

2.2.3.3 Immunoperoxidase staining

Cells were plated into 8-well chamber slides and grown up as for immunofluorescence. The cells were then rinsed twice with ice-cold PBS, and fixed by incubating on ice for 15 min in ice-cold 1:1 methanol:acetone. After fixing, the slides were air-dried for 20 min and rinsed twice in PBS for two periods of 10 min. To quench endogenous peroxidase activity, the cells were then blocked for 10 min at RT in 3% (v/v) hydrogen peroxide in methanol. After again rinsing in PBS for two periods of 10 min the cells were blocked again, this time for 20 min at RT in goat serum/blocking buffer (1 drop of goat serum per 3.3 ml of 0.1% (w/v) BSA in PBS). The cells were then incubated overnight at 4 °C in primary antibody diluted in 0.1% BSA in PBS (Table 2.3). No primary antibody was added to control wells. Following this incubation, the cells were rinsed in wash buffer (0.15 M sodium chloride and 0.05% (v/v) Tween 20 in PBS) for three periods of 5 min, and then incubated for 1 hr at RT in the biotinylated secondary antibody provided with the kit (diluted in 0.5% BSA in PBS; Table 2.3). The cells were then rinsed as before and incubated for 1 hr at RT in biotinylated peroxidase that had been allowed to complex with avidin (ABC complex: 20 µl of kit reagent A, 2 ml of 0.5% BSA in PBS, and 20 µl of reagent B, incubated at RT for 30 min). Slides were rinsed for three periods of 10 min in wash buffer, and incubated in ABC complex at RT for 1 hr. After three further 10 min rinses in wash buffer, the cells were incubated for 7.5 min in the dark in substrate solution (1 DAB tablet and 10 µl of 30% (v/v) hydrogen peroxide in 16.7 ml of PBS), before rinsing thoroughly with dH_20. Coverslips were

mounted using mounting solution (50% (v/v) glycerol and 0.025% (w/v) sodium azide in PBS), and sealed in place with clear nail varnish. The slides were viewed using a Nikon Diaphot phase-contrast microscope.

NB: PBS and wash buffer at pH 7.6.

Antibody	Dilution
anti-mortalin (goat polyclonal)	1/500
FITC-labelled anti-goat	1/25
anti-caveolin (rabbit polyclonal)	1/100
biotinylated anti-rabbit	1/200

Table 2.3 Antibody dilutions for immunocytochemistry

2.2.4 Extraction, synthesis and quantitation of DNA

2.2.4.1 Extraction of genomic DNA from human cell lines

Genomic DNA was prepared from mammalian cell lines according to (Laird et al., 1991). Cells were first harvested by trypsinisation and then pelleted by brief centrifugation in a microcentrifuge tube. Cells were resuspended and simultaneously lysed in 1 ml of lysis buffer (Section 2.2.2.2), followed by incubation for several hours at 37 °C with constant agitation. DNA was precipitated and redissolved as in Section 2.2.2.2.

2.2.4.2 Extraction of plasmid DNA from bacteria

Plasmid DNA was isolated from overnight cultures of transformed bacteria by alkaline lysis. Cultures were first refrigerated for 20 min before being pelleted by centrifugation, and resuspended in the appropriate volume of solution I (100 mM Tris.HCl pH 8.0 and 100 mM EDTA; Table 2.4). Bacteria were then lysed by addition of solution II (0.2 M NaOH and 1% (w/v) SDS; Table 2.4) and gentle mixing by inversion. After a 5 min incubation on wet ice, detergent and protein were precipitated by addition of ice-cold solution III (3 M KOAc pH 4.8; Table 2.4) and momentary vigorous shaking. After incubating on ice for a further 15 min, the flocculate was removed by centrifugation at 10 000 g. Supernatant containing plasmid DNA was decanted into a fresh polypropylene tube. (For small to medium scale preparations of plasmid DNA,

RNA'ase was added at this stage at a final concentration of 10 μg/ml and the lysate incubated for 15 min at 37 °C.) 1/10 volume of chloroform was then added to the lysate to remove residual protein, and the two phases were mixed by shaking and separated by centrifugation. The upper aqueous phase was decanted, and the DNA precipitated by addition of 0.6 volumes of isopropanol. The DNA was then rinsed with 70% (v/v) ethanol, and resuspended in TE (pH 8.0).

	50 ml culture	500 ml culture
Solution I	1 ml	20 ml
Solution II	2 ml	40 ml
Solution III	1.5 ml	30 ml

Table 2.4. Plasmid prep solution quantities

Plasmid DNA was further purified by equilibrium centrifugation through a continuous caesium chloride density gradient prepared by dissolving caesium chloride in the DNA solution to a concentration of 1 g/ml and then adding ethidium bromide to a final concentration of 740 μg/ml. Centrifugation was performed at 80 000 rpm in polycarbonate tubes for 16 hr using a TLA100.3 rotor in a Beckman TL-100 ultracentrifuge. Following centrifugation, the lowermost (supercoiled) DNA band was removed from the gradient using a syringe and an 18½ gauge needle. Ethidium bromide was extracted by addition of equal volumes of water-saturated butan-1-ol and the volume increased by addition of water. DNA was recovered by addition of 1/10 volume of 3 M NaOAc and $2^1/2$ volumes of ethanol followed by centrifugation at 10 000 g, washing in 70% (v/v) ethanol and resuspension in TE (pH 8.0).

2.2.4.3 Synthesis and purification of oligonucleotides

Oligonucleotides were synthesised at the BICR as a core service on an Applied Biosystems model 392 or 394 RNA/DNA synthesiser using phosphoramidite chemistry according to the manufacturer's instructions. 5′ trityl groups were removed as part of the synthesis and the oligonucleotides eluted into a solution of 29% (v/v) ammonia. This eluate was then incubated at 55 °C overnight in order to 'de-protect' the oligonucleotides. Vials were then chilled on ice and the DNA-ammonia solutions transferred to 15 ml Falcon tubes. Oligonucleotides were precipitated by the addition of 0.1 volumes of 7.5 M ammonium acetate and 3 volumes of ethanol, followed by

incubation on dry ice for 30 min. The DNA was then pelleted by centrifugation in a Sorvall HB-6 rotor at 10 000 g for 15 min. The pellet was washed in 70% (v/v) ethanol, air-dried and dissolved in 0.5 ml of dH_2O. DNA concentrations were calculated (Section 2.2.4.4) and the oligonucleotides stored at -20 °C until required.

2.2.4.4 Quantitation of DNA concentrations

DNA was quantified by spectrophotometric determination of its UV light absorbency. 5 µl of sample was added to 495 µl of de-ionised water and the absorbency of the solution measured at 260 nm and 280 nm in a quartz cuvette, using dH_2O as a blank. The concentration of the solution was calculated using de Beer's law on the basis that an optical density of 1.0 at 260 nm corresponds to a concentration of 50 µg/ml for double-stranded DNA and 33 µg/ml for single-stranded oligonucleotides. Pure preparations of DNA and RNA have a ratio of A_{260}/A_{280} readings between 1.8 and 2.0.

2.2.5 PCR and analysis of amplification products

2.2.5.1 PCR

The following conditions were used to perform the majority of PCR amplifications. Occasional slight alterations to the annealing temperature or concentration of magnesium ions were required for optimal results. 1 µl of 0.5 mg/ml template DNA was added to 24 µl of PCR mix (1 µM oligonucleotide primers, 1.5 mM $MgCl_2$, 50 µM dNTPs, 1 x reaction buffer (50 mM KCl and 10 mM Tris.HCl pH 8.0), and 1 unit *Taq* DNA polymerase). dH_20 instead of template was added to control reactions. When required, 1 µCi [α^{32}P]dCTP per reaction was included for radiolabelling of amplification products. The thermal cycling parameters consisted of 30 rounds of 1 min denaturation at 94 °C, 30 s annealing at 55 °C, and 30 s extension at 72 °C, using an MJC Research PTC-200 thermal cycler (Genetic Research Instrumentation Ltd, Dunmow, Essex, UK).

Primers: see Table A1 (Appendix) for details of primers used to amplify polymorphic STSs mapping to human chromosome 7

Primers used to amplify a portion of the hygromycin B resistance cassette from pBabe Hygro:
L: CTGGGGCGCCCTCTGGTAAG
R: GCGTGGATATGTCCTGCGGG
product size: 209 bp

Primers used to amplify a portion of the neomycin resistance cassette from psp72 containing a corrected PMC1 neo polA cassette:
L: ATGCTCTTCGTCCAGATCAT
R: AAAGTATCCATCATGGCTGAT
product size: 150 bp

2.2.5.2 Allele loss analysis of microcell hybrid cell clones and tumours

100 ng of template DNA from parental cells and hybrid cell clones or tumours was subjected to PCR using the standard reaction conditions (Section 2.2.5.1); amplification products were radiolabelled *in situ*. PCR products were resolved on 6% (w/v) polyacrylamide denaturing gels and visualised by autoradiography (Section 2.2.5.3). Allele loss was determined by visual examination of autoradiographs. Amplification of the restriction fragment length polymorphism GH220/324, which lies within the c-*MET* locus, was performed in the absence of radiolabelling. The PCR product was digested with *Msp* 1 to allow RFLP detection, and resolved on an agarose gel.

2.2.5.3 Denaturing polyacrylamide gel electrophoresis

The radiolabelled PCR products of polymorphic markers were resolved on 6% (w/v) polyacrylamide gels under denaturing conditions. A gel solution (6% (w/v) acrylamide, 0.2% (w/v) bisacrylamide and 8 M urea in 1 x TBE (see below)) was prepared from Sequagel stock solutions. The solution was polymerised by the addition of 150 µl of 20% (w/v) ammonium persulphate and 75 µl of TEMED per 60 ml of gel. This solution was then poured between glass plates separated by 0.4 mm spacers and allowed to set at room temperature. Gels were pre-run at 100 W for 45 min in 1 x TBE (90 mM Tris-borate and 2 mM EDTA pH 8.0) to warm the gels to approximately 50 °C before the loading of samples. 4 µl of PCR product was mixed with 6 µl of STOP buffer (95% formamide, 20 mM EDTA pH 8.0, 0.05% (w/v) xylene cyanol and 0.05% (w/v)

bromophenol blue) and denatured by heating at 94 °C for 10 min followed by quenching on ice. 4 µl of this mixture was subjected to electrophoresis at 100 W for 2-3 hr depending upon the size of the PCR product. The gel was then transferred to a sheet of Whatman 3MM paper and dried under vacuum at 80 °C for 45 min. The PCR products were detected by autoradiography using X-ray film.

2.2.5.4 Agarose gel electrophoresis of unlabelled PCR products

Unlabelled (cold) PCR products were separated on non-denaturing agarose gels and visualised by staining with ethidium bromide and UV transillumination. Gels were prepared by dissolving electrophoresis grade agarose in 1 x TAE (40 mM Tris-acetate and 2 mM EDTA pH 8). After being heated in a microwave to dissolve the agarose, molten gels were cooled to approximately 60 °C and ethidium bromide added to a final concentration of 5 µg/ml before being poured into an appropriate gel former. Once solid, gels were placed into electrophoresis tanks containing 1 x TAE. Samples were mixed with one-fifth volume of gel-loading buffer (40% (w/v) sucrose, 0.05% (w/v) bromophenol blue and 0.05% (w/v) xylene cyanol) before being loaded into the wells of the gel. Electrophoresis was performed at 5 V/cm. In order to estimate the size of fragments resolved by electrophoresis, samples were run alongside aliquots of molecular weight marker, normally the *Hae* III digest of bacteriophage ϕX174 DNA. DNA was visualised by UV transillumination and the gel photographed.

2.2.6 Microbiological techniques

2.2.6.1 Transformation of bacteria with plasmid DNA

Competent *E. coli* (DH5α) were thawed on ice, and aliquots transferred to pre-chilled 1.5 ml screw-cap microcentrifuge tubes. Approximately 10 ng of plasmid DNA was added and gently mixed with the cells by stirring with a pipette tip. After incubation on ice for 30 min, cells were heat-shocked at 42 °C for 45 s and then placed on ice for a further 2 min. 400 µl of SOC medium (2% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 20 mM glucose, 10 mM NaCl and 10 mM $MgCl_2$) was then added to the mixture, and the cells were incubated at 37°C for 1 hr in an orbital shaker at 240 rpm. After this time cells were pelleted by pulsing in a microcentrifuge. 450 µl of supernatant was discarded and the cells resuspended in the remaining 50 µl. Cells were then spread on LB medium plates containing 1.5% (w/v) agar supplemented with the

appropriate antibiotic. Plates were incubated in an inverted position at 37 °C, and colonies transferred into LB medium the following day.

For the preservation of bacterial stocks, a 0.5 ml aliquot of overnight culture in LB medium (1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract and 1% (w/v) NaCl, pH 7.0) was mixed with an equivalent volume of 50% (w/v) glycerol, chilled on ice and then stored at -70° C in plastic cryotubes. Cultures were subsequently re-established by inoculation of 5 ml of LB medium containing the appropriate antibiotic with 20 µl of the glycerol stock.

Figure 2.1

Figure 2.1
Microcell mediated monochromosome transfer procedure

Microcells are prepared from donor rodent cells that contain a single *Hytk*-tagged copy of a particular human chromosome. The microcells are then fused to recipient cells using polyethylene glycol. Hygromycin B-resistant colonies are examined microscopically for growth potential and, when macroscopic, ring-cloned and expanded.

Microcell mediated monochromosome transfer procedure

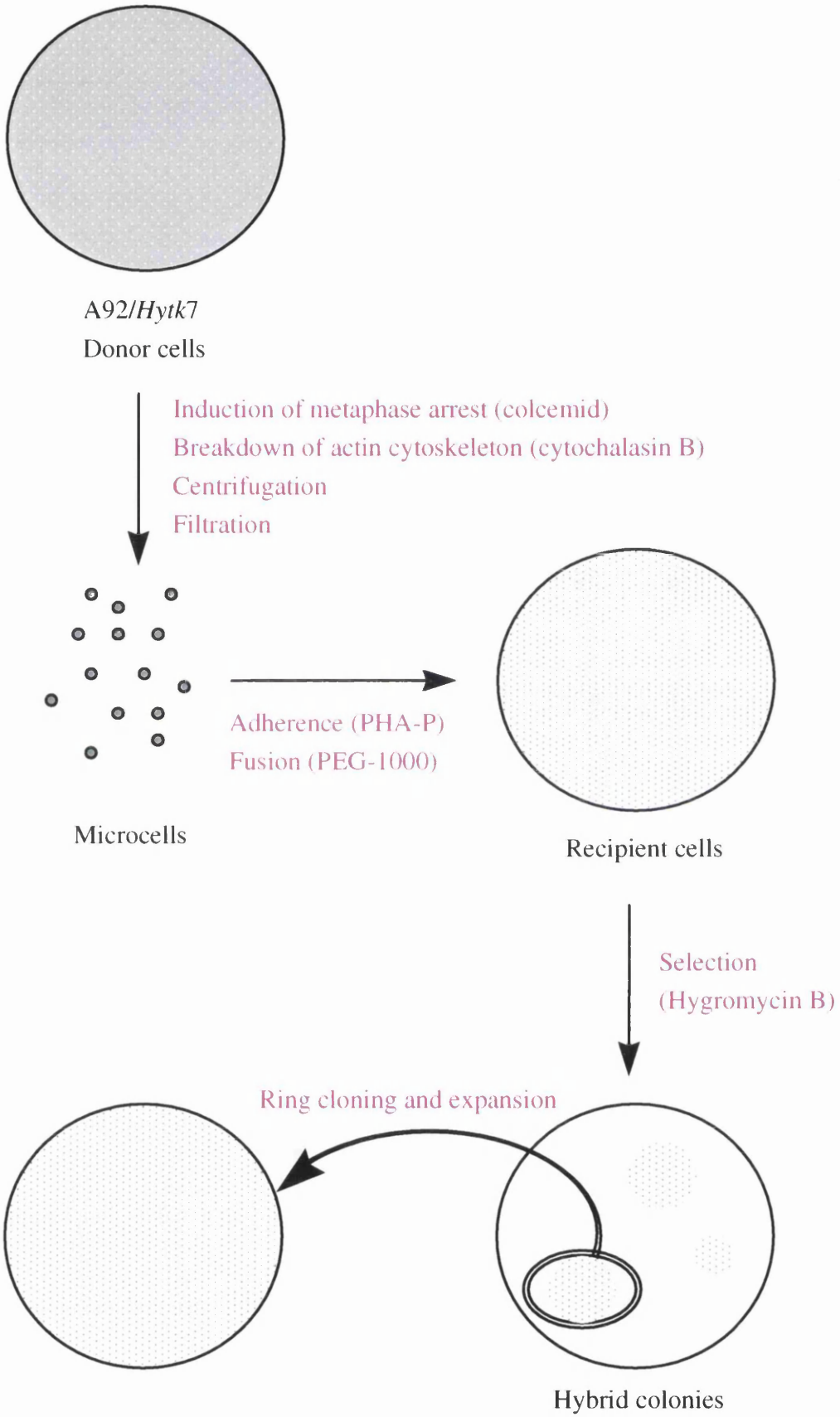


Figure 2.1

CHAPTER 3

RESULTS

Assignment of tumour cell lines to senescence complementation group D

3. Results

Assignment of tumour cell lines to senescence complementation group D: identification of recipient cell lines for use in MMCT

3.1 Purpose

The technique of microcell mediated monochromosome transfer (MMCT) was the cornerstone of this study into the role of genes on chromosome 7 in cellular senescence and tumour suppression. The first task, therefore, was to select appropriate recipient cell lines for use in this assay. Previously, MMCT had been used to introduce chromosome 7 into two immortal, but non-tumourigenic, cell lines that belong to immortality complementation group D and have defects at 7q31. This induced senescence in a proportion of hybrids, thus mapping the group D gene to this chromosome (Ogata et al., 1993). However, we wished to use the technique both to determine whether chromosome 7 could reverse the immortal phenotype of tumourigenic cell lines of the appropriate complementation group, and to investigate the tumour suppressive effects of the chromosome: it was therefore necessary to select tumourigenic as opposed to purely immortal cell lines.

The only tumour cell lines originally documented as belonging to complementation group D are A2182 and A1698, derived from lung and bladder carcinomas respectively (Pereira-Smith & Smith, 1988). However, since our group is primarily interested in breast and ovarian cancer, both of which show very high frequencies of LOH at 7q31 (Reviewed in Zenklusen & Conti, 1996), we chose to identify for ourselves group D cell lines of these tumour types. Three complementary approaches were used to do this. The first approach involved searching for general evidence of a gene inactivation event on 7q, while the second two aimed to specifically identify tumour cell lines belonging to senescence complementation group D.

3.2 Homozygosity at 7q

Extrapolating from Knudson's two hit model, loss of all or part of a chromosome arm in a tumour or tumour cell line is indicative of loss of a tumour suppressor gene in that region. The retained allele is presumed to contain an inactivating mutation (Knudson, 1971). Replicative senescence is believed to be an important mechanism of tumour suppression, and the genes involved—including the complementation group genes—

may be critical targets for inactivation during tumourigenesis. I therefore analysed 12 tumour cell lines for evidence of loss of all or part of a copy of the long arm of chromosome 7. Three of these cell lines (SUIT2, TMSG and COLO357) were derived from pancreas, four (MDA453, MCF7, MDA-MB-231 and ZR75) from breast, and five (OVIP, OVCAR3, OVCAR4, OVCAR5 and CH1) from ovarian tumours. Highly polymorphic simple sequence repeats (SSRs), DNA sequences comprising direct tandem repeats of two, three or four nucleotides (Sheffield et al., 1995; Weissenbach et al., 1992), were used to carry out the study. Variation in the number of repeat units at a particular SSR locus translates into length variation; the fact that a particular SSR can potentially have many allelomorphic forms accounts for the high frequency of heterozygosity among this class of polymorphic marker. Several genetic and physical maps that use these markers have been established for the genome, including chromosome 7 (Bouffard et al., 1997). These maps, available in electronic form at various websites (Section 2.1.12), are proving to be invaluable research resources.

I selected a number of SSR polymorphic markers from the length of 7q and, using appropriate primers, PCR-amplified them from DNA extracted from the 12 tumour cell lines; the products were run out under denaturing conditions on a polyacrylamide gel and scored as homozygous or heterozygous. Representative autoradiographs are shown in Figure 3.1, and the results are summarised in Table 3.1 (See Figure 4.8 for an ideogram of 7q giving approximate positions of markers.) In four of the cell lines (the breast tumour cell line MDA-MB-231, the ovarian tumour cell line OVCAR5 and the pancreatic tumour cell lines COLO357 and TMSG), nearly every marker mapping to 7q was found to be homozygous: despite the lack of availability of normal paired tissue, it was apparent that one copy of the chromosome arm had been lost. Four markers mapping to the p arm were then PCR-amplified from MDA-MB-231 and OVCAR5, and run out under the same conditions. Most of these markers were heterozygous, indicating that the loss was confined to the long arm.

These results are indicative of inactivation of a tumour suppressor gene on 7q in four of the 12 cell lines. In three of the four cell lines homozygous for 7q, we observed the occasional heterozygous marker amidst the background of homozygosity (D7S821 in COLO357, D7S687 in TMSG and D7S633 in MDA-MB-231). This suggests that loss of the chromosome arm was followed by endo-reduplication of the remaining copy of the arm; subsequent to this duplication a replication slippage event has presumably occurred in each of the above cell lines, giving rise to new alleles. This demonstrates

the advantage of this approach over fluorescent in situ hybridisation (FISH), where chromosome loss in these circumstances would be undetectable.

Markers were also PCR-amplified from DNA extracted from the bladder cell line A1698, which is known to belong to immortality complementation group D (Pereira Smith and Smith, 1988). The cell line was found to be mainly heterozygous for the markers analysed (Figure 3.1 for representative autoradiographs). Thus, while we hypothesise that cell lines that are homozygous for 7q are likely to belong to complementation group D, it does not follow that *all* group D cell lines are homozygous for this region.

3.3 The cellular distribution of mortalin

3.3.1 Cell lines of known senescence complementation group assignment

Mortalin, a member of the HSP70 family, was identified in a protein screen as a 66 kDa protein present in the cytosolic fraction of normal mouse embryonic fibroblasts (MEFs), but absent from the cytosolic fraction of immortal MEFs (Wadhwa et al., 1993a). The protein may have a functional role in senescence: micro-injection of an antibody against it can transiently stimulate cell division in senescent MEFs (Wadhwa et al., 1993a), and overexpression of cytosolic mortalin in NIH 3T3 cells induces senescence (Wadhwa et al., 1993c). Mortalin *is* present in immortal mice and human cells; however, while in mortal cells its distribution is diffuse and cytoplasmic (pancytosolic), in immortal cells the protein is less diffuse and more perinuclear (Wadhwa et al., 1993b). Intriguingly, four such perinuclear distributions of mortalin exist in immortal human cells and these enigmatically correlate with immortality complementation group assignment (Wadhwa et al., 1995). Group A cells exhibit a granular juxtanuclear cap, group B cells a granular gradient from nuclear to cell membrane, group C cells a granular juxtanuclear arch, and group D cells a fibrous perinuclear distribution (Figure 3.2). In 17 of 18 cell lines tested that had been assigned to a single complementation group by somatic cell hybridisation, the mortalin distribution was perinuclear rather than pancytosolic and correlated with the original complementation group assignment. Although the molecular basis of this correlation remains unknown, staining for mortalin appears a theoretically simple way to both distinguish mortal from immortal cells and to assign a cell line to a particular complementation group.

To confirm that we too could detect the correlation of mortalin distribution with complementation group assignment, I used an antibody against mortalin to carry out

indirect immunofluorescence on pre-senescent normal fibroblasts and on a cell line representative of each complementation group. The chosen representative of group A was the fibrosarcoma cell line HT1080, of group B the cervical tumour cell line HeLa, of group C the osteosarcoma cell line 143BTK, and of group D the immortal fibroblast cell line SUSM1. Confocal images of the stained cells are shown in Figure 3.3. In accordance with Wadhwa's findings the protein appeared to be pancytosolic in fibroblasts, but perinuclear in each of the immortal cell lines. In the representatives of immortality complementation groups B and D the protein showed the predicted granular gradient and fibrous distribution respectively. The representatives of groups A and C also showed the predicted distribution, with a concentration of the protein on one side of the nucleus, but as had been suspected it was not possible to tell the difference between the two distributions. In each case over 60% of the cells analysed showed the appropriate distribution, while phase contrast microscopy of unstained cells indicated that the differential distributions were not simply a consequence of different cellular morphologies (data not shown). Thus it appeared that we could reliably use the distribution of mortalin to assign our cell lines of unknown complementation group to group B, group D or group A/C.

3.3.2 Cell lines of unknown senescence complementation group assignment

Immunofluorescence with the mortalin antibody was then carried out on the tumour cell lines that I had previously shown to be homozygous for chromosome 7q. Unfortunately, only the breast tumour cell line MDA-MB-231 and the ovarian tumour cell line OVCAR5 gave reliable, reproducible staining (and in the case of MDA-MB-231 it was necessary to carry out the experiment on plastic rather than glass slides to achieve a reasonable degree of cell adherence). Images of the stained cells are shown in Figure 3.4. Both of these cell lines showed the highly distinctive fibrous, perinuclear distribution of mortalin which is associated with cells from immortality complementation group D. I also investigated the distribution of mortalin in the pancreatic tumour cell line SUIT2, which was not hemizygous for 7q (Table 3.1). Rather than showing the group D-associated localisation, the distribution of mortalin in this cell line resembled that of cells from complementation group B (Figure 3.5).

3.4 The replicative lifespan of cell-cell hybrids

3.4.1 Generation of hybrids

As a definitive test of the immortality complementation group assignment of the OVCAR5 and MDA-MB-231 cell lines, somatic cell hybridisation studies were performed. Both cell lines were fused with SUSM1, a known representative of group D, and HeLa, a known representative of group B (Pereira-Smith & Smith, 1988). As a control, it was also necessary to fuse each of these cell lines to itself. The four cell lines were each transfected with a plasmid encoding resistance to hygromycin B and a plasmid encoding resistance to neomycin. At least eight transfectant clones of each type were expanded and pooled (thus minimising the risk of extrapolating results from a non-representative sub-clone) to produce the parent cell populations. Each of these populations was then fused to each of the other parent cell populations, taking care to always fuse a hygromycin B-resistant parent to a neomycin-resistant parent. The subsequent use of dual hygro and neo selection permitted the expansion of hybrid colonies only. The morphology and colony size of hybrids was examined microscopically, and where possible several hybrids of each type were ring-cloned and expanded. Multiplex PCR-amplification of the two dominant drug resistance cassettes (ratio of Hyg^R primers to Neo^R primers 1:10) was then used to confirm that clones that survived dual selection and that proliferated to the extent that their DNA could be extracted, were true hybrids: 63 of the 64 clones analysed proved positive for both these markers (Figure 3.6 for representative gel images). The replicative potential of individual hybrid clones was then determined. Hybrids were maintained either until senescence, or until 50 population doublings had been achieved; cells that achieved 50 population doublings were defined as being immortal.

3.4.2 Replicative lifespan of hybrids

Table 3.2 summarises the replicative potential of all hybrids generated. When either OVCAR5 or MDA-MB-231 were fused to SUSM1 (group D), all expanded hybrid clones achieved 50 population doublings; this indicates non-complementation of senescence genes, suggesting that the cell lines share a defect in the same senescence gene and belong to the same complementation group (Figure 3.7 shows images of these hybrids). In support, all expanded OVCAR5/MDA-MB-231 hybrids also achieved 50 population doublings.

Images of HeLa/SUSM1, HeLa/MDA-MB-231, and HeLa/OVCAR5 hybrids are shown in Figure 3.8. The majority of clones of each of these hybrid types had limited proliferative potential (Table 3.2), the point at which senescence occurred ranging from 5 to 27 population doublings. A minority of hybrids of each type were immortal; this is presumably due to chromosomal segregation with the loss of critical growth regulatory genes. As mortal HeLa/SUSM1 and HeLa/MDA-MB-231 hybrids approached the end of their replicative lifespan the cells enlarged and flattened, giving a morphology characteristic of senescent cells. Furthermore, these hybrids stained positive for expression of senescence-associated endogenous acidic β-galactosidase, while hybrids of all types that showed indefinite replicative potential (and maintained their youthful morphology) did not. While some growth arrested HeLa/OVCAR5 hybrids adopted the morphology characteristic of senescent cells and stained positive for acidic β-galactosidase expression, others appeared to undergo some form of cell death. These results are indicative of complementation of different senescence genes, and confirm that OVCAR5, MDA-MB-231 and SUSM1 do belong to a different complementation group from HeLa.

As a further control, each cell line was also fused to itself, to ensure that loss of proliferative potential was not a general, non-specific feature of hybridisation. Nearly all homotypic hybrids proliferated well and reached 50 population doublings (Table 3.2). Some evidence of senescence was observed in HeLa/HeLa hybrids, but to a much smaller extent than that observed in HeLa/SUSM1, HeLa/MDA-MB-231 and HeLa/OVCAR5 hybrids.

I had intended to use the bladder cell line A1698 as the known representative of immortality complementation group D, rather than SUSM1. When A1698 was fused to HeLa, however, all of eight hybrids that were ring-cloned proliferated through to the cut-off point of 50 population doublings (data not shown). This result is not in accordance with those of Pereira Smith and Smith (1988).

Figure 3.1

Figure 3.1
Representative examples of tumour cell line microsatellite marker analysis

Autoradiographs from radiolabelled PCR products of three chromosome 7 microsatellite markers, amplified from cell line DNA. All cell lines except A1698 are homozygous for each of the three markers.
A92/*Hytk7*: MMCT donor. COLO357, TMSG: pancreatic carcinoma. OVCAR5: ovarian carcinoma. MDA-MB-231: breast carcinoma. A1698: bladder carcinoma.

Representative examples of tumour cell line microsatellite marker analysis

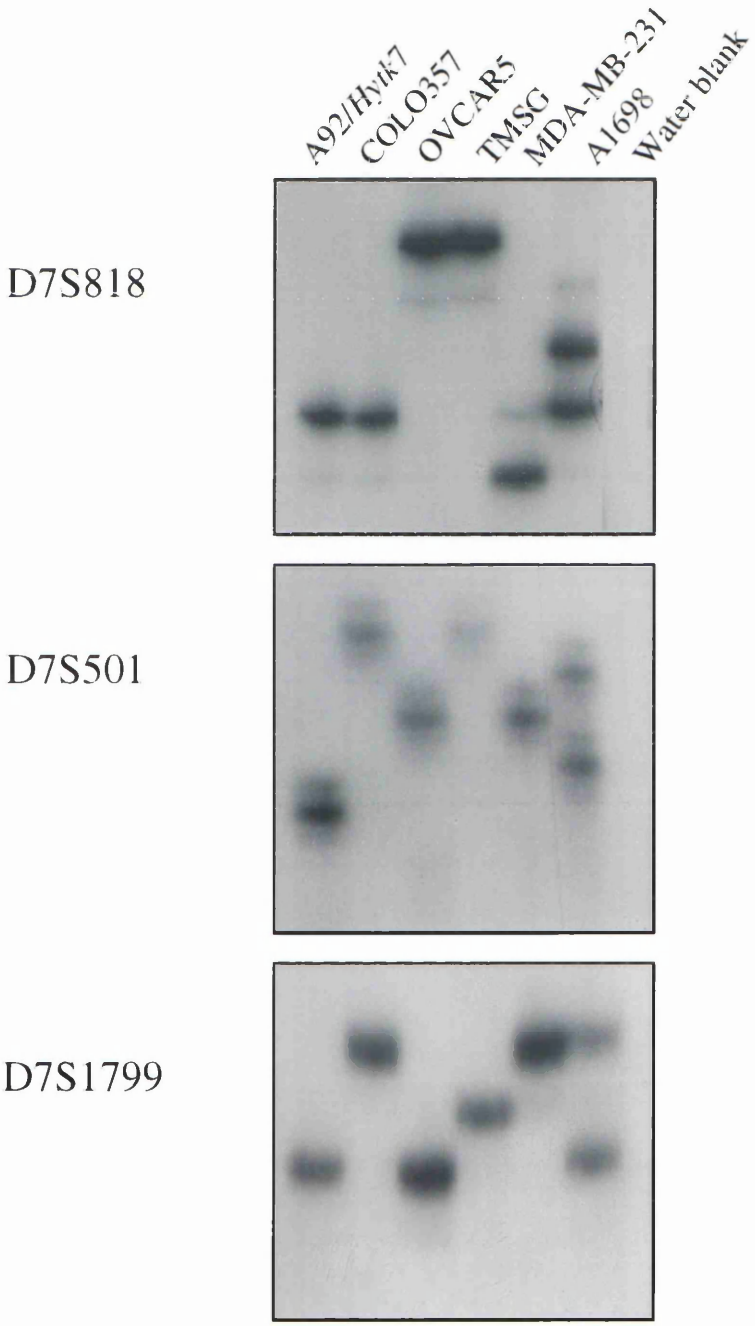


Figure 3.1

Table 3.1

Table 3.1
Summary of tumour cell line microsatellite marker analysis

Summary of analysis of microsatellite markers mapping to chromosome 7.
Markers are given in order of chromosomal location.
Hom: homozygous. Het: heterozygous.
SUIT2, COLO357, TMSG: pancreatic carcinoma.
MDA-MB-453, MCF7, MDA-MB-231, ZRF75: breast carcinoma.
OVIP, OVCAR3, OVCAR4, OVCAR5, CH1: ovarian carcinoma.

Summary of tumour cell line microsatellite marker analysis

	MARKER	SUIT2	COLO357	TMSG	MDA-453	MCF7	MDA-231*	ZR75	OVIP	OVCAR3	OVCAR4	OVCAR5	CH1
p arm	D7S1819						Het					Het	
	P34087						Het					Hom	
	P19270						Het					Het	
	P28070						Het						
	D7S1831											Het	
q arm	D7S1797		Hom	Hom			Hom					Hom	
	D7S820		Hom	Hom			Hom					Hom	
	D7S821		Het	Hom			Hom					Hom	
	D7S1841		Hom	Hom			Hom					Hom	
	D7S501		Hom	Hom			Hom					Hom	
	D7S796		Hom	Hom			Hom					Hom	
	D7S818		Hom	Hom			Hom					Hom	
	D7S1799		Hom	Hom			Hom					Hom	
	D7S692		Hom	Hom			Hom					Hom	
	D7S525		Hom	Hom			Hom					Hom	
	D7S471			Hom			Hom					Hom	
	D7S523		Hom	Hom			Hom					Hom	
	D7S687	Hom	Hom	Het	Hom	Het	Hom	Het	Het	Het	Het	Hom	Hom
	D7S816		Hom	Hom			Hom					Hom	
	D7S1811		Hom	Hom			Hom					Hom	
	D7S486	Hom	Hom	Hom	Het	Het	Hom	Hom	Het	Hom	Hom	Hom	Hom
	D7S522	Hom	Hom	Hom	Het	Hom	Hom	Het	Het	Hom	Het	Hom	Hom
	D7S2460		Hom	Hom			Hom					Hom	
	727CA	Het	Hom	Hom	Het	Het	Hom	Hom	Het	Het	Hom	Hom	Het
	778CA	Het	Hom	Hom	Het	Hom	Hom	Het		Hom	Het	Hom	Het
	724CA	Het	Hom	Hom	Het	Hom	Hom	Het	Het	Het	Het	Hom	Het
	740CA	Hom	Hom	Hom	Het	Hom	Hom	Het	Het	Het	Hom	Hom	Het
	D7S633	Het	Hom	Hom	Het	Het	Het	Hom	Het	Het	Het	Hom	Het
	D7S677		Hom	Hom			Hom					Hom	
	17TA-5/17B-PE3	Het	Hom	Hom	Het	Hom		Het	Hom	Hom		Hom	Het
	786CA	Het	Hom	Hom	Het	Het	Hom	Hom		Het	Het	Hom	Het
	ARMA073ZB9	Hom	Hom	Hom	Het	Het	Hom		Het	Hom	Het	Hom	Het
	D7S643		Hom	Hom			Hom					Hom	
	D7S650		Hom	Hom			Hom					Hom	
	D7S685		Hom	Hom			Hom					Hom	
	D7S1809		Hom	Hom			Hom					Hom	
	D7S1801		Hom	Hom			Hom					Hom	
	D7S1822		Hom	Hom			Hom					Hom	
	D7S2203		Hom	Hom			Hom					Hom	
	D7S2197			Hom			Hom					Hom	
	D7S649		Hom	Hom			Hom					Hom	

* MDA-MB-453/MDA-MB-231

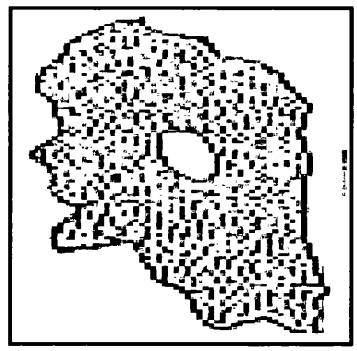
Table 3.1

Figure 3.2

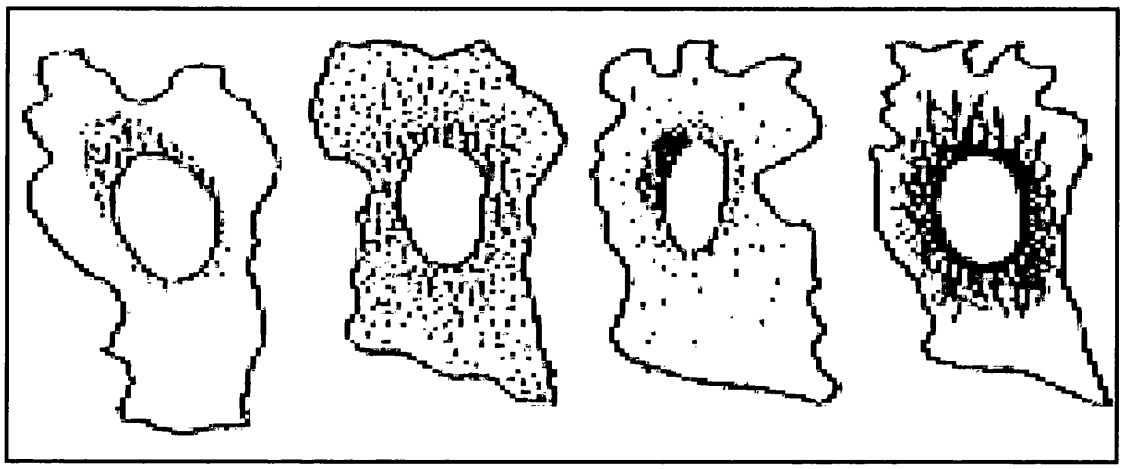
Figure 3.2
Correlation of the cellular distribution of mortalin with immortality complementation group assignment

Schematic representation of the intracellular distribution of mortalin in mortal and immortal human cells. The four types of perinuclear distribution found in immortal cells correlate with immortality complementation group assignment. Wadhwa et al., 1995.

Correlation of the cellular distribution of mortalin with immortality complementation group assignment



Mortal
(eg normal fibroblast)



Complementation
group A B C D

Wadhwa et al., 1995

Figure 3.2

Figure 3.3

Figure 3.3
The cellular distribution of mortalin in cell lines representative of immortality complementation groups A-D

Confocal immunofluorescence micrographs of normal fibroblasts (a) and representatives of each complementation group (b-e) stained for mortalin. The intracellular distribution of the protein in mortal and immortal cells assigned to complementation groups A-D is as described by Wadhwa et al. (1995). Scale bars represent 25 μm.
Normal fibroblasts, early passage. HT1080: fibrosarcoma.
HeLa: cervical carcinoma. 143BTK: osteosarcoma.
SUSM1: fibroblast (immortalised, non-tumourigenic).

The cellular distribution of mortalin in cell lines representative of immortality complementation groups A-D

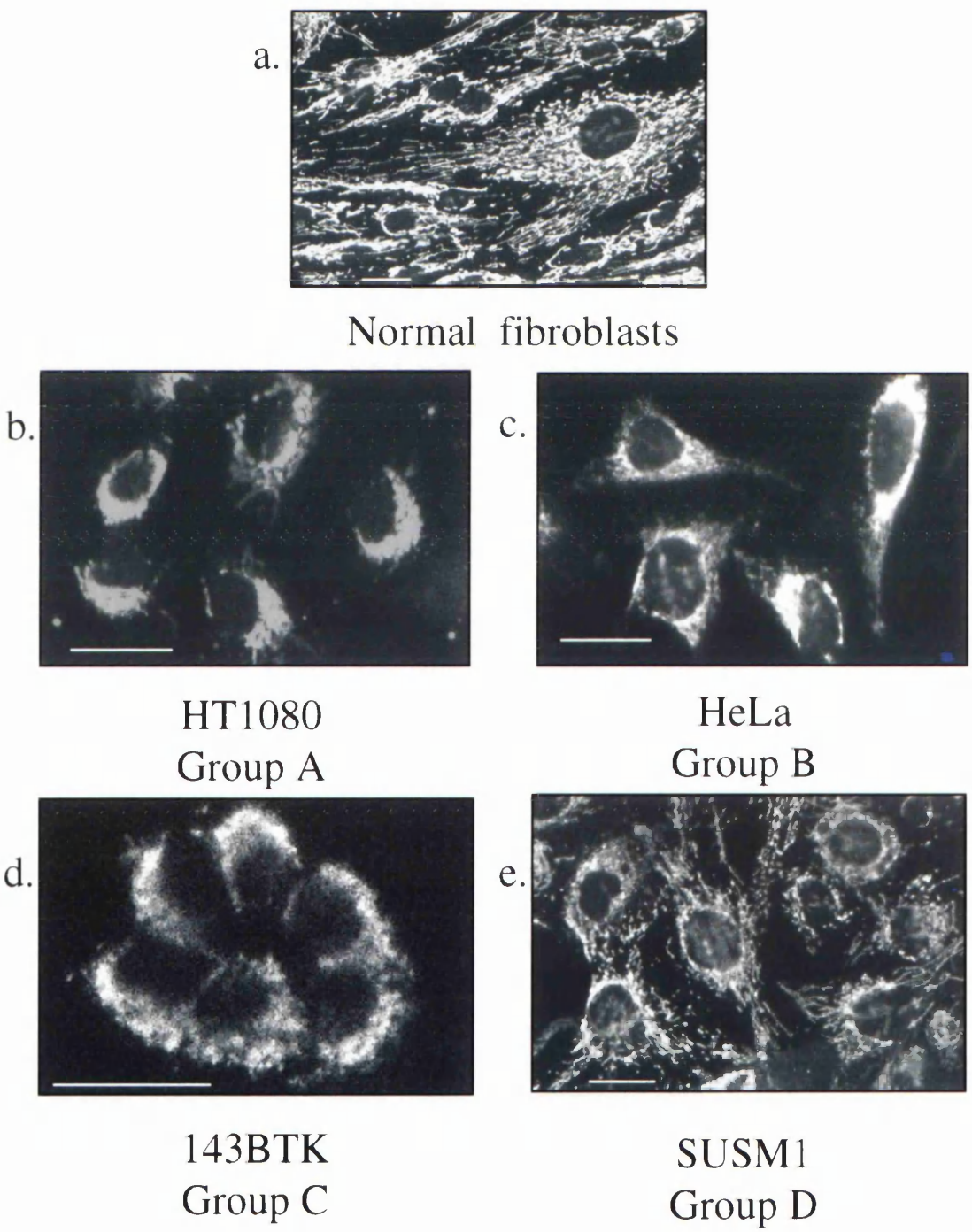


Figure 3.3

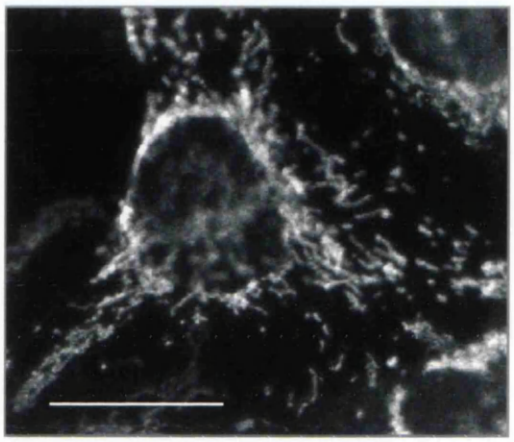
Figure 3.4

Figure 3.4
The cellular distribution of mortalin in tumour cell lines homozygous for 7q

Confocal immunofluorescence micrographs of cells stained for mortalin. SUSM1 (a) is known to belong to complementation group D, and OVCAR5 and MDA-MB-231 (b & c) are homozygous for 7q. All three cells show the perinuclear, fibrous distribution of mortalin associated with cells of complementation group D (Wadha et al., 1995).
Scale bars represent 25 μm.
SUSM1: fibroblast (immortalised, non-tumourigenic).
OVCAR5: ovarian carcinoma.
MDA-MB-231: breast carcinoma.

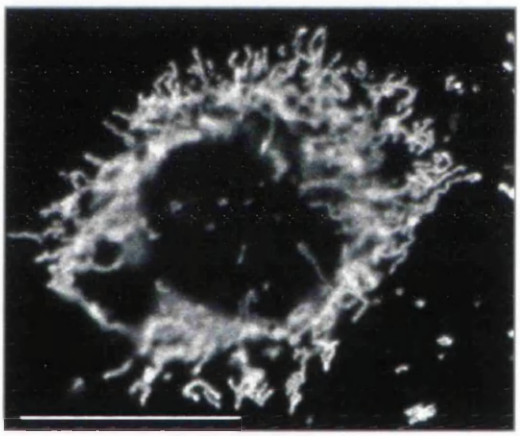
The cellular distribution of mortalin in tumour cell lines homozygous for 7q

a.



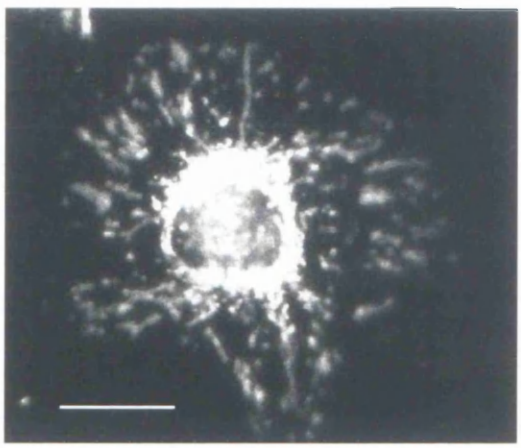
SUSM1: Group D

b.



OVCAR5

c.



MDA-MB-231

Figure 3.4

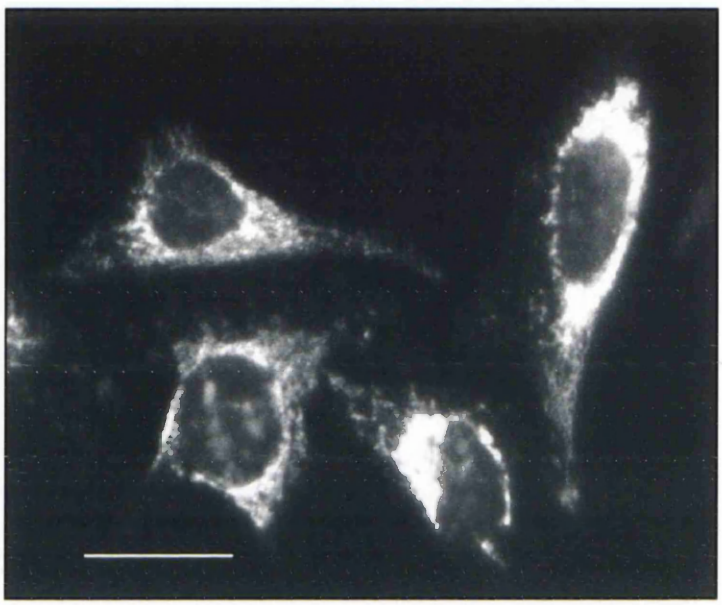
Figure 3.5

Figure 3.5
The cellular distribution of mortalin in a tumour cell line heterozygous for 7q

Confocal immunofluorescence micrographs of cells stained for mortalin. HeLa (a) is known to belong to complementation group B. SUIT2 (b) has not previously been assigned to an immortality complementation group and is heterozygous for 7q. Cells of each type show the granular gradient of mortalin from nuclear to cell membrane associated with cells of complementation group B (Wadhwa et al., 1995).
Scale bars represent 25 μm.
HeLa: cervical carcinoma. SUIT2: pancreatic carcinoma.

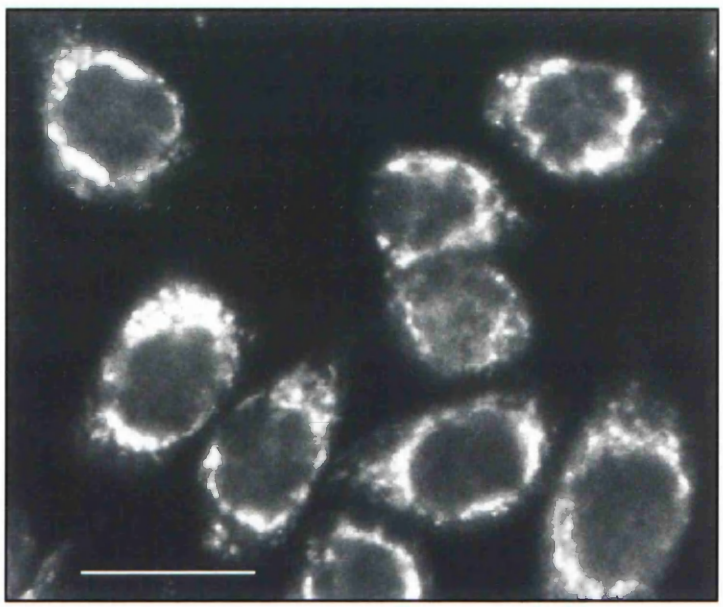
The cellular distribution of mortalin in a tumour cell line heterozygous for 7q

a.



HeLa: Group B

b.



SUIT2

Figure 3.5

Figure 3.6

Figure 3.6
PCR analysis of cell-cell hybrids

Products of multiplex PCR reactions, using primers to amplify a portion of the hygromycin B resistance cassette (209 bp) and the neomycin resistance cassette (156 bp) from DNA extracted from cell-cell hybrid clones and their parental cell populations. PCR products were resolved on 3% (w/v) agarose gels and stained with ethidium bromide before visualisation on a UV transilluminator.
M: ϕX174 RF DNA/Hae III fragments.
Template DNA:
a: hygromycin B resistant parent. b: neomycin resistant parent.
1-10: cell-cell hybrid clones.
w: water blank.

PCR analysis of cell-cell hybrids

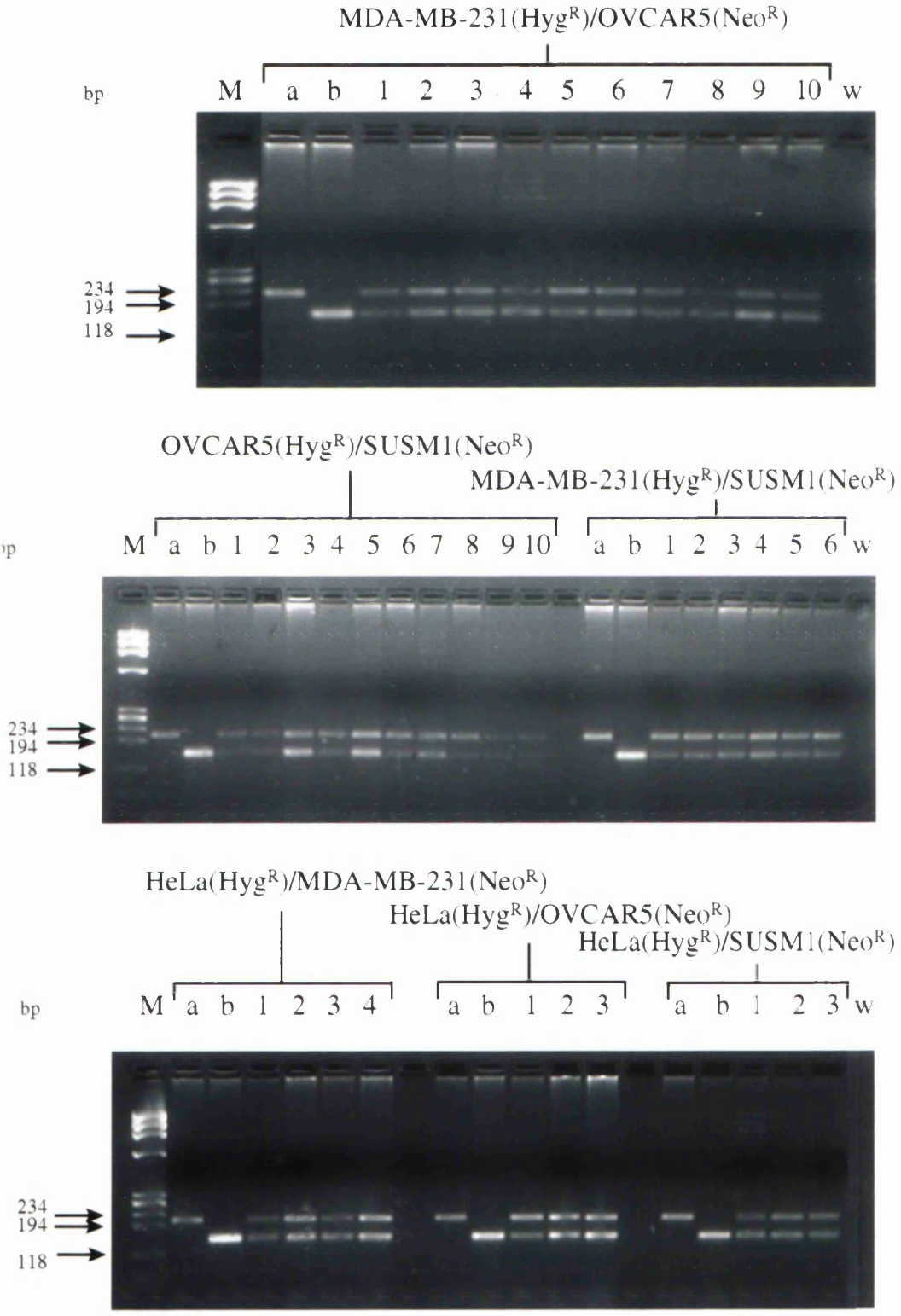


Figure 3.6

Table 3.2

Table 3.2
The replicative potential of cell-cell hybrids

Summary of the replicative fate of cell-cell hybrids generated from tumour/immortal cell lines. pd: population doublings MDA-MB-231: breast carcinoma. SUSM1: fibroblast (immortalised, non tumourigenic). OVCAR5: ovarian carcinoma. HeLa: cervical carcinoma.

The replicative potential of cell-cell hybrids

Hybrid	Total no. of colonies if <50	No. of colonies that senesced before picking	No. of colonies picked	No. of picked colonies that senesced before 50 pd	Total percentage senescence
MDA-MB-231/SUSM1	♣	0	10	0	0
OVCAR5/SUSM1	♣	0	10	0	0
MDA-MB-231/OVCAR5	♣	0	10	0	0
HeLa/SUSM1	30	20	9	8	97
HeLa/MDA-MB-231	16	7	6	2	56
HeLa/OVCAR5	10	♦6	4	2	♦80
HeLa/HeLa	22	4	6	1	23
MDA-MB-231/MDA-MB-231	30	0	6	0	0
OVCAR5/OVCAR5	♣	0	10	0	0
SUSM1/SUSM1	♣	0	6	0	0

♣ > 50 colonies

♦ some of these colonies underwent a form of cell death rather than classical senescence

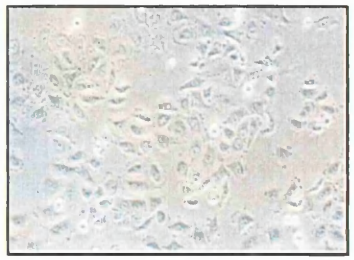
Table 3.2

Figure 3.7

Figure 3.7
Cell-cell hybrids with indefinite replicative capacity

Phase-contrast photomicrographs of parental cell lines (a-c) and their cell-cell hybrids (d-e). The hybrid cells are of similar morphology to their parental cells. All images are at the same magnification.
OVCAR5: ovarian carcinoma. SUSM1: fibroblast (immortalised, non-tumourigenic). MDA-MB-231: breast carcinoma.

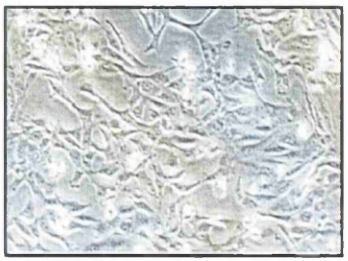
Cell-cell hybrids with indefinite replicative capacity

a. 

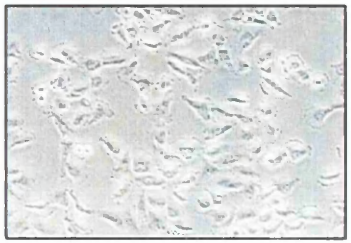
OVCAR5

b. 

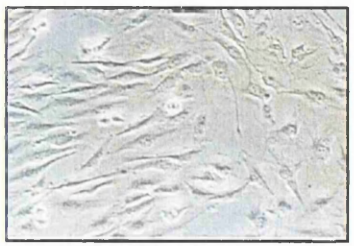
SUSM1

c. 

MDA-MB-231

d. 

OVCAR5/SUSM1

e. 

MDA-MB-231/SUSM1

Figure 3.7

Figure 3.8

Figure 3.8
Cell-cell hybrids with limited replicative capacity

Phase-contrast photomicrographs of parental cell lines (a-d) and a sub-set of their cell-cell hybrids (e-g). The hybrid cells are enlarged and flattened compared to their parental cells, and stain positive for expression of senescence-associated endogenous β-galactosidase. All images are at the same magnification.
HeLa: cervical carcinoma. OVCAR5: ovarian carcinoma. SUSM1: fibroblast (immortalised, non-tumourigenic). MDA-MB-231: breast carcinoma.

Cell-cell hybrids with limited replicative capacity

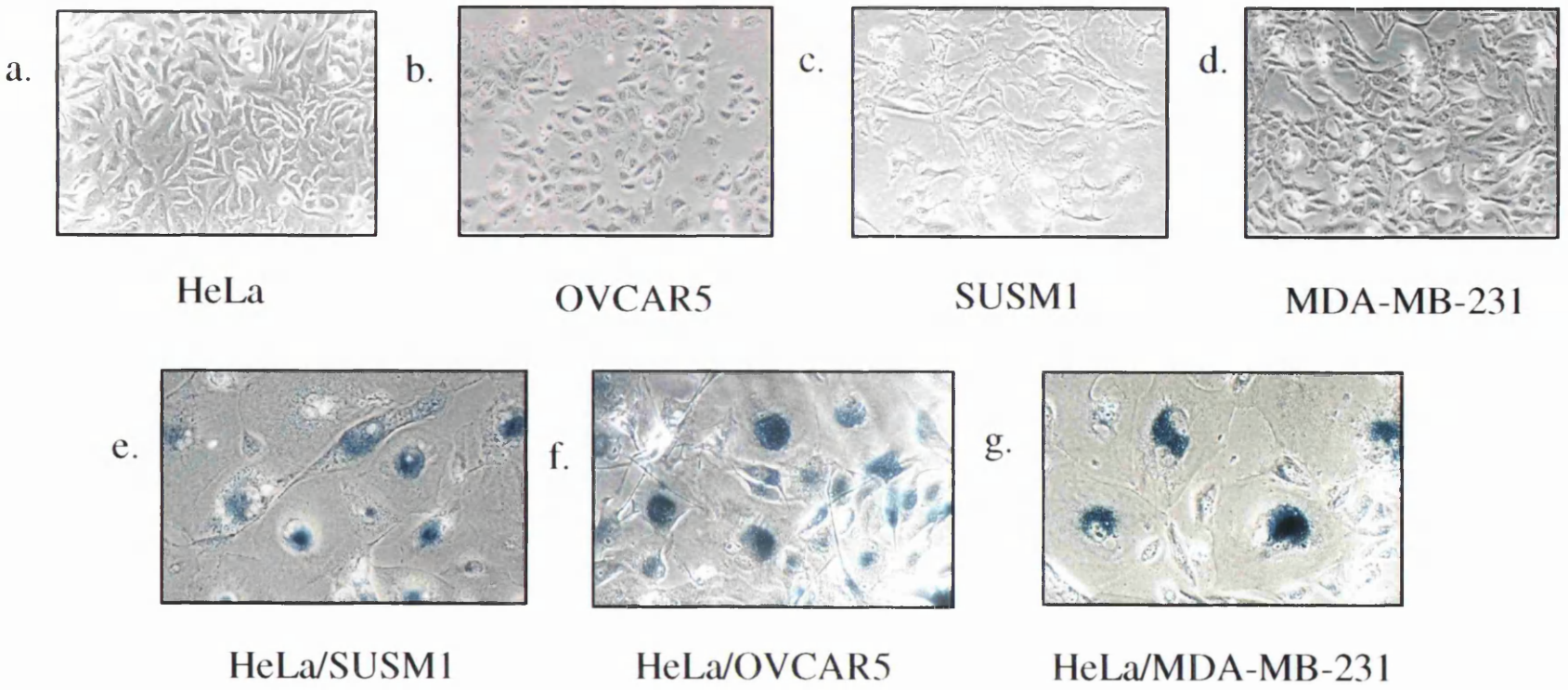


Figure 3.8

CHAPTER 4

RESULTS

Introduction of human chromosome 7 into tumour cell lines and the analysis of immortal hybrids

4. Results

Introduction of human chromosome 7 into tumour cell lines and the analysis of immortal hybrids

4.1 Purpose

Transfer of an intact copy of chromosome 7 into immortal fibroblast cell lines that belong to immortality complementation group D induces senescence (Ogata et al., 1993). The cell lines used in this study, SUSM1 and KMST6, were immortalised by *in vitro* mutagenesis (Namba et al., 1988; Namba et al., 1985); however, the cells do not form colonies in soft agar or tumours in nude mice. We wished to determine whether a normal copy of chromosome 7 could induce senescence even in group D cells with a greater variety of genetic defects. The only reported incidence of chromosome 7-induced senescence in a tumourigenic cell line was for the human hepatoma cell line HepG2 (Ogata et al., 1995). We decided to perform similar experiments using the breast tumour cell line MDA-MB-231 and the ovarian tumour cell line OVCAR5, both of which we had mapped to immortality complementation group D (Chapter 3).

It was hoped that this study could also be used to map the senescence gene to a specific sub-chromosomal region. In Ogata's experiments a proportion of chromosome 7-containing hybrids escaped replicative senescence and could be passaged indefinitely: they appeared to retain, or revert to, their immortal phenotype (1993; 1995). This evasion of senescence was most likely caused by inactivation of the senescence gene on the donor chromosome. If such inactivation resulted from deletion or mitotic recombination then it should have been possible to map these events by determining the pattern of retention of informative SSR markers on the donor chromosome. We planned to pursue this mapping strategy for any immortal MDA-MB-231/Chromosome 7 and OVCAR5/Chromosome 7 hybrids that we obtained.

Based upon the probable role of replicative senescence in tumour suppression, and the finding that SUSM1 and KMST6 both display loss of genetic material from the 7q31 region (Ogata, 1993), it is possible that the group D senescence gene is the target of the 7q31 LOH observed in tumours. However, the deletion mapping strategy outlined above potentially offers several advantages over LOH mapping: one, the unequivocal nature of the data (there is no DNA from normal stromal cells to complicate interpretation of banding patterns); two, the allele loss can be associated

with an observable phenotypic effect; and three, since the end product is an immortal cell line, there is an indefinite source of nucleic acid and proteins for further analysis.

4.2 Induction of senescence

4.2.1 In MDA-MB-231 and OVCAR5 cells

Microcell mediated monochromosome transfer was used to introduce intact copies of human chromosome 7—and, as a control, human chromosome 15—into MDA-MB-231 and OVCAR5 cells. The donor chromosomes were tagged with a selectable fusion gene, *Hytk*; this is derived from both the hygromycin phosphotransferase gene *Hph*, conferring hygromycin resistance and allowing positive selection of transfected cells, and the herpes simplex virus thymidine kinase gene *tk*, allowing negative selection (Cuthbert et al., 1995). After two weeks under hygromycin B selection, the fusion plates were examined microscopically for both proliferating and growth-arrested colonies. The progress of individual colonies was then monitored periodically.

A summary of the effects of the chromosomes on the growth potential of hybrids is given in Table 4.1. Approximately 30% of MDA-MB-231/Chromosome 7 and OVCAR5/Chromosome 7 hybrids underwent growth arrest. This arrest occurred after between five and eight population doublings, resulting in colonies of around 30 to 250 cells; despite weekly re-feeding, in most cases no increase in cell number was subsequently observed. No growth arrest was observed after introduction of chromosome 15, indicating that the chromosome 7-induced growth arrest was not a general, non-specific effect initiated by an excess of chromosomal material. Furthermore, introduction of chromosome 7 into the pancreatic tumour cell line SUIT2, which is heterozygous for 7q and does not show the fibrous mortalin distribution associated with complementation group D cells, also failed to induce growth-arrest (Table 4.1).

The cells of the growth-arrested hybrids were enlarged and flattened, a morphology characteristic of senescent cells (Figure 4.1 shows an OVCAR5/Chromosome 7 hybrid). To confirm the nature of the growth-arrest the hybrids were stained for senescence-associated endogenous β-galactosidase activity. Growth-arrested hybrids of both recipient cell types showed positive blue staining (Figure 4.2 shows a stained MDA-MB-231/Chromosome 7 hybrid), detectable after hybrids had been maintained in culture for around seven weeks; immortal hybrids of the same age did not stain positive. Interestingly, growth-arrested OVCAR5/Chromosome

7 hybrids often underwent some form of cell death, and could often not be maintained in culture long enough to detect staining. This cell death is reminiscent of that observed in OVCAR5/HeLa cell-cell hybrids (Section 3.4.2).

Some growth-arrested hybrids were subjected to immunofluorescence using an antibody against mortalin. In addition to varying with complementation group assignment, the distribution of mortalin varies according to whether a cell is mortal or immortal (Section 3.3.1). Growth-arrested hybrids of both recipient cell types showed reversion of mortalin from its immortal (perinuclear) to its mortal (pancytosolic) distribution (Figure 4.3 shows MDA-MB-231/Chromosome 7 hybrid cells). Significantly, within a single growth-arrested OVCAR5/Chromosome 7 colony that spontaneously immortalised (presumably following the appearance of a variant immortal cell), the enlarged, growth-arrested cells showed reversion of mortalin to the mortal distribution while the smaller, proliferating cells did not (data not shown).

4.2.2 In HeLa cells

I introduced chromosome 7 into HeLa cells (complementation group B) as a potential control. Published reports indicate that the senescence-inducing effect of chromosome 7 is specific to recipients that belong to complementation group D (Ogata et al., 1993), and that that of chromosome 4 is specific to recipients that belong to group B (Ning et al., 1991). Unexpectedly, all six HeLa/Chromosome 7 hybrids generated underwent growth arrest after between seven and twenty one population doublings, and stained strongly positive for senescence-associated endogenous β-galactosidase activity (Figure 4.4).

We suspected this growth arrest to be a result of expression of the *CAVEOLIN-1* gene, which maps to chromosome 7 (Hurlstone et al., 1999). *CAVEOLIN-1* is expressed at very low levels in HeLa cells (Figure 4.5), and previous work in the institute had shown that it induces a senescence-like growth arrest in this cell line when expressed under the control of a heterologous promoter (Ken Parkinson, personal communication). I therefore carried out immunoperoxidase staining with an antibody against Caveolin-1 on growth-arrested HeLa/Chromosome 7 hybrids and on normal HeLa cells. Much greater levels of staining were observed in the growth-arrested hybrids than in the normal HeLa cells (Figure 4.6). This indicated that the *CAVEOLIN-1* gene on the donor chromosome was being expressed and could therefore be responsible for the growth arrest.

As the normal level of Caveolin-1 expression in OVCAR5 and MDA-MB-231 cells is much greater than that in HeLa cells (Figure 4.5), we considered it unlikely that expression of this protein was the cause of the chromosome 7-induced growth arrest in these cell lines. Caveolin-1 expression clearly shows no correlation with immortality complementation group assignment or 7q LOH, arguing against it being the group D senescence gene. It is interesting that chromosome 7 had no growth-inhibitory effects when introduced into the SUIT2 cell line, which, like HeLa, expresses very low levels of Caveolin-1 (Figure 4.5). It may be that Caveolin-1-induced senescence is something of an oddity specific to HeLa cells. However, it is clear that the existence of genes such as *CAVEOLIN-1* complicates both interpretation of monochromosome transfer experiments and the senescence complementation group picture in general.

4.3 Microsatellite deletion analysis of immortal hybrids

In all, 45 immortal OVCAR5/Chromosome 7 hybrids and 34 immortal MDA-MB-231/Chromosome 7 hybrids were generated. Many SSR markers mapping to 7q were assayed to determine whether alleles were informative, namely whether the allele size differed between the donor and endogenous chromosome. 34 of the markers assayed were clearly informative for OVCAR5/Chromosome 7 hybrids, and 17 for MDA-MB-231/Chromosome 7 hybrids. These markers were then PCR-amplified from DNA extracted from the immortal hybrids and run out under denaturing conditions on a polyacrylamide gel. The products were visualised by autoradiography and scored for loss or retention of the donor allele.

Representative examples of the deletion analysis are shown in Figure 4.7 and the results are summarised in Tables 4.2 and 4.3. Figure 4.8 shows an ideogram of the long arm of chromosome 7 with the approximate positions of markers. The presence of donor alleles in the majority of the DNA samples confirmed that the immortal colonies did indeed contain an additional chromosome 7. Overall, more than half the immortal hybrids of both cell types had lost one or more marker from the long arm of the donor chromosome, consistent with the hypothesis that the immortality of these hybrids was a result of loss or inactivation of the senescence gene on the introduced chromosome. However, it is very apparent that this mode of analysis is hampered by noise, with losses not restricted to one particular region of the chromosome.

62% (28/45) of immortal OVCAR5/Chromosome 7 hybrid samples had lost or partially lost at least one marker from the long arm. 11% (5/45) of hybrids—for

example hybrids 17 and 24—appeared unequivocally to have lost the entire long arm, the hygromycin resistance marker having presumably integrated elsewhere in the genome. Several hybrids—for example hybrids 14 and 29—instead displayed multiple interstitial losses of the q arm; not including loss of the most telomeric markers, 27% (12/45) of hybrids displayed this pattern of loss. The most frequently lost q arm marker was the tetranucleotide repeat D7S818 (114 cM from 7pter; 7q22), which was lost or showed reduced intensity in 42% (19/45) of hybrid samples.

56% (19/34) of MDA-MB-231/Chromosome 7 hybrids had lost one or more markers from the long arm. None had lost every marker assayed, but 9% (3/34) had lost all but two. Multiple interstitial losses of the q arm, not including loss of the most telomeric markers, were displayed by 21% (7/34) of hybrids (for example hybrids 8 and 11). The most frequently lost q arm markers were the dinucleotide repeat AFMA073ZB9, the tetranucleotide repeat D7S2847 (both approximately 125 cM from 7pter; 7q31), and the telomeric tetranucleotide repeat D7S1807; these were each lost in 35% (12/34) of hybrids.

A possible allele-loss 'hot-spot' in immortal OVCAR5/Chromosome 7 hybrids spans the markers 17TA-5/17B-RE3 to D7S650 (7q31; Table 4.2). A possible hot-spot in immortal MDA-MB-231/Chromosome 7 hybrids, spanning AFMA073ZB9 and D7S2847, lies within this region (Table 4.3). Moreover, the 17TA-5/17B-RE3 to D7S650 region largely lies within the minimum region of loss defined by six separate LOH studies (Takahashi et al., 1995; Achille et al., 1996; Koike et al., 1997; Shridhar et al., 1997; Edelson et al., 1997; Zenklusen et al., 1994). The hot-spots, however, do not stand out very distinctly, and in OVCAR5/Chromosome 7 hybrids there is a further possible hot-spot spanning the markers D7S1797 to D7S818.

For OVCAR5/Chromosome 7 hybrids the analysis was extended to the p arm of the donor chromosome, to determine whether losses were specific to the q arm. Again, over half the immortal hybrids (56%; 25/45) were found to have lost at least one of the two informative markers analysed from this arm (Table 4.2), raising doubts as to the significance of losses found on the q arm. Indeed, the frequency of loss (53%; 24/45) of the p arm marker GATAP19270 was greater than that of any long-arm marker analysed. In addition, a limited number of q arm markers were analysed from hybrids generated from the control SUIT2 line (Table 4.4). Again, a high frequency of apparently random losses was found: 37% of hybrids (7/19) had lost at least one of the four markers

assayed. These results call into question the significance of the allele-loss hot-spot identified on the long arm.

In summary, it difficult to draw conclusions from this analysis as to the sub-chromosomal location of the chromosome 7 senescence gene. For the combinations of donor chromosome and recipient cell lines used here, this mapping strategy appears inappropriate (see Discussion).

Table 4.1

Table 4.1
The proliferative capacity of tumour cell line clones after introduction of human chromosomes 7 and 15
The percentage of OVCAR5, MDA-MDA-231 and SUIT 2 clones that undergo growth arrest after introduction of chromosomes 7 and 15 by MMCT.
OVCAR5: ovarian carcinoma. MDA-MB-231: breast carcinoma. SUIT2: pancreatic carcinoma.

The proliferative capacity of tumour cell line clones after introduction of human chromosomes 7 and 15

	Chromosome 7		Chromosome 15	
	Total no. of colonies	**Percentage of hybrids that undergo growth arrest**	**Total no. of colonies**	**Percentage of hybrids that undergo growth arrest**
MDA-MB-231	*	30	20	0
OVCAR5	*	30	9	0
SUIT2	20	0	-	-

* >50 colonies

Table 4.1

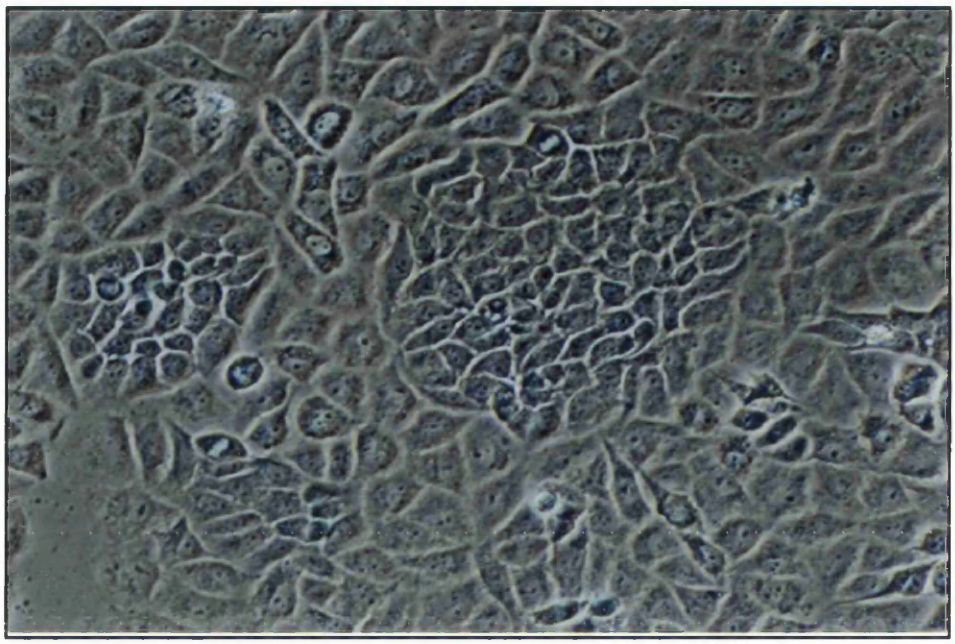
Figure 4.1

Figure 4.1
OVCAR5/Chromosome 7 hybrids

Phase-contrast photomicrographs of proliferating (a) and growth-arrested (b) OVCAR5/Chromosome 7 hybrid colonies at approximately 4 weeks after introduction of the chromosome. Cells in the growth-arrested colony are enlarged and flattened compared to those in the proliferating colony. Both images are at the same magnification.

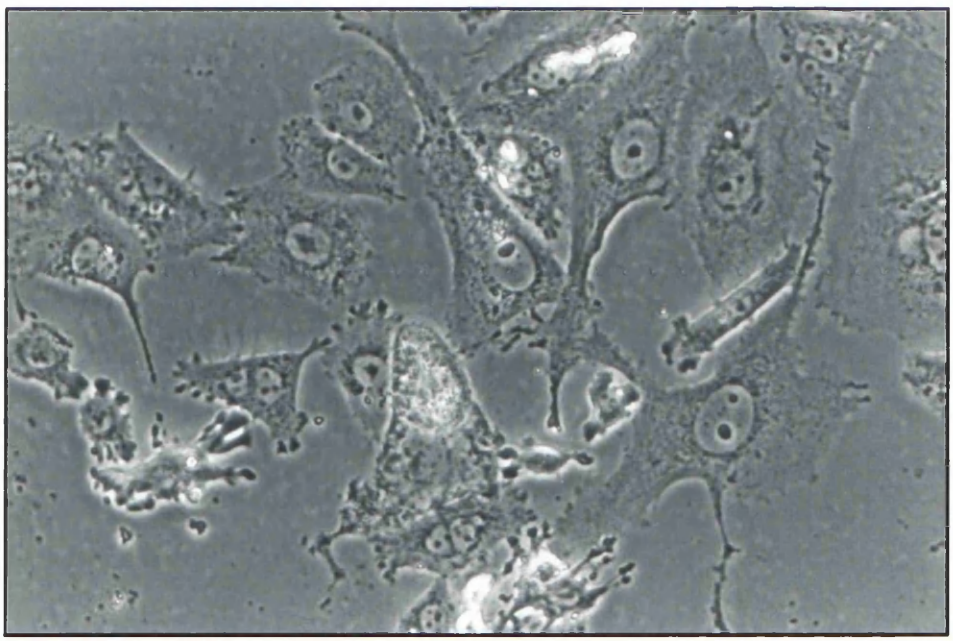
OVCAR5/Chromosome 7 hybrids

a.



Proliferating OVCAR5/Chr7 colony

b.



Growth-arrested OVCAR5/Chr7 colony

Figure 4.1

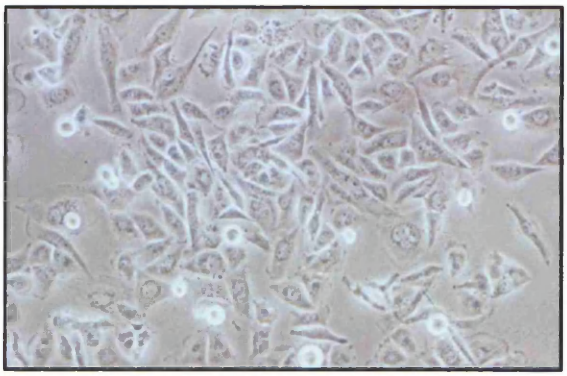
Figure 4.2

Figure 4.2
MDA-MB-231/Chromosome 7 hybrids

Phase-contrast photomicrographs of proliferating (a) and growth-arrested (b & c) MDA-MB-231/Chromosome 7 hybrid colonies. Cells in the growth-arrested colonies are enlarged and flattened compared to those in the proliferating colony, and stain positive for expression of senescence-associated endogenous β-galactosidase. The β-gal staining was performed approximately 9 weeks after introduction of the chromosome. All images are at the same magnification.

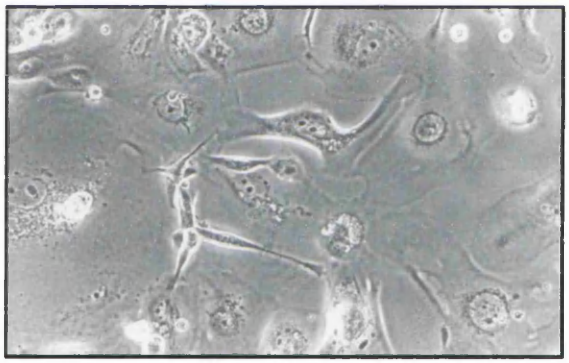
MDA-MB-231/Chromosome 7 hybrids

a.



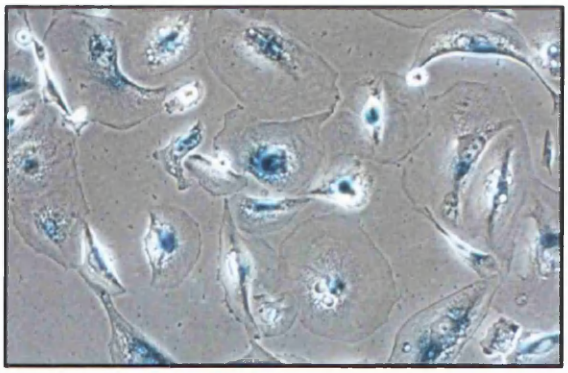
Proliferating MDA-MB-231/Chr7 colony

b.



Growth arrested MDA-MB-231/Chr7 colony

c.



Growth-arrested MDA-MB-231/Chr7 colony
β-gal stained

Figure 4.2

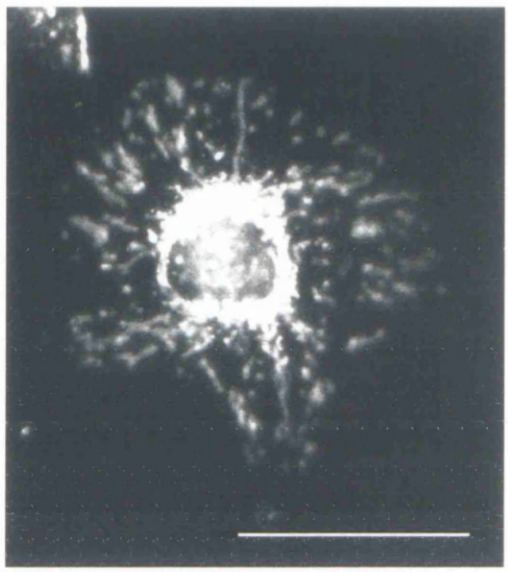
Figure 4.3

Figure 4.3
The cellular distribution of mortalin in growth arrested MDA-MB-231/Chromosome 7 hybrid cells

Confocal immunofluorescence micrographs of an MDA-MDA-231 cell (a) and growth-arrested MDA-MB-231/Chromosome 7 hybrid cells (b & c) stained for mortalin. The intracellular distribution of the protein in growth-arrested cells has reverted from the fibrous, perinuclear distribution characteristic of immortal cells belonging to complementation group D to the pancytosolic distribution characteristic of mortal cells (Wadhwa et al., 1995).
Scale bars represent 50 μm.

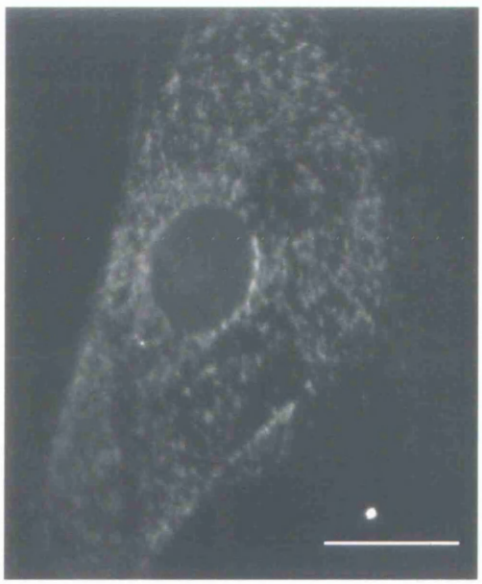
The cellular distribution of mortalin in growth arrested MDA-MB-231/Chromosome 7 hybrid cells

a.

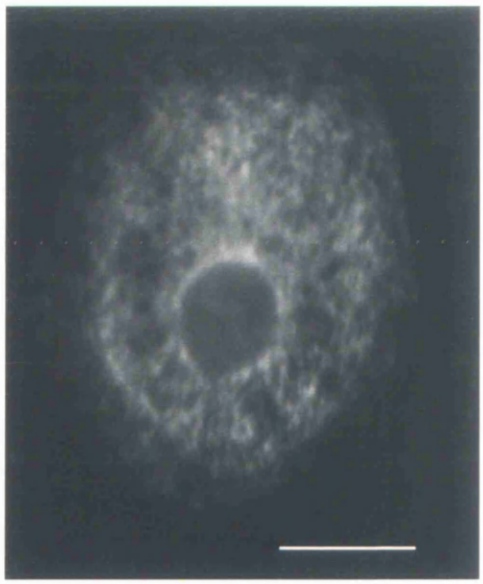


MDA-MB-231

b.



c.



Growth-arrested MDA-MB-231/Chr7 cells

Figure 4.3

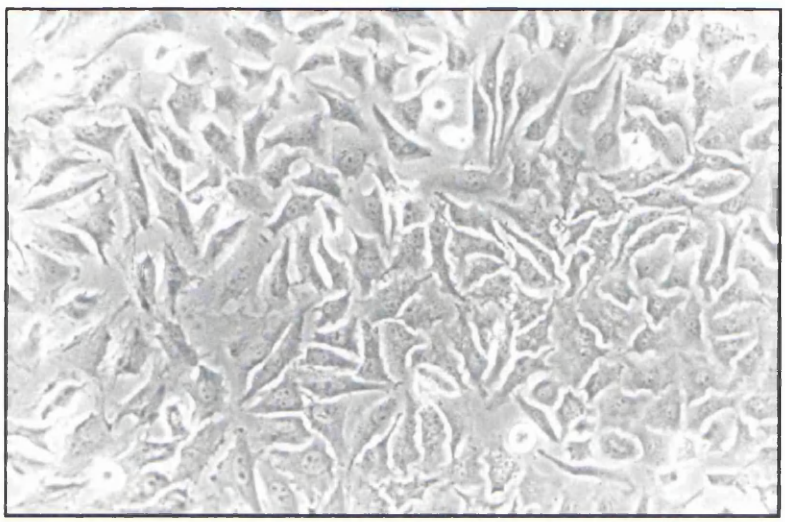
Figure 4.4

Figure 4.4
HeLa/Chromosome 7 hybrid

Phase-contrast photomicrographs of HeLa cells (a) and a growth-arrested HeLa/Chromosome 7 hybrid colony (b). The cells in the growth-arrested colony are enlarged and flattened compared to normal HeLa cells, and stain positive for expression of senescence-associated endogenous β-galactosidase. The colony was stained approximately 10 weeks after introduction of the chromosome. Both images are at the same magnification.

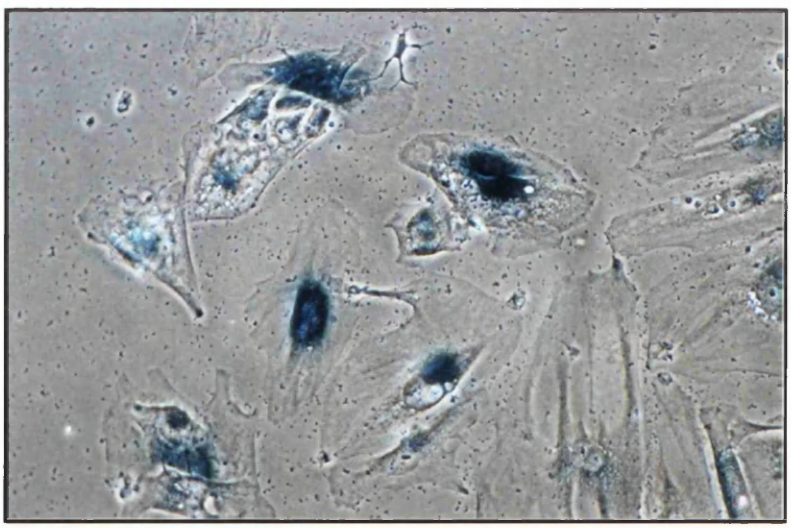
HeLa/Chromosome 7 hybrid

a.



HeLa

b.



Growth-arrested HeLa/Chr7 colony
β-gal stained

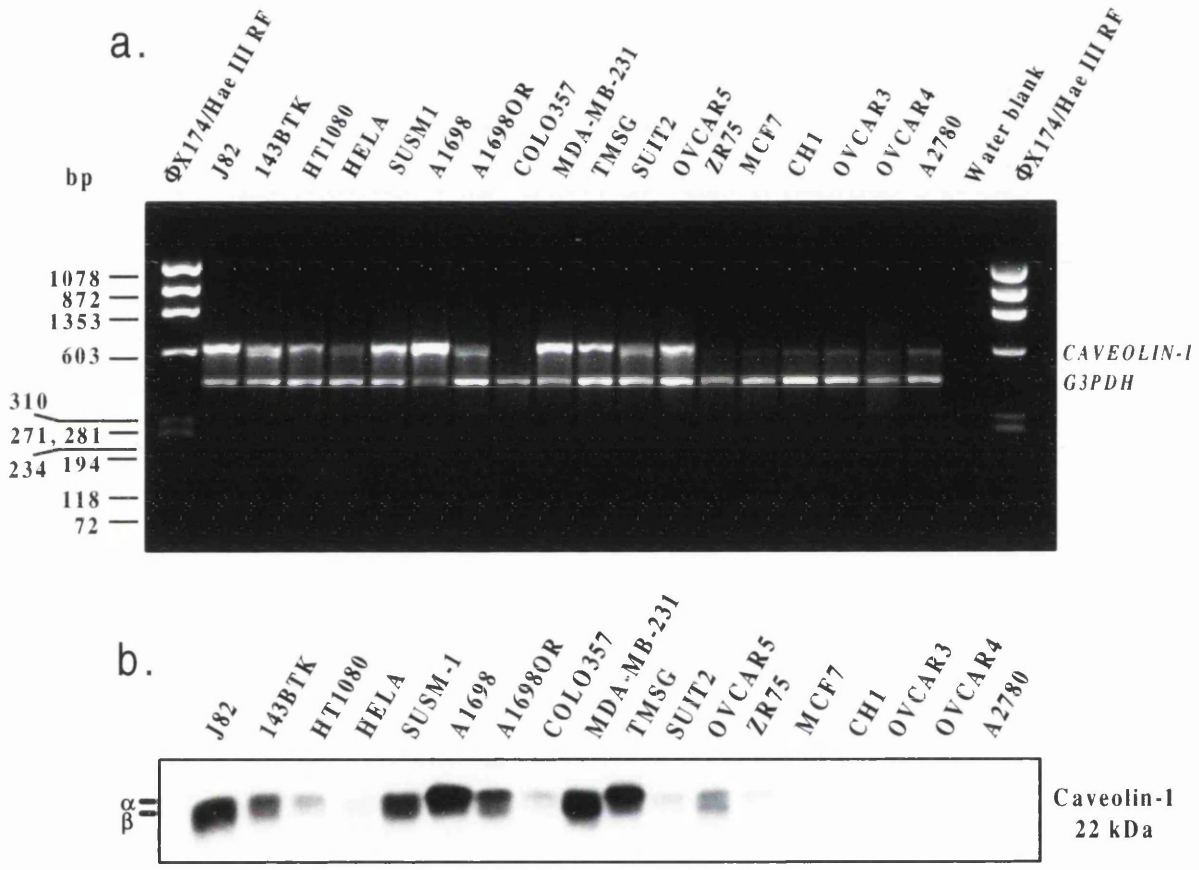
Figure 4.4

Figure 4.5

Figure 4.5
Caveolin-1 expression in tumour cell lines

RT-PCR analysis (a) and western blot analysis (b) of CAVEOLIN-1 expression in tumour cell lines. HeLa cells express very low levels of both transcript and protein. Primers that amplify a region of G3PDH cDNA were included in the RT-PCR reactions as an internal control for amplification efficiency. α and β refer to the two protein isoforms of Caveolin-1.

Caveolin-1 expression in tumour cell lines



This work was performed by A. Hurlstone

Figure 4.5

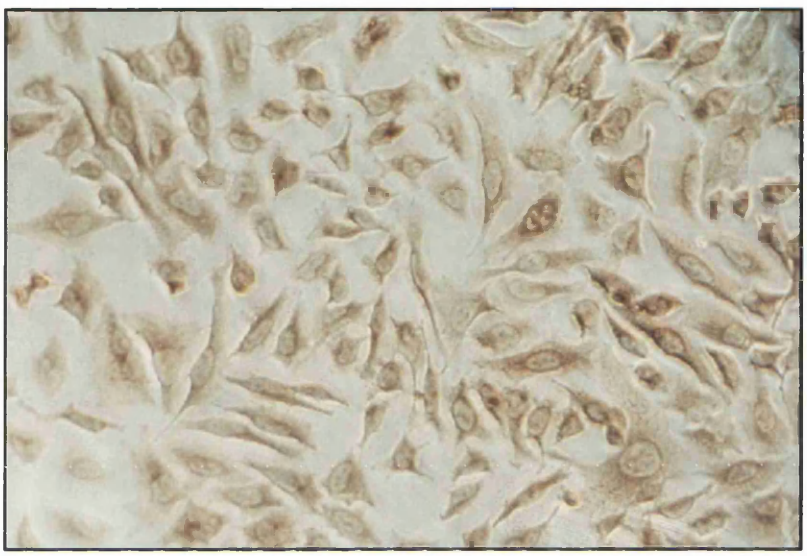
Figure 4.6

Figure 4.6
Caveolin-1 expression in HeLa/Chromosome 7 hybrid cells

Phase-contrast photomicrographs of HeLa cells (a) and a growth-arrested HeLa/Chromosome 7 hybrid colony (b), immunoperoxidase stained for Caveolin-1. Cells of the growth-arrested colony express greater levels of Caveolin-1 than normal HeLa cells. The colony was stained approximately 7 weeks after introduction of the chromosome. Both images are at the same magnification.

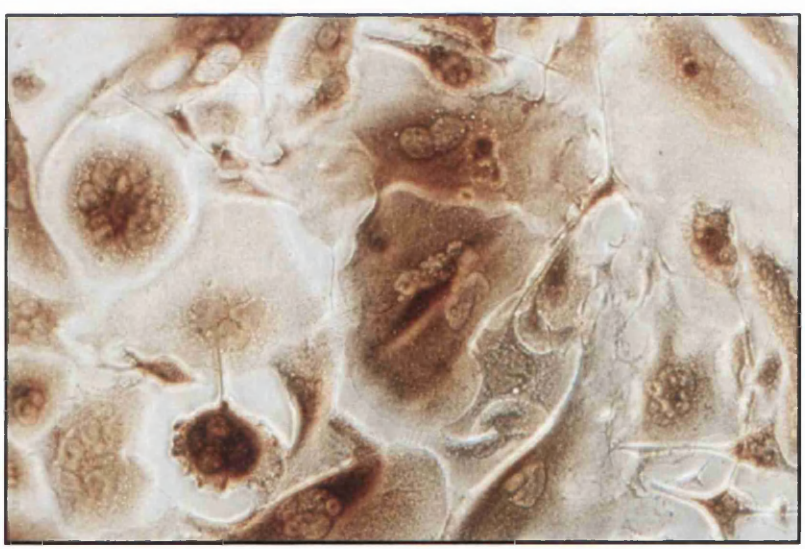
Caveolin-1 expression in HeLa/Chromosome 7 hybrid cells

a.



HeLa

b.



Growth-arrested HeLa/Chr7 colony

Figure 4.6

Figure 4.7

Figure 4.7
Representative examples of allele loss analysis in immortal tumour cell line/Chromosome 7 hybrids

Autoradiographs from radiolabelled PCR products of the chromosome 7 microsatellite markers D7S692, D7S1807, D7S1809 and D7S821, amplified from the DNA of immortal OVCAR5/Chromosome 7 hybrids (a) and MDA-MB-231/Chromosome 7 hybrids (b).
A92/*Hytk*7: MMCT donor. EA: endogenous allele.
DA: donor allele. IB: invariant band (PCR artefact).

Representative examples of allele loss analysis in immortal tumour cell line/Chromosome 7 hybrids

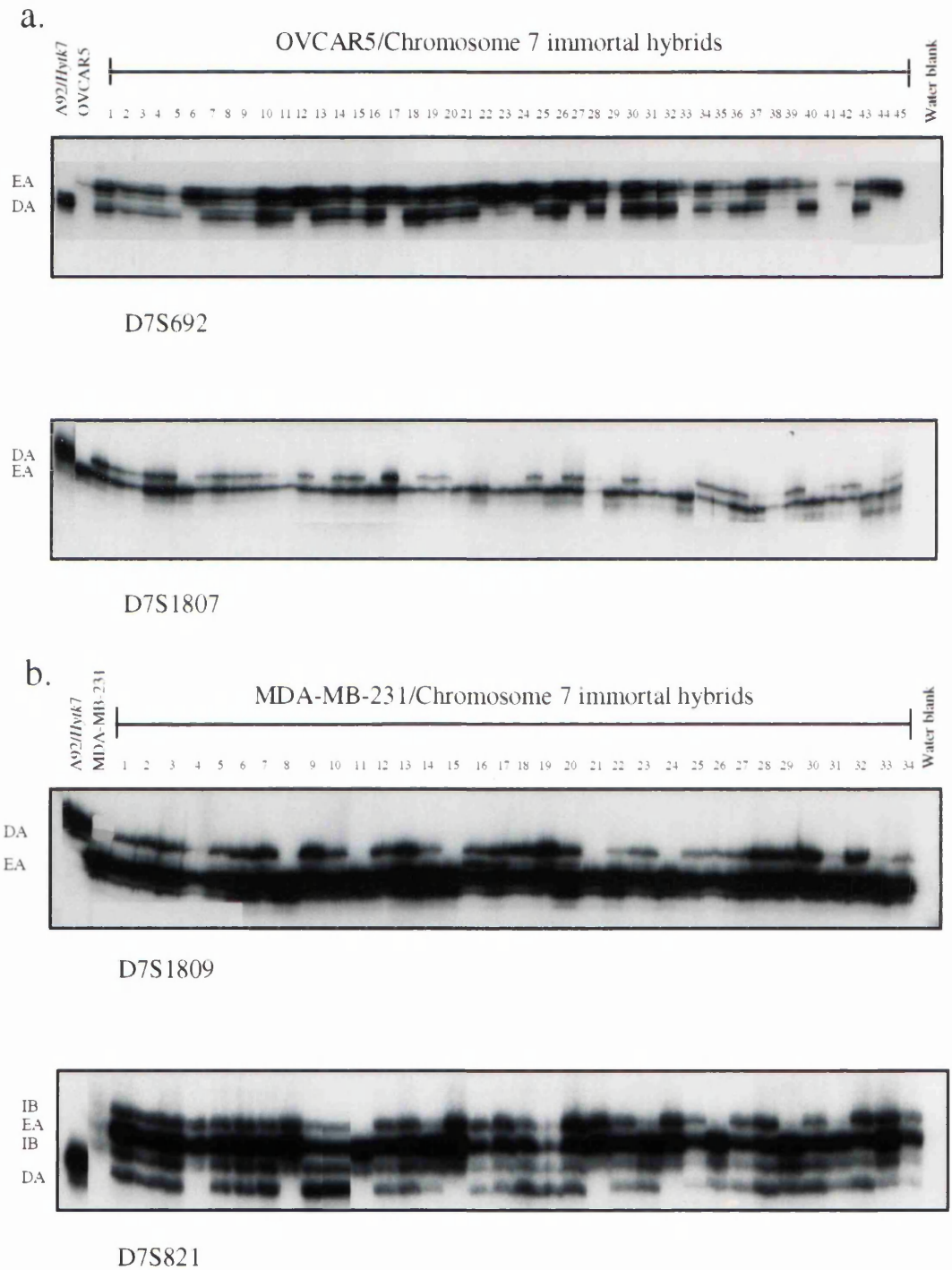


Figure 4.7

Table 4.2

Table 4.2
Summary of allele loss in immortal OVCAR5/Chromosome 7 hybrids

Summary of chromosome 7 microsatellite deletion analysis of immortal OVCAR5/Chromosome 7 hybrids. Only hybrids that exhibited loss of one or more marker are shown. Markers are given in order of chromosomal location. -: donor allele lost. +: donor allele retained. /: equivocal (band is of reduced intensity). ^: new allele. '% loss' column gives the percentage of hybrids that have lost the marker (either fully or equivocally).

Summary of allele loss in immortal OVCAR5/Chromosome 7 hybrids

p arm

q arm

	4	5	6	7	9	10	11	12	14	17	19	21	22	23	24	25	27	29	30	32	33	34	35	37	38	39	41	42	44	45	% loss
GATAP34087	-	+	+	-	+	-	+	+	-		-	+	-	/	-	-	-	+	-	+	-	-	+	+	/	+	-	+	-	+	36
GATAP19270	+	-	/	-	+	-	+	/	-	-	-	+	-	/	-	-	-	-	-	/	-	-	+	+	/	-	-	-	-	-	53
D7S1797	+	+	+	-	+	+	/	-	+	-	+	+	-	/	-	+	-	/	-	+	-	+	+	+	/	/	-	/	-	-	38
D7S820	+	+	+	-	+	+	+	/	+	-	+	/	-	/	-	+	-	+	/		-	+	+	+	/	+		+		+	29
D7S821	+	+	+	-	+	+	+	/	+	-	+	+	-	-	-	+	-	-	-	+	-	+	+	+	/	/		/	-	-	36
D7S1841	+	+	-	+	+	+	+	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	+	-	-	38
D7S501	+	+	-	+	+	+	+	-	+	-	-	+	-	-	-	-	-	+	-	+	+	-	+	+	+	+	-	-	-	-	33
D7S818	+	+	+	-	+	+	+	-	+	-	-	+	-	-	-	-	-	-	-	/	-	-	+	+	/	+	-	/	-	-	42
D7S692	+	+	-	+	+	+	+	-	+	-	+	+	-	/	-	+	-	-	+	+	-	+	+	+	/	/		-	-	-	33
D7S1817	+	+	-	+	+	+	+	-	+	-	+	+	-	-	-	+	-	-	+	+	-	+	+	+	/	+	-	-	-	-	31
D7S525	+	+	-	+	+	+	+	-	+	-	+	+	-	-	-	+	-	-	+	+	-	+	+	+	+	+	-	-	-	-	29
D7S523	+	+	-	-	+	+	+	-	-	-	+	+	-	^	-	+	-	-	+	+	-	+	+	+	^	-	-	-	+	-	31
D7S1811	+	+	+	-	+	+	+	-	+	-	+	+	-	-	-	+	-	+	+	+	-	+	/	-	-	+	-	-	-	+	31
D7S486	+	+	+	-	+	+	+	-	+	-	+	+	-	-	-	+	-	+	+	+	-	+	/	-	/	+	-	-	-	+	31
GH220/324	+	+	+	-	+	+	+	-	-	-	+	+	-	-	-	+	-	+	+	+	-	+	/	-	+	+	-	-	-	+	31
778CA	+	+	+	-	+	+	+	-	-	-	+	+	-	/	-	+	-	+	+	+	-	+	/	-	-	+	-	-	-	+	33
724CA	+	+	+	-	+	+	+	-	-	-	+	+	-	-	-	+	-	+	+	+	-	+	/	-	/	+	-	-	-	+	33
17TA-5/17B-RE3	+	+	+	-	+	+	+	-	+	-	+	+	-	-	-	+	-	-	+	+	-	+	/	-		/			-	-	38
786CA	+	+	+	-	-	+	+	-	+	-	+	+	-	-	-	+	-	-	+	+	-	+	+	-	-	+	-	-	-	-	36
AFMA073ZB9	+	+	+	-	+	+	+	-	+	-	+	+	-	-	-	+	-	-	+	+	-	+	+	-	-	+	-	+	-	-	31
D7S2847	+	+	+	-	+	-	+	-	-	-	+	+	-	-	-	+	-	-	+	+	-	+	+	-	+	/	-	+	-	-	36
GGAAP7358	+	+	+	-	+	+	+	-	-	-	+	+	-	-	-	+	-	-	+	+	-	+	/	-	/	/	-	+	-	-	38
D7S643	+	+	+	-	+	/	+	-	+	-	+	+	-	-	-	+	-	-	+	+	-	+	+	-	-	-	-	+	-	-	36
D7S650	+	+	+	-	+	+	+	-	-	-	+	+	-	-	-	+	-	-	+	+	-	+	+	-	/	/	-	+	-	-	36
D7S1835	+	+	+	-	+	+	+	-	+	-	+	+	-	/	-	+	-	+	+	+	-	+	+	+	+	+	-	+	-	+	22
D7S685	+	+	+	-	+	+	+	-	+	-	+	+	-	-	-	+	-	-	+	+	-	+	/	-	/	/	-	+	-	-	36
D7S1809	+	+	+	-	+	+	+	-	^	-	+	+	-	-	-	+	-	+	+	+	-	+	+	+	*	+		*	-	+	22
D7S1822	+	+	+	-	+	+	+	-	+	-	+	+	-	-	-	+	-	+	+	+	-	+	+	+	+	+	-	+	-	+	22
D7S530	+	+	+	-	+	+	+	-	/	-	+	+	-	-	-	+	-	-	+	+	-	+	+	-	/	+	-	+	-	-	33
D7S2203	+	+	+	-	+	+	+	-	+	-	+	+	-	-	-	+	-	+	+	+	-	+	+	/	/	+	-	+	-	+	27
D7S2197	+	+	+	-	+	+	+	-	-	-	+	+	-	-	-	+	-	^	+	+	-	+	+	+	/	^	-	-	-	^	29
D7S1804	+	+	+	-	+	+	+	-	+	-	+	+	-	-	-	+	-	-	+	+	-	+	+	+	+	+	-	+	-	+	24
D7S2202	+	+	-	+	+	+	+	-	+	-	+	+	-	-	-	+	-	-	+	/	-	+	+	+	/	/	-	+	-	/	33
D7S794	+	+	-	-	+	+	+	/	+	-	/	+	-	-	-	+	-	+	-	-	-	+	+	+	/	/	-	+	-	+	36
D7S1805	+		-	-	+	+	+	/	-	-	/	+	-	-	-	+	-	+	-	/	-	+	+	+	/	+		/		+	38
D7S1807	+	+	-	+	+	+	+	/	-	-	-	+	-	-	-	-	-	+	-	/	-	-	+	+	/	+	-	/	-	+	38

Table 4.2

Table 4.3

Table 4.3
Summary of allele loss in immortal MDA-MB-231/Chromosome 7 hybrids

Summary of chromosome 7 microsatellite deletion analysis of immortal MDA-MB-231/Chromosome 7 hybrids. Only hybrids that exhibited loss of one or more marker are shown. Markers are given in order of chromosomal location. _: donor allele lost. +: donor allele retained. /: equivocal (band is of reduced intensity). ^: new allele. '% loss' column gives the percentage of hybrids that have lost the marker (either fully or equivocally).

Summary of allele loss in immortal MDA-MB-231/Chromosome 7 hybrids

	3	4	6	8	10	11	14	15	16	21	22	24	25	26	27	29	31	33	34	% loss
D7S1797	+	+	+	+	+	-	+	+	+	+	+	+		-	-	+	+			
D7S820	+	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	9
D7S821	+	-	+	-	+	-	+	-	+	-	+	-	+	+	+	+	+	+	+	18
D7S1841	+	-	+	-	+	-	/	-	+	-	+	-		/	+	+	+	-	/	29
D7S501	-	-	+	-	+	+	+	-	+	-	+	-	+	+	+	+	+	+	+	18
D7S818	+	-	+	+	+		+	-	+	-	+	+	+	+	+	+	+	+	+	12
D7S1799	+	-	+	-	+	-	+	-	+	-	+	+		+	+	+	+	+	+	15
D7S471	+	-	+	-	/	+	-	-	+	-	+	-	+	+	+	+	+	+	+	21
D7S523	+	-	-	-	+	+	-	-	+		+	-	-	+	+	+	+		+	24
D7S687	+	-	+	+	+	+	+	-	+	-	-	+		+	+	+	-	+	-	18
724CA	+	-	+	-	+	-	+	-	+	-	-	-	-	+	+	+	-	-	-	32
AFMA073ZB9	+	-	+	-	+	-	+	-	+	-	-	-	-	+	+	-	-	-	-	35
D7S2847	+	-	+	-	+	+	-	-	-	-	-	-		+	+	+	-	-	-	35
D7S1809	+	-	+	-	+	-	+	-	+	-	+	-	+	+	+	+	-	-	+	24
D7S1801	+	-	+	-	+	-	+	-	+	-	-	-		+	-	+	-	-	+	29
D7S1804	+	-	+	-	+	-	+	-	+	-	-	-	+	+	+	+	-	-	+	26
D7S1807	+	-	+	-	+	-	+	-	+	-	-	-		-	+	-	-	-	-	35

Table 4.3

Table 4.4

Table 4.4
Summary of allele loss in SUIT2/Chromosome 7 hybrids

Summary of chromosome 7 microsatellite deletion analysis of SUIT2/Chromosome 7 hybrids. Only hybrids that exhibited loss of one or more marker are shown. Markers are given in order of chromosomal location. -: donor allele lost. +: donor allele retained. /: equivocal (band is of reduced intensity). ^: new allele. '% loss' column gives the percentage of hybrids that have lost the marker (either fully or equivocally).

Summary of allele loss in SUIT2/Chromosome 7 hybrids

	2	3	4	9	10	16	18	% loss
D7S821	-	-	+	-	+	-	-	25
D7S818	-	+	+	-	/	-	-	25
D7S486			-		-	-	-	20
724CA	+	+	+	+	-	-	-	15

Table 4.4

Figure 4.8

Figure 4.8
Ideogram of the long arm of chromosome 7

The long arm of chromosome 7 is depicted as an ideogram showing cytogenetic banding. Alongside are shown the polymorphic SSR (and RFLP) markers used to perform allele loss analysis. Those in blue represent Genethon markers, in red CHLC markers, in green CA.GT markers identified by A. Hurlstone (BICR, unpublished data), and in black an RFLP (GH220/324) within the c-*MET* locus and a microsatellite marker (17TA-5/17B-RE3) in intron 17b of the *CFTR* locus. See Table A1 for more details of these markers.

Ideogram of the long arm of chromosome 7

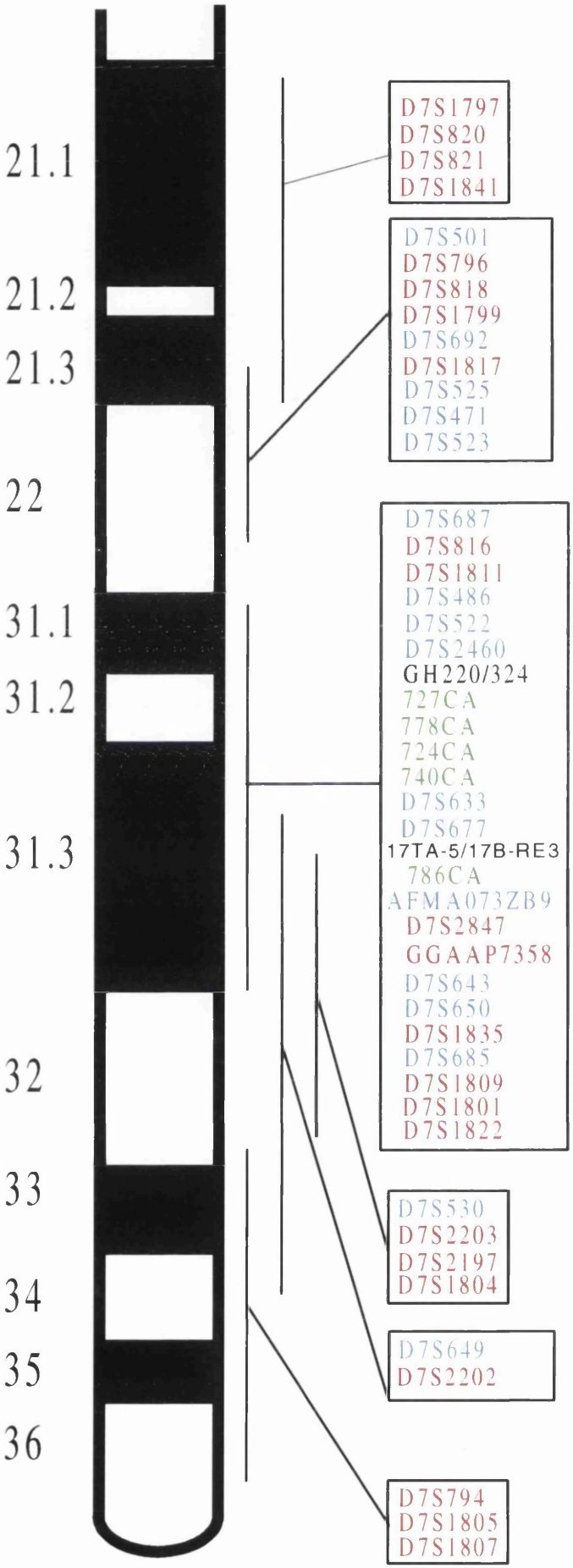


Figure 4.8

CHAPTER 5

RESULTS

Analysis of the tumour suppressive effects of human chromosome 7

5. Results

Analysis of the tumour suppressive effects of human chromosome 7

5.1 Purpose

As detailed in the introduction, high frequencies of LOH are observed at 7q31 in a variety of human tumours (Reviewed in Zenklusen & Conti, 1996). This indicates the existence of a tumour suppressor gene in this region. Introduction of human chromosome 7 by MMCT was found to reduce the tumour-forming ability of a murine squamous cell carcinoma cell line, giving functional evidence for this gene (Zenklusen et al., 1994b). Significantly, one of the two hybrid clones that retained the tumourigenicity of the parent cell line had a deletion at 7q31.1-7q31.2. The observation that introduction of human chromosome 7 induces replicative senescence in two immortal, non-tumourigenic cell lines that have 7q31 defects (Ogata et al., 1993) also suggests the existence of a gene in this region with a role in replicative senescence. Given the probable role of replicative senescence in tumour suppression, it is tempting to speculate that the 7q31 tumour suppressor and replicative senescence gene are one and the same.

We chose to test this hypothesis, by investigating whether the senescence-inducing effect of chromosome 7 is separable from its tumour suppressive effect. To do this, we analysed the tumourigenicity of some of the immortal chromosome 7-containing hybrid clones already generated (Chapter 4). As these clones have presumably deleted or otherwise inactivated the senescence gene on the donor chromosome, any reduction in their tumourigenicity compared to the parent cell line would suggest the existence of an additional chromosome 7 tumour suppressor gene. Specifically, we used immortal hybrids for which no SSR losses on the donor chromosome had been found, as in such hybrids disruption to genes other than the senescence gene will have been minimised. To gain an *in vitro* measure of the tumourigenic potential of these clones we analysed their anchorage independent growth in soft agar, while as a more definitive test we determined their ability to form tumours in nude mice.

Should evidence be found for the existence of a separate tumour suppressor gene, we anticipated that the strategy we had pursued would facilitate relatively rapid gene mapping. SSR deletion analysis of DNA extracted from revertant soft agar

colonies or tumours, and cDNA or DNA subtractions between tumourigenic and non-tumourigenic hybrid clones, would be two obvious mapping approaches.

5.2 Immortal OVCAR5/Chromosome 7 hybrids: their growth in soft agar

Pilot experiments confirmed that the OVCAR5 parent cell line was capable of anchorage independent growth in soft agar, with approximately 80 colonies forming per 5000 cells plated. The colony-forming efficiency in agar of the MDA-MB-231 parent cell line, however, was rather lower, and the colonies that did form were irregular in terms of shape and size (data not shown).

We therefore proceeded with immortal hybrids generated from the OVCAR5 parent cell line. First, the growth in soft agar of hybrids 3, 20 and 40, none of which showed any SRS losses on the donor chromosome (Table 4.2), was compared to that of OVCAR5. Two of the hybrids, hybrids 20 and 40, displayed a very marked (20-fold) reduction in colony-formation compared to the parent cell line, while the remaining hybrid, hybrid 3, exhibited a slight reduction (Figure 5.1a). These results could not be explained simply by variations in surface growth rates: while the doubling time of all three hybrids was slightly lower than that of the parent cell line (perhaps because they contained an additional chromosome), the hybrid with the fastest doubling time, hybrid 40 (Figure 5.2), was one of the two hybrids with very low colony-forming ability in agar.

Further studies were then performed to determine whether the low colony-forming efficiency of hybrids 20 and 40 was within that of the normal clonal variation of the parent cell line. The growth in soft agar of six individual OVCAR5/p-babe Hygro transfectant clones (hyg 1-6), and of two OVCAR5/Chromosome 7 immortal hybrids that had lost every SSR marker analysed from the donor chromosome (hybrids 22 and 41; Table 4.2), was measured. Two additional OVCAR5/Chromosome 7 immortal hybrids were included in this assay: these were hybrids 6 and 7, which from the SSR analysis appeared to have lost mutually exclusive regions of the donor chromosome (Table 4.2).

The results are summarised in Figure 5.1b. One of the p-babe Hygro transfected clones, hyg 3, and one of the immortal hybrids that had lost all markers from the donor chromosome, hybrid 22, showed an even greater reduction in anchorage-independent growth compared to the parent cell line than hybrids 20 and 40. Four p-babe Hygro transfected clones, hyg 1, 2, 4 and 5, showed a slighter (three to five-fold) reduction in

colony-formation, while one, hyg 6, exhibited a 3-fold increase (subsequent experiments suggested that this increase may be closer to 10-fold—data not shown). The other OVCAR5/Chromosome 7 hybrid that appeared to have lost all donor chromosome SSR markers, hybrid 41, displayed a level of anchorage independent growth comparable to that of the parent cell line.

From these results it is apparent that there is a huge amount of clonal variation within this cell line in the ability to form colonies in soft agar; again, this variation does not appear to correlate with differences in surface growth rates (data not shown). Indeed, the 20-fold reduction in colony forming ability of hybrids 20 and 40 (compared to the parent cell population as a whole) lies within the normal clonal variation of the cell line. However, in the absence of knowledge of the colony-forming efficiency of the specific OVCAR5 cells from which hybrids 20 and 40 were generated, it cannot be concluded that chromosome 7 has no tumour suppressive effect in OVCAR5. The fact that two out of three of the test hybrids showed a very marked reduction in anchorage independent growth, compared to just two out of eight of the control cell lines, perhaps argues in favour of a tumour suppressive effect. As further circumstantial support one of the two hybrids that appeared to have retained mutually exclusive regions of the donor chromosome exhibited a marked reduction in colony-forming ability (hybrid 7), while the other (hybrid 6) did not (Figure 5.1c).

5.3 Immortal OVCAR5/Chromosome 7 hybrids: their growth in nude mice

To help determine whether the donor chromosome 7 really was exerting a tumour suppressive effect in hybrids 20 and 40, the growth of these hybrids in athymic nude mice was analysed. The aim was both to ascertain whether tumour-forming ability correlated with colony growth in soft agar, and to extract and analyse DNA from any tumours that formed. If the donor chromosome *did* have tumour suppressive properties, there would be selection pressure for tumour cells to lose all or part of it—and such loss may be detectable by SRS deletion analysis.

Accordingly, 500 000 cells of the parent cell line and of each of hybrids 3, 20 and 40 (the 'test hybrids') were injected subcutaneously into nude mice. In a subsequent experiment, the tumour-forming ability of the p-babe Hygro transfected clones hyg 2 and hyg 3, and of the immortal hybrids 22, 41, 6 and 7, was also analysed. The results of both studies, in terms of the length of time it took for tumours to form, are summarised in Table 5.1. Perhaps unexpectedly, it is apparent that the tumour-forming

ability of these hybrids and clones does not correlate particularly well with their ability to form colonies in soft agar. While hybrid 20 was very suppressed for growth in soft agar, it formed tumours in nude mice more rapidly than any of the other hybrids and clones tested (with the exception of the parent cell line). Hybrid 41 formed colonies in agar as readily as the parent cell line, but 3 of the 5 mice injected with it still showed no signs of tumours after 23 weeks. Similarly, of the two hybrids retaining mutually exclusive regions of the donor chromosome, hybrid 7 exhibited little growth in soft agar but formed tumours reasonably rapidly, while hybrid 6 displayed reasonable growth in soft agar yet took several months to form tumours. In agreement with the soft agar experiments, however, the tumour studies clearly demonstrate much clonal variation within the OVCAR5 parent cell line. The studies also show that the time taken for hybrids 20 and 40 to form tumours, although greater than that for the parent cell population as a whole, lies within that clonal variation.

DNA was then extracted from all five tumours formed by each of the three test hybrids, and the loss or retention of informative SSR markers on the donor chromosome was investigated. One microsatellite marker mapping to the p arm, and nine markers mapping to the q arm, were analysed; Figure 5.3 shows a representative autoradiograph. Hybrid 40 tumour 2 had lost every marker analysed. This, however, was the only one of the 15 tumour samples analysed that displayed any losses at all (Table 5.2a; see Figure 4.8 for an ideogram of 7q giving approximate positions of markers.) This argues against there being selection pressure for tumours to lose the chromosome. DNA was also extracted from a limited number of cell clones expanded from agar colonies of the three test hybrids. This DNA was also subjected to microsatellite marker deletion analysis. Interestingly, both the clones expanded from colonies of hybrid 20 had lost all the markers analysed (Table 5.2b). While this is perhaps a random event there may be some selection pressure in soft agar, but not in tumours, for hybrids to lose the donor chromosome.

5.4 Immortal OVCAR5/Chromosome 7 hybrids derived from a single OVCAR5 cell clone: their growth in soft agar and nude mice

We had therefore found no conclusive evidence for the existence of a gene on chromosome 7, separate to the senescence gene, that suppresses the tumourigenicity of the OVCAR5 cell line. However, as a final test we investigated the soft agar growth and tumourigenicity of a panel of OVCAR5/Chromosome 7 hybrids that were all

derived from the same single OVCAR5 cell clone. This approach eliminated the difficulty in interpretation of results caused by the large clonal variation in the OVCAR5 cell line.

A single OVCAR5 colony was extracted from soft agar and expanded. Chromosomes 7 and 15 were then introduced into this clone by MMCT, and six hybrids of each type were expanded. The growth of these 12 hybrid clones in soft agar was compared to that of the parent cell clone and of the OVCAR5 cell line. Interestingly, five of the six chromosome 7-containing hybrids showed a marked reduction in colony formation compared to the parent cell clone (Figure 5.4); the sixth clone showed slightly greater growth than the parent cell clone. No differences in the surface growth rates of the parental clone and the six hybrids were noticed. Of the six chromosome 15-containing hybrids, five showed much greater colony formation in agar than the parent cell clone (Figure 5.5): this suggests that there may be a gene or genes on chromosome 15 with anchorage independent growth promoting activity. The sixth displayed the same level of colony formation as the parent cell clone.

Overall, these results suggest that there is a gene or genes on chromosome 7, independent of the senescence gene, that suppresses the anchorage independent growth of the OVCAR5 cell line. The single chromosome 7-containing hybrid that is not suppressed for anchorage independent growth in soft agar (Chr 7/6) may have lost the region of the donor chromosome that contains this gene(s). Two million cells of the parent clone and of four of the chromosome 7-containing hybrids (Chr7/1, Chr7/3, Chr7/4 and Chr7/6) were then injected subcutaneously into nude mice, to determine whether those clones suppressed for anchorage independent growth in soft agar were also suppressed for tumour formation. The results are summarised in Table 5.3. Two of the hybrids suppressed for growth in soft agar, Chr7/3 and Chr7/4, were also suppressed for tumourigenicity: after eight weeks none of the mice injected with these hybrids had developed a tumour, while every mouse injected with the parent cell clone had. This suggests that there is indeed a gene on chromosome 7, separate to the senescence gene, that suppresses the tumourigenicity of the OVCAR5 cell line. Hybrid Chr7/6, which was not suppressed for growth in soft agar, was similarly unsuppressed for tumour growth in nude mice: every mouse injected with this hybrid had developed a tumour within eight weeks, the average time of tumour formation being only a week greater than for the parent cell clone. This supports the hypothesis that in this hybrid the region of the donor chromosome that contains the suppressor gene has been lost. Hybrid

Chr7/1, on the other hand, was suppressed for growth in soft agar but not for tumourigenicity, the average time of tumour formation being even less than that of the parent cell clone. Possible reasons for this anomaly are given in the Discussion.

Figure 5.1

Figure 5.1
The growth in soft agar of immortal OVCAR5/Chromosome 7 hybrids and OVCAR5 single cell clones

The colony-growth in soft agar of three immortal OVCAR5/Chromosome 7 hybrids that have retained markers from the donor chromosome (a), six hygromycin B-resistant OVCAR5 single cell clones and two immortal OVCAR5/Chromosome 7 hybrids that have lost markers from the donor chromosome (b), and two immortal OVCAR5/Chromosome 7 hybrids that have retained mutually exclusive markers from the donor chromosome (c). Values are expressed as colony-formation percentages, relative to the mean colony-formation of the OVCAR5 parent cell line. Colony-formation refers to the number of colonies of $\geq$ 0.1 mm diameter formed per 5000 cells plated, after 25 days incubation.

The growth in soft agar of immortal OVCAR5/Chromosome 7 hybrids and OVCAR5 single cell clones

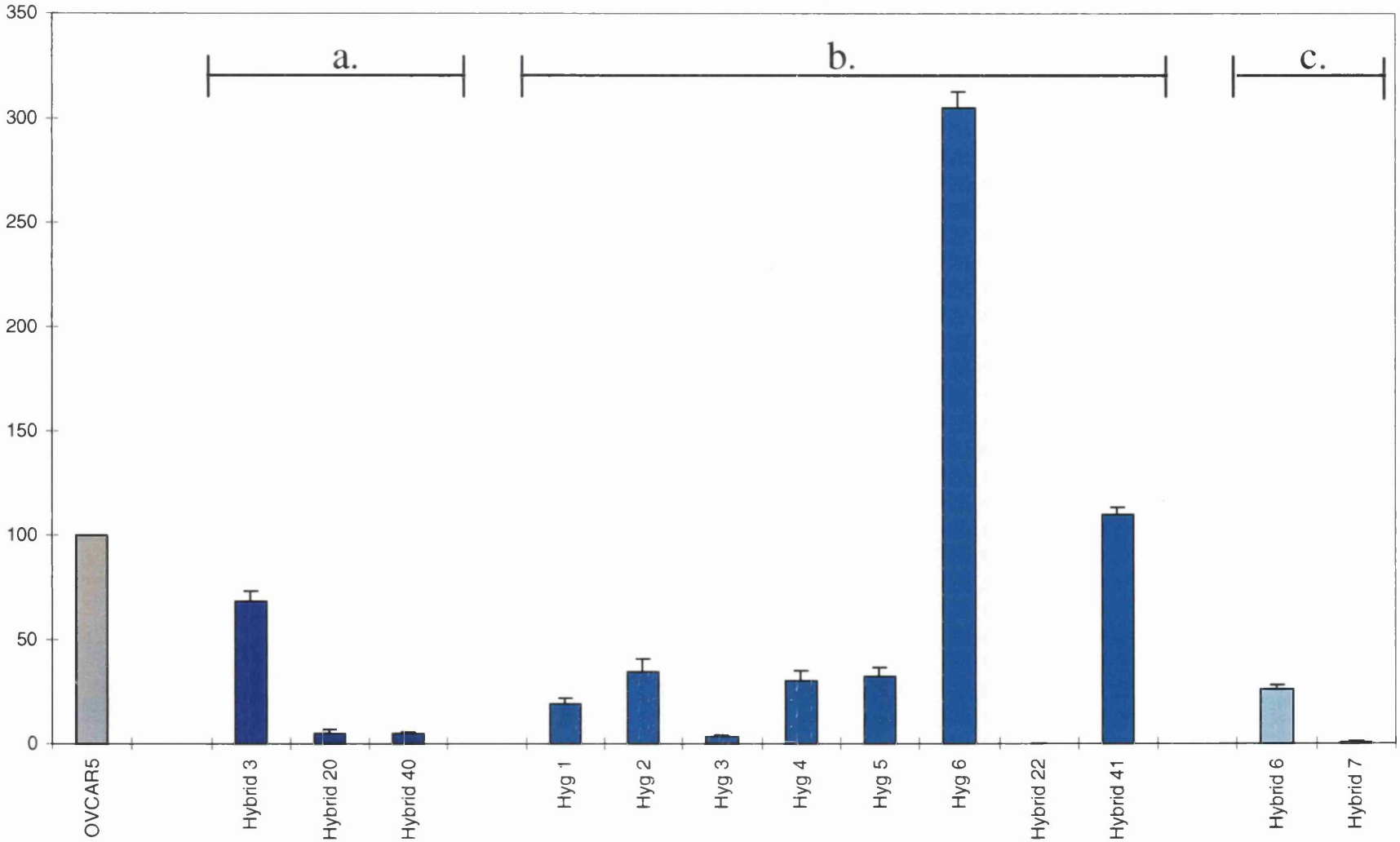


Figure 5.1

Figure 5.2

Figure 5.2
The surface growth rates of OVCAR5 and three immortal OVCAR5/Chromosome 7 hybrids

The *in vitro* proliferation of OVCAR5 and immortal OVCAR5/Chromosome 7 hybrids. 5000 cells of each cell type were plated into replica tissue culture dishes on day one and cultured in the absence of drug selection. The total number of cells per dish was estimated on days 6, 9 and 15. Cell counts were performed with a haemocytometer.

The surface growth rates of OVCAR5 and three immortal OVCAR5/Chromosome 7 hybrids

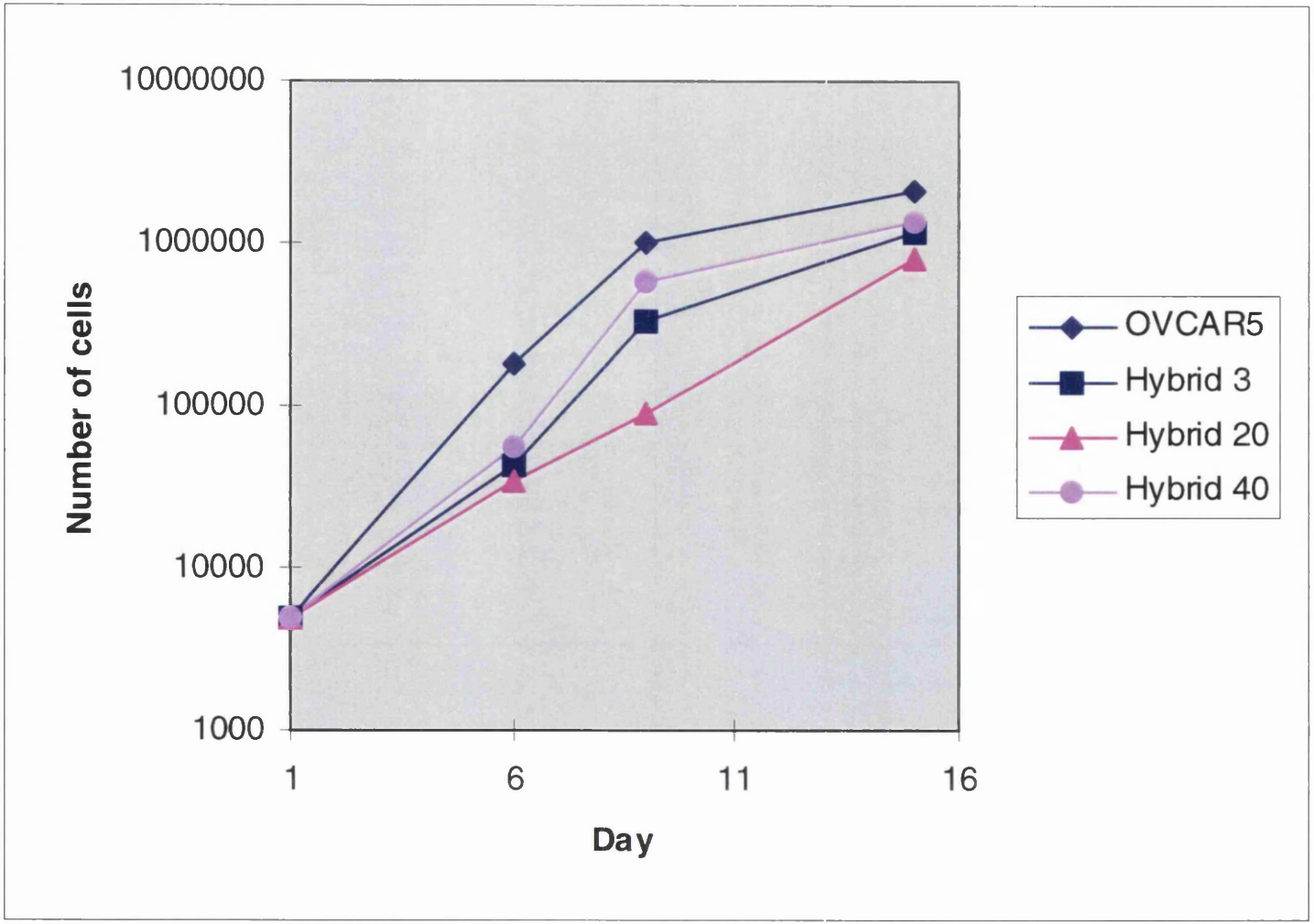


Figure 5.2

Table 5.1

Table 5.1
Tumour growth of immortal OVCAR5/Chromosome 7 hybrids and of OVCAR5 single cell clones

Summary of tumour formation in athymic nude mice of the OVCAR5 cell line, immortal OVCAR5/Chromosome 7 hybrids and hygromycin B -resistant OVCAR5 single cell clones. 0.5 million cells were subcutaneously injected above the renal capsule (one injection site per animal). 5 animals were used for each of OVCAR5 and hybrids 3, 20 and 40; 4 animals were used for all other cell hybrids/clones. The times stated represent the average number of weeks taken for tumours to *first* appear.

Tumour growth of immortal OVCAR5/Chromosome 7 hybrids and of OVCAR5 single cell clones

Cell line/Clone	Average time for tumours to form (weeks)
OVCAR5 (parent cell line) -	3.0
Hybrid 3	7.2
Hybrid 20	5.6
Hybrid 40	11.4
Hyg 2	9.4
Hyg 3	9.0
Hybrid 22	23.0+ ♠
Hybrid 41	19.0+ ♣
Hybrid 6	18.0+ ♦
Hybrid 7	6.6

♠ No tumours after 23 weeks in 4/4 mice
♣ No tumours after 23 weeks in 3/4 mice
♦ No tumours after 23 weeks in 2/4 mice

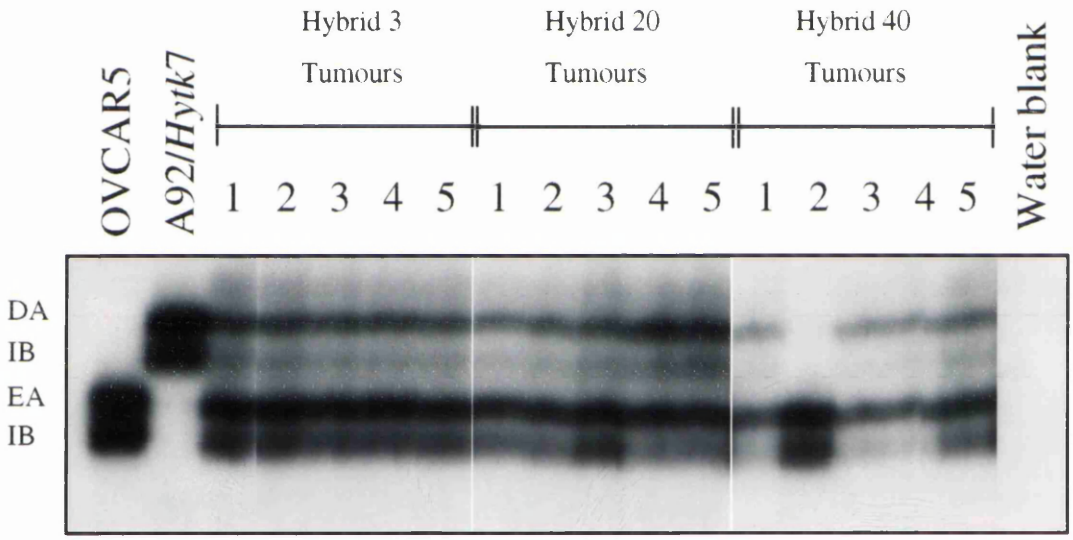
Table 5.1

Figure 5.3

Figure 5.3
Representative example of tumour allele loss analysis

Autoradiographs from radiolabelled PCR products of the chromosome 7 microsatellite marker D7S1805, amplified from the DNA of tumours formed by immortal OVCAR5/Chromosome 7 hybrids. Loss of the donor allele is visible in only one tumour (hybrid 40 tumour 2).
A92/*Hytk7*: MMCT donor. DA, donor allele;
IB, invariant band (PCR artefact); EA, endogenous allele.

Representative example of tumour allele loss analysis



D7S1805

Figure 5.3

Table 5.2

Table 5.2
Summary of tumour and agar clone allele loss analysis

Summary of chromosome 7 microsatellite deletion analysis of tumours (a), and agar colonies (b), formed by OVCAR5/Chromosome 7 immortal hybrids. Markers are given in order of chromosomal location. -: donor allele lost. +: donor allele retained. /: equivocal.

Summary of tumour and agar clone allele loss analysis

a.

	Hybrid 3						Hybrid 20						Hybrid 40				
	T1	T2	T3	T4	T5		T1	T2	T3	T4	T5		T1	T2	T3	T4	T5
p arm																	
	GATAP19270	+	+	+	+	+		+	+	+	+	+		+	-	+	+	+
q arm	D7S820	+	+	+	+	+		+	+	+	+	+		+	-	+	+	+
	D7S1841	+		+	+	+		+	+	+	+	+		+	-	+	+	+
	D7S523	+	+	+	+	+		+	+	+	+	+		+	+	+	+	+
	778CA	+	+	+	+	+		+	+	+	+	+		+	+	+	+	+
	786CA	+	+	+	+	+		+	+	+	+	+		+	+	+	+	+
	D7S2847	+	+	+	+	+		+	+	+	+	+		+	+	+	+	+
	D7S685	+	+	+	+	+		+	+	+	+	+		+	+		+	+
	D7S530	+	+	+	+	+		+	+	+	+	+		+	-	+	+	+
	D7S1805	+	+	+	+	+		+	+	+	+	+		+	-	+	+	+

b.

Hybrid 3			Hybrid 20			Hybrid 40	
A1	A2		A1	A2		A1	A2
+	+		-	-		+	+
+	+		-	-		+	+
+	+		-	-		+	+
+	+		-	-		+	+
+	+		-	-		+	+
+	+		-	-		+	+
+	+		-	-		+	+
+	+		-	-		+	+
+	/		-	-		+	+
+	/		-	-		+	+

Table 5.2

Figure 5.4

Figure 5.4
The growth in soft agar of an OVCAR5 single cell clone after introduction of chromosome 7

The colony-growth in soft agar of an OVCAR5 single cell clone and six immortal OVCAR5/Chromosome 7 hybrids generated from this clone (Chr7/1 to Chr7/6). Values are expressed as colony-formation percentages, relative to the mean colony-formation of the OVCAR5 single cell clone. Colony-formation refers to the number of colonies of ≥ 0.1 mm diameter formed per 5000 cells plated, after 18 days incubation. Hybrid Chr7/1 formed no colonies.

The growth in soft agar of an OVCAR5 single cell clone after introduction of chromosome 7

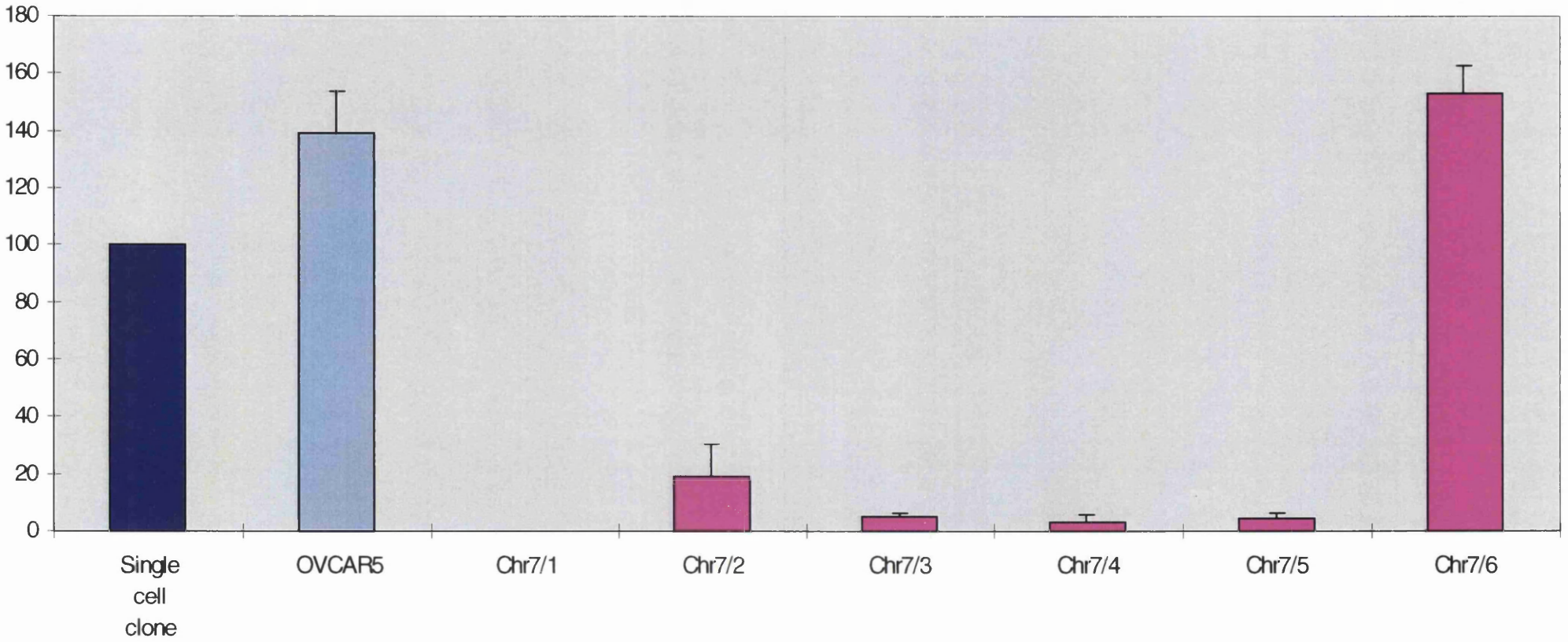


Figure 5.4

Figure 5.5

Figure 5.5
The growth in soft agar of an OVCAR5 single cell clone after introduction of chromosome 15

The colony-growth in soft agar of an OVCAR5 single cell clone and six OVCAR5/Chromosome 15 hybrids generated from this clone (Chr15/1 to Chr15/6). Values are expressed as colony-formation percentages, relative to the mean colony-formation of the OVCAR5 single cell clone. Colony-formation refers to the number of colonies of ≥ 0.1 mm diameter formed per 5000 cells plated, after 18 days incubation.

The growth in soft agar of an OVCAR5 single cell clone after introduction of chromosome 15

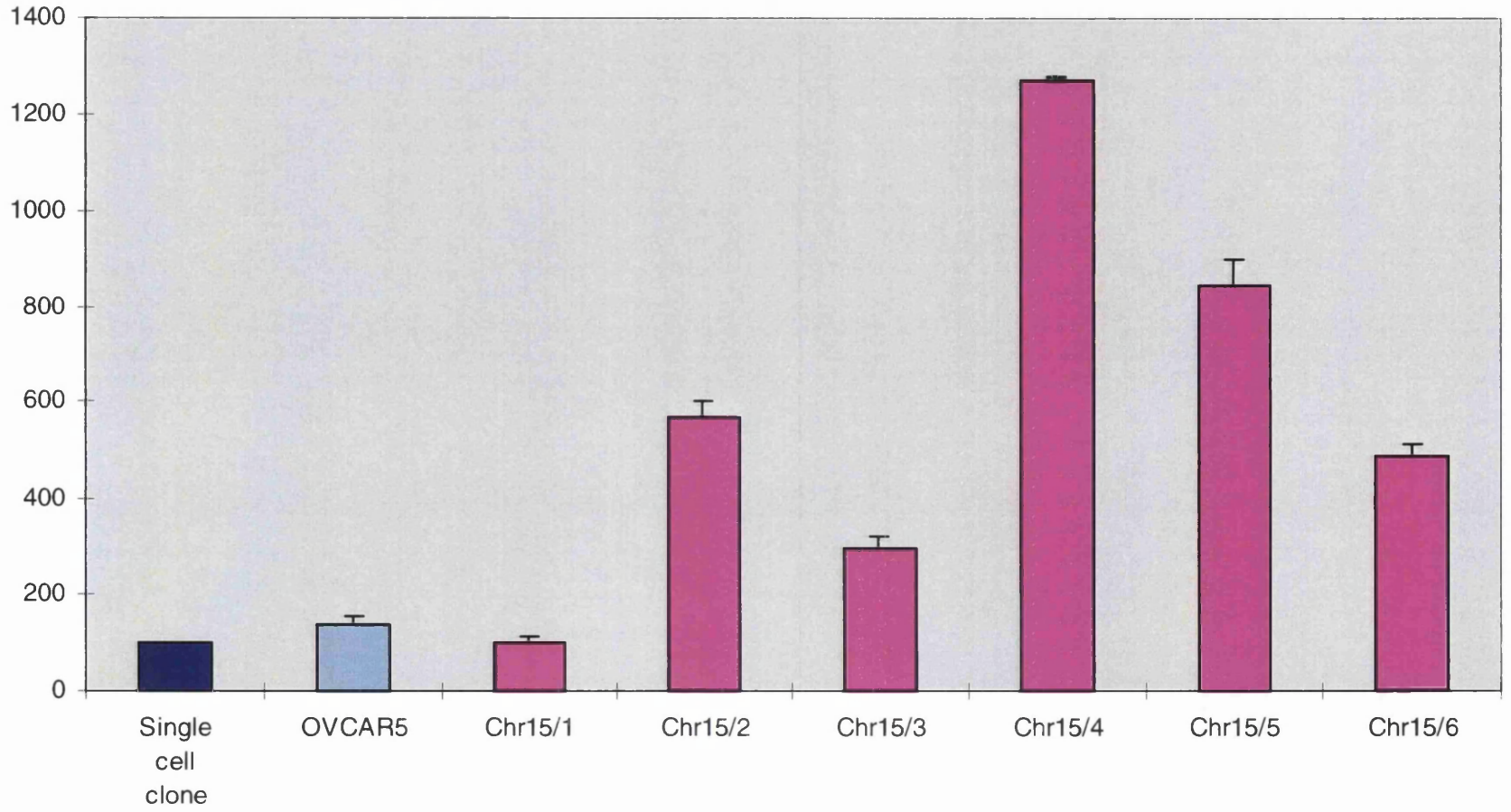


Figure 5.5

Table 5.3

Table 5.3
Tumour growth of an OVCAR5 single cell clone after introduction of chromosome 7

Summary of tumour formation in athymic nude mice of an OVCAR5 single cell clone and of four immortal OVCAR5/Chromosome 7 hybrids generated from this clone (Chr7/1, Chr7/3, Chr7/4 and Chr7/6). 2 million cells were subcutaneously injected above the renal capsule (one injection-site per animal). 4 animals were used for Chr7/3; 5 animals were used for all others. The times stated represent the average number of weeks taken for tumours to *first* appear.

Tumour growth of an OVCAR5 single cell clone after introduction of chromosome 7

Cell clone	Average time for tumours to form (weeks)
OVCAR5 single cell clone	5.0
Chr 7/1	4.2
Chr7/3	8.0+♠
Chr7/4	8.0+♣
Chr7/6	6.2

♠ No tumours after 8 weeks in 4/4 mice
♣ No tumours after 8 weeks in 5/5 mice

Table 5.3

CHAPTER 6

DISCUSSION

6. Discussion

Microcell mediated transfer of chromosome 7 into the immortal, non-tumourigenic fibroblast cell lines SUSM1 and KMST6, and into the hepatoma cell line HepG2, induces senescence in a proportion of hybrids (Ogata et al., 1993; Ogata et al., 1995). These findings provided the first functional evidence for the existence of a chromosome 7 senescence gene that is capable of reversing the immortal phenotype of human cells. In my own studies, chromosome 7 induced growth arrest with features of senescence in a proportion of hybrids of both the ovarian tumour cell line OVCAR5 and the breast tumour cell line MDA-MB-231. Introduction of chromosome 15 into these cell lines, and of chromosome 7 into the control pancreatic cell line SUIT2, had no effect. These results provide further support for the existence of a chromosome 7 senescence gene. Although the forced expression of a variety of genes can result in a non-specific, senescence-like growth arrest, genes introduced by MMCT are under the control of their normal endogenous promoter. This, coupled with my observation that mortalin reverts to its mortal distribution and that the chromosome has no effect in a control cell line, makes it unlikely that the chromosome 7-induced growth arrest is simply a consequence of increased expression of a gene that normally has no role in senescence. However, because of the sheer number of genes introduced during the procedure—chromosome 7 is 150 MB and represents 5% of the genome—non-specific gene dosage effects cannot be entirely ruled out. Ultimately, it will be necessary to show that specific chromosome 7-derived sequences—for example BAC or PAC clones of a particular 7q region—can also induce the senescence phenotype.

My observations of chromosome 7-induced senescence differ from those published in two main aspects: the percentage of hybrids that senesce, and the speed with which they senesce. Approximately 60% of chromosome 7-containing SUSM1 and KMST6 hybrids underwent senescence (Ogata et al., 1993), compared to approximately 30% of MDA-MB-231 and OVCAR5 hybrids. (The precise proportion was difficult to quantify, particularly for the OVCAR5 cell line, because of rapid seeding of new colonies by the immortal hybrids). This difference may reflect the greater variety of genetic defects in tumourigenic as opposed to non-tumourigenic recipients: the growth-inhibitory effect of the senescence gene may be partially swamped by the growth-promoting effects of a multitude of oncogenic loci. This does not provide the complete answer, however, since chromosome 7 also induced senescence in approximately 60% of hybrids generated from the tumourigenic cell line

HepG2 (Ogata et al., 1995). It is possible that small alterations to the experimental procedure can increase or decrease the extent of chromosome breakage/recombination that occurs, and hence alter the proportion of hybrids that delete the senescence gene on the donor chromosome. The position of the senescence gene relative to the integration site of the drug resistance marker would also affect the yield of clones with limited division potential. The activity itself of the senescence gene may also vary, particularly given that the donor chromosome used in my study is of different origin to that used in Ogata's experiments. In one published study the efficiency with which chromosome 7-derived radiation hybrids induced senescence was increased by treating the donor cells with azacytidine (Nakabayashi et al., 1997); the extent of methylation of the donor senescence gene may thus be at least partly responsible for variations in the percentage of hybrids that senesce. Differences in the proportion of donor cells that contain an intact donor chromosome may also provide a partial explanation.

The OVCAR5/Chromosome 7 and MDA-MB-231/Chromosome 7 hybrids that underwent growth-arrest did so just 5-8 population doublings after introduction of the chromosome. This compares to 10-30 population doublings for SUSM1 and KMST6 hybrids and 10-24 doublings for HepG2 hybrids (Ogata et al., 1993; Ogata et al., 1995). I have not determined whether telomere shortening is associated with chromosome 7-induced senescence of OVCAR5 and MDA-MB-231. However, the appropriate experiments have been performed for hybrids generated from SUSM1, demonstrating that telomere shortening does indeed take place (Nakabayashi et al., 1997). It is possible, therefore, that the difference in the timing of the growth arrest is a result of differences in the length of the recipients' telomeres. SUSM1 cells have extremely long telomeres (Bryan et al., 1995) which, after introduction of the chromosome, may take a greater number of population doublings to shorten to the critical length at which senescence is triggered. It is also possible that the critical telomere length itself is cell-type specific.

It is interesting that a proportion of growth-arrested OVCAR5/Chromosome 7 hybrids appeared to undergo some form of cell death, particularly given that senescent cells can be very resistant to apoptosis (Wang, 1995). A similar phenomenon occurs following introduction of chromosome 4—which is also believed to carry a senescence gene (Ning et al., 1991)—into HeLa cells: although cells in the growth-arrested hybrids stain positive for expression of senescence-associated β-galactosidase, TUNEL assays demonstrate a four-fold increase in apoptosis compared to HeLa cells alone (Steven

Bryce and Hazel Ireland, BICR, personal communication). It is possible that the chromosome 4 gene and the chromosome 7 gene both restore crisis (M2) rather than true senescence (M1). (This is consistent with evidence that the complementation group B and D gene products act in the same biochemical pathway (Loughran et al., 1997).) The fact that the telomeres of SUSM1 shorten following introduction of chromosome 7 indicates that the senescence gene product suppresses telomere maintenance—and *is* thus involved in M2. It is significant that expression of the endogenous β-galactosidase gene is not specific to cells in M1: SV40-infected human fibroblasts undergoing crisis (M2) also stain positive for its expression (Dimri et al., 1995).

The chromosome 7 senescence gene is believed to represent the immortality complementation group D gene, its inactivation giving rise to immortal cell lines belonging to this group. Pereira Smith and Smith (1998) identified four complementation groups, A-D, by interfusing 30 immortal cell lines and observing whether or not the resulting hybrids senesced. The groups do not correlate with the activity of known immortality suppressor genes, giving support for the existence of at least four more senescence genes or critical steps in the pathway to immortalisation. However, some controversy still surrounds the existence of the complementation groups. My own cell-cell fusion studies, while not intended to be a comprehensive test of Pereira-Smith and Smith's results, indicate that HeLa (which they assigned to group B) and SUSM1 (which they assigned to group D) do indeed belong to different immortality complementation groups. Further supporting the theory that immortal cell lines belong specifically to one of the four groups, the cell lines OVCAR5 and MDA-MB-231 formed mortal hybrids when fused to HeLa, but immortal hybrids when fused to SUSM1 and to each other. (However, without knowing the status of, for example, pRB and p53 in OVCAR5 and MDA-MB-231, complementation of genes known to be involved in senescence cannot be ruled out as an explanation of the mortality of OVCAR5/HeLa and MDA-MB-231/HeLa hybrids.) Also as expected, the proportion of cell-cell hybrids that underwent growth arrest was higher than that of microcell hybrids (the generation of which causes damage to the donor chromosome). However, when I fused HeLa to the bladder cell line A1698, mapped by Pereira Smith and Smith to group D, no signs of senescence were observed in the hybrids: although the cells in some hybrid colonies underwent a fairly rapid form of cell death, all the eight colonies that were ring-cloned and expanded were successfully propagated through to fifty population doublings. Segregation and loss of chromosomes is a common feature of cell-cell

hybrids but, although I haven't tested this, it seems unlikely that *all* eight hybrids would have ejected the relevant chromosome 4 or 7 (the chromosomes carrying functional copies of the group B and group D gene respectively).

It is perhaps significant that other investigators have also failed to reproduce some of the findings of Pereira-Smith and Smith. One group reported that 38 of 39 hybrids generated from the fusion of immortal cell lines that were reported to complement each other failed to senesce (Ryan, Maher & McCormick, 1994). To explain this discrepancy, the authors suggest that the 'senescence' observed in Pereira-Smith and Smith's experiments resulted from the long-term use of drug selection, rather than from genetic complementation. However, this seems unlikely, as all Pereira-Smith and Smith's homotypic hybrids (those generated by fusing a cell line to itself) proliferated indefinitely, despite presumably being kept under the same drug selection conditions as the heterotypic hybrids. Furthermore in my own studies, where drug selection was discontinued after initial selection for hybrid colonies, senescence of HeLa/SUSM1, HeLa/OVCAR5 and HeLa/MDA-MB-231 hybrids was still observed. It is difficult, however, to disregard the fact that so many of Ryan et al.'s hybrids failed to senesce. A further study assigns two human epidermal squamous cell carcinoma cell lines to more than one senescence complementation group: almost without exception, hybrids generated from fusing these cell lines to known representatives of three of the four complementation groups proliferated indefinitely (Berry, Burns & Parkinson, 1994). As the authors suggest, it is possible that the two cell lines have inactivated all three of the complementation group genes involved. Similarly, it is feasible that the A1698 cell line used in my study had undergone further genetic changes in culture, inactivating the group B gene in addition to the group D gene. However, this explanation is inconsistent with evidence that the complementation group gene products act in a linear pathway: in this scenario, inactivation of a second, third or fourth complementation group gene would not be expected to confer any further selective advantage.

The senescence-inducing effect of chromosome 7 is reportedly restricted to cell lines that belong to complementation group D. Indeed, such specificity was the basis for assigning the group D gene to this chromosome. Ogata et al. (1993) claim that chromosome 7 has no effect when introduced into the cell lines HT1080, HeLa, and TE85, representative of complementation groups A-C respectively. However, I noticed a strong growth-inhibitory effect of chromosome 7 in HeLa cells. Moreover, the

growth-arrested hybrids exhibited features of senescence. Chromosome 4 has also been observed to induce features of senescence in a non-complementation group-dependent manner (Nick Forsyth, BICR, personal communication): of fifteen hybrids generated from the group A osteosarcoma cell line HT1080, half proliferated significantly more slowly than the parent cell line and one ceased to proliferate completely, staining positive for expression of senescence-associated endogenous β-galactosidase. Again, the chromosome is reported to have no effect in non-group B cell lines (Ning et al., 1991). Although the genes responsible for this non-complementation group-specific growth arrest may be entirely different from the complementation group genes themselves (as suspected for the case of chromosome 7-induced growth arrest in HeLa—see Chapter 4), these findings do call into question the clear-cut results of Pereira-Smith and Smith. Any gene that can induce features of senescence in a non-complementation group-dependent manner would be predicted to complicate the results, and interpretation, of cell-cell fusion analysis.

One phenomenon that supports the findings of Pereira-Smith and Smith is the correlation of the distribution of mortalin with complementation group assignment (Wadhwa et al., 1995). In Wadhwa's study, 17 of 18 cell lines tested that had been assigned to a single complementation group by cell-cell fusion experiments exhibited this correlation. In my limited analysis, four cell lines (one representative of each complementation group) showed the predicted distribution; moreover, the two cell lines that I assigned to group D by cell-cell fusion, MDA-MB-231 and OVCAR5, also exhibited the group D distribution. The molecular basis of this correlation remains unknown, but the mortalin distributions may reflect a complementation group gene product pathway. If the complementation group gene products in some way support a system of mortalin transport, loss of a particular complementation group gene would disrupt this; the four different perinuclear distributions may each reflect disruption at a different point in the transport pathway. In conclusion, the existence of four different complementation groups for senescence has been neither satisfactorily confirmed nor refuted. What for the sake of my project is more important (and less contentious) is that a gene exists on chromosome 7 that can induce senescence in certain immortal cell lines.

My attempt to map this gene by SSR deletion analysis of immortal hybrids has proved unsuccessful. This is perhaps not suprising: to my knowledge, *SURF1* is the only gene so far cloned by the microcell hybrid deletion mapping strategy (Zhu et al.,

1998). In my immortal hybrids a great variety of losses were found scattered throughout the donor chromosomes, with several chromosomes exhibiting multiple interstitial losses. It is probable that chromosome breakage occurs during the actual preparation of microcells, resulting in random deletions that confer no proliferative advantage to the hybrid. For certain markers and in certain hybrids, loss of the endogenous allele, rather than the donor allele, was detected. This implies that, after fusion with microcells, mitotic recombination may also act to induce losses in the donor chromosome. Such random losses, and indeed losses resulting from selective pressures other than the evasion of replicative senescence, would mask the regions of loss that contain the senescence gene. A further complication is that the frequency of 'random' losses is not uniform along the chromosome. The frequency of loss of a particular region depends upon its position relative to the integration site of the selectable marker: if the selectable marker is situated near the centromere then the frequency of loss of either telomeric region will be inflated, and if near a telomere the frequency of loss of the other telomere will be inflated. It would be easy to interpret frequencies of loss exaggerated in such manner as meaningful. To avoid this, and to eliminate other 'random' regions of loss from the analysis, it is necessary to analyse a control cell line. In such cell lines there should be no evidence for endogenous inactivation of the gene of interest and hence little selective pressure to inactivate the gene on the donor chromosome. The true region, that containing the gene of interest, should thus be lost at a higher frequency in hybrids of the test cell line than in the control cell line, while other regions should be lost at equal frequency. This type of analysis, however, can require a very large number of hybrids, and the technique is still limited by the density of informative markers. In my own hybrids, the pattern of loss was so complex that the utility of the control line SUIT2 was limited.

While several hybrids complicated the analysis by containing multiple interstitial deletions, the opposite situation also proved problematic. A large percentage of the hybrids I generated were entirely uninformative for mapping, with every marker analysed either being lost (the hygromycin B resistance marker presumably integrating elsewhere in the genome) or retained. In the latter hybrids, where the donor chromosome does not undergo extensive breakage or recombination, the gene is presumably inactivated by microdeletions, mutations, or epigenetic mechanisms such as promoter silencing by methylation—none of which are likely to be detected by SSR analysis. When the inactivation of known growth suppressor genes in immortal

microcell hybrids is analysed, many losses found *are* very small; after chromosome 9 was introduced into mouse cells, for example, many deletions were found to encompass just a single exon of $p16^{INK4a}$ (England et al., 1996). In my own hybrids, it may be worth returning to those for which no microsatellite losses were found, and to try to detect microdeletions using alternative techniques such as RDA.

It is possible that the success of an SSR deletion mapping strategy depends at least partly on the particular chromosome used. Certainly, similar studies performed in this institute with chromosome 6 have not generated such a complex pattern of loss (Sara Fitzsimmons, personal communication). Chromosome 7 is known to contain both an aphidicolin-inducible fragile site and a recombination hot-spot (discovered in the hunt for CFTR, when it was noticed that some markers underwent recombination more often than expected), which may or may not be related to each other (Huang et al., 1998; Kerem et al., 1989). These features may make chromosome 7 particularly predisposed to breakage and recombination, complicating this type of analysis. Alternatively, it is possible that recipient cell type-specific differences may affect the frequency of losses incurred post-fusion.

The fact that high frequency of deletion of known growth suppressor genes is detected in the appropriate hybrids suggests that the strategy is sound in principle (England et al., 1996). However, to be used successfully when no prior positional information is available, some modification to the procedure may be required. One approach would be to initially introduce smaller chromosome segments, for example fragments contained within radiation hybrids, and thus eliminate large regions of the chromosome that do not induce the required phenotype. Having identified the smallest fragment capable of inducing senescence, deletion analysis could then be performed on immortal hybrids generated with it.

There is independent evidence for the existence on chromosome 7 of a tumour suppressor gene; this may or may not be the same as the chromosome 7 senescence gene. As discussed in the introduction, high frequencies of LOH centring on 7q31 are found in a wide range of human cancers, including breast, ovarian, head and neck, colon, gastric and prostate tumours (Reviewed in Zenklusen & Conti, 1996). Work carried out in our laboratory supports these findings: Adam Hurlstone found 40% 7q LOH in a panel of breast tumour samples centring on the 7q31 marker 17TA-5/17B-RE3, and Vincent O'Neill found high frequencies of LOH on chromosome 6 in murine skin carcinomas centring on a region syntenic to 7q31 (unpublished data). However, the

significance of LOH data is not always easy to judge. A wide spectrum of LOH frequency at 7q31has been reported, ranging from less than 10% to 80% at a single marker and within a single tumour type (Kerangueven et al., 1995; Zenklusen et al., 1994a). It is difficult to fully account for this variation in terms of factors such as tumour stage, sample processing and data analysis, and such studies do raise some doubts about the 7q LOH data. The recombination hot-spots and aphidicolin-inducible fragile site found at 7q31 pose additional complications. It is possible that in cancer cells such inherently unstable regions undergo deletions and rearrangements without actually contributing to the disease process itself, and are simply co-selected with other mutations that do confer a selective growth advantage. However, the fact that LOH at 7q31 is not observed in all tumour types (for example bladder, cervix and glioma), and that LOH of 7q31 and 4q32-34 are not observed in the same tumour samples (Loughran et al., 1997), both suggest that LOH at 7q31 *is* directly selected for during tumour development.

It is also encouraging that functional evidence for the existence of a tumour suppressor gene at 7q31 has been obtained. Introduction of chromosome 7 into a murine squamous cell carcinoma cell line was found to reduce the tumour-forming ability of five of the seven hybrids generated (Zenklusen et al., 1994b). Furthermore, one of the non-suppressed hybrids was found to have a deletion in the donor chromosome at 7q31. My own observation, that introduction of chromosome 7 suppresses the anchorage-independent growth and tumourigenicity of an OVCAR5 single cell clone, provides additional functional evidence for the existence of a tumour suppressor gene on this chromosome. Five of the six hybrids generated in this study exhibited significantly reduced colony formation in agar compared to the parent cell clone. This proportion of suppression is very similar to that (five out of seven) observed in Zenklusen's tumour experiments. The single hybrid whose anchorage independent growth was not suppressed had presumably deleted or otherwise inactivated the putative tumour suppressor gene on the donor chromosome. Introduction of chromosome 15 into the same parent cell clone did not suppress anchorage independent growth, indicating that the effect of chromosome 7 was due to DNA sequences specific to that chromosome. Of three suppressed chromosome 7 hybrids that I tested for tumour formation in nude mice, two proved less tumourigenic than the parent cell line. To my knowledge, this is the first demonstration of chromosome 7-induced suppression of tumourigenicity in a human ovarian tumour cell line.

It is perhaps odd that one of the three clones was suppressed for anchorage independent growth in soft agar but not for tumour growth in nude mice. In my previous studies hybrids 20 and 7 were also largely incapable of growth in soft agar yet able to form tumours in nude mice (Chapter 5). A group employing a similar strategy to investigate a putative colorectal tumour suppressor gene on chromosome 8 also generated a hybrid with these properties (Gustafson et al., 1996). Although some investigators have demonstrated a perfect correlation between anchorage independent growth and tumourigenicity (Newbold et al., 1982), studies of non-tumourigenic HeLa/fibroblast hybrids and of tumourigenic segregants derived from them indicate that the two phenotypes *are* separable in certain cell systems (Stanbridge & Wilkinson, 1980). Gustafson et al. suggest that anchorage independence is associated with metastatic ability, not tumourigenicity *per se*. However, this does not explain why introduction of chromosome 7 should suppress both the anchorage independent growth and tumourigenicity of two hybrids, but the anchorage independent growth only of one (Chr7/1). Introduction of chromosome 8 in Gustafson et al.'s experiments resulted in similarly differentially suppressed hybrids (1996). They suggest that the pattern of endogenous mutations present in the parental cell line may determine the precise response to the suppressor gene. Although my hybrids were all generated from the same single cell clone, this explanation could still apply if any relevant mutations occurred after the clone's isolation. Alternatively, two different genes on chromosome 7 could be involved, one with the capacity to suppress anchorage independent growth and one with that to suppress tumourigenicity *per se*. The latter gene may have been lost from the donor chromosome of the clone that was not suppressed for tumour formation.

Given the evidence for a gene(s) on chromosome 7 that suppresses the transformed phenotype of OVCAR5 cells, why did there appear to be no selection pressure *in vivo* for tumours of hybrids 20 and 40 to lose all or part of the donor chromosome? It is feasible that these hybrids were not suppressed by chromosome 7 to begin with: the colony-forming ability in agar of the parental clones from which these hybrids were generated is unknown, and, given the high level of clonal variation observed within the cell line, it is possible that these clones were not themselves capable of appreciable levels of anchorage-independent growth. Alternatively the gene could, as described above, have acted in these hybrids in such a way as to suppress anchorage independent growth but not tumourigenicity. In this instance there would have been selection pressure for agar colonies, but not for tumours, to lose the gene; the fact that

both hybrid 20 agar colonies analysed *had* lost the donor chromosome supports this explanation. A scenario where two different genes are responsible for suppression of anchorage independence and tumourigenicity, with hybrid 20 retaining only the former, would also explain these findings. It is possible too that the putative tumour suppressor gene *had* been lost from the donor chromosome of these hybrids, but in such a way as to be undetectable by SSR deletion analysis; inactivation of tumour suppressor genes in this type of study by no means always occurs by way of large chromosomal deletions (Gustafson et al., 1996). A more reliable way to test for loss of a tumour suppressor gene is to re-inject cells derived from the relevant tumour; if the suppressor gene has been deleted or otherwise inactivated, cells derived from the tumour should form a new tumour at the same speed as the parent cell line.

There are no published studies addressing the issue of whether the group D senescence gene and the tumour suppressor gene defined by LOH studies and Zenklusen's mouse experiments are one and the same. Certainly the genomic abnormalities of SUSM1 and KMST6, the region of LOH in tumours and the deletion in Zenklusen's non-suppressed hybrid clone all involve 7q31. In my own studies, at least two of the cell lines homozygous for chromosome 7 also belonged to complementation group D. But although these findings are consistent with the hypothesis that the two genes are one and the same, they do not prove it. The fact that certain bladder cell lines have been assigned to complementation group D, but that LOH at 7q31 is not seen in bladder tumours, perhaps argues against the senescence gene being the target of tumour LOH. My agar studies showing suppression of anchorage independent growth and tumourigenicity in immortal, chromosome 7-containing hybrids of an OVCAR5 single cell clone suggest that there *is* a gene on chromosome 7, separate to the senescence gene, that can suppress the transformed phenotype. However, this interpretation relies on the assumption that the chromosome 7-containing hybrids have indeed deleted or inactivated the group D senescence gene. It is formally possible that the immortal hybrids had instead inactivated a different, *endogenous* complementation group gene, and that the group D gene on the donor chromosome was intact and responsible for suppressing the transformed phenotype of the hybrids. However, for this to have occurred in all five hybrids suppressed for anchorage independent growth seems unlikely. Perhaps a more serious argument against the existence of two separate genes is that, despite the use of a control cell line and chromosome in the MMCT experiments, the chromosome 7 senescence gene was not responsible for the growth arrest observed

in OVCAR5. In this scenario there would be no selection pressure for the hybrids to lose this gene. However, although it is easy to envisage how a gene involved in replicative senescence could be a target of LOH in tumours, it is less easy to imagine that a senescence gene could act to suppress anchorage independent growth in soft agar or tumour growth in nude mice without actually inducing senescence. On balance, the simplest interpretation of my data is that there is indeed a gene on chromosome 7, separate to the senescence gene, that suppresses the transformed phenotype of OVCAR5. It is not yet possible to say whether this gene maps to the minimum region of LOH in tumours. It could be that both genes—the chromosome 7 senescence gene and the separate tumour suppressor gene—are targets for LOH in tumours. This would perhaps explain the particularly high frequencies of loss found on chromosome 7 in tumours, and the fact that some 7q LOH studies have defined non-overlapping minimal regions (Kerr et al., 1996; Hilary Russell, Queen's University Belfast, personal communication).

Cloning and characterisation of the chromosome 7 senescence and tumour suppressor genes would give further insight into the mechanisms of cellular immortalisation and tumourigenesis. Several experiments might facilitate the cloning of these genes. Efforts to identify the senescence gene should probably focus on the immortal microcell hybrids that do not show any microsatellite losses: it may be possible to isolate DNA (or cDNA derived from it) mapping within microdeletions on the donor chromosome. To do so, RDA could be performed between genomic DNA extracted from immortal OVCAR5/*Hytk7* hybrids that show no microsatellite losses mixed with A92 mouse genomic DNA, and that extracted from OVCAR5 mixed with A92/*Hytk7* DNA. Success would, however, depend upon there being a sufficient number of RFLPs that discriminate between endogenous and exogenous copies of the chromosome. Alternatively, MMCT could be used to transfer the donor chromosome of immortal OVCAR5/*Hytk7* hybrids that have no obvious losses into rodent A92 cells. After checking that the transferred chromosome had not undergone further deletions, RDA could be performed between DNA extracted from the new hybrid cells and that extracted from normal A92/*Hytk7* donor cells. Any material obtained would be screened for candidate genes. It may additionally be possible to carry out subtractive hybridisation between cDNA PCR-amplified from senescent OVCAR5/*Hytk7* hybrid cells and that amplified from a comparable number of immortal OVCAR5/*Hytk7* cells; the technology to perform RT-PCR cDNA subtraction on a very small number of cells

already exists (Weaver et al., 1999). Any cDNA expressed in senescent but not in immortal hybrids that mapped to chromosome 7 would then be a candidate for the senescence gene. An alternative approach, which assumes that the protein is upregulated during senescence, would be to carry out subtractive hybridisation between cDNA derived from pre-senescent fibroblasts and that derived from senescent fibroblasts. Again, any cDNA mapping to chromosome 7 would be a candidate.

It would be necessary to screen any candidates for the ability to induce senescence in cell lines that belong to immortality complementation group D. Ultimate proof would depend on the demonstration of inactivating mutations (or gene silencing mechanisms) in complementation group D cell lines and immortal microcell hybrids: RNA blots prepared from such cell lines could be screened for aberrant transcripts or loss of expression; Southern blot analysis could be used to screen candidate genes for microdeletions and methylation differences; and SSCP could be used to carry out mutational analysis of cDNA and exonic regions of genomic DNA. Once the senescence gene had been identified, its ability to suppress anchorage independent growth and tumourigenicity in target cell lines could be tested; this would help resolve the issue of whether or not there really is a separate tumour suppressor gene.

The chromosome 7-containing hybrids generated from the single cell clone of OVCAR5 may prove a useful resource in the mapping of the tumour suppressor gene. The hybrid that was not suppressed for either anchorage independent growth in soft agar or tumour formation in nude mice has presumably deleted or otherwise inactivated the gene on the donor chromosome. Although, based on my previous experience, there is little reason to suppose that SSR deletion analysis would help delineate a specific region, such an approach would show whether or not the donor chromosome is largely intact. If it *is* intact, RDA could be performed between genomic DNA prepared from this hybrid and from one of the five suppressed hybrids. Again, should the technique fail to discriminate between endogenous and exogenous copies of chromosome 7, the donor chromosome of the non-suppressed hybrid could be transferred into A92 cells. RDA would then be performed between DNA extracted from this new hybrid and from the normal A92/*Hytk*7 donor. Alternatively, subtractive hybridisation could be performed between cDNA derived from suppressed and non-suppressed hybrids. Any cDNAs obtained that map to chromosome 7 would again be candidates. Microsatellite deletion analysis *could* help delineate regions of loss in revertant agar colonies or tumours formed by suppressed hybrids; as the generation of these revertants from the

parent hybrids did not involve any microcell preparation, the number of 'random' losses on the donor chromosome should be minimal. For those hybrids which do not show any SSR losses, RDA or a cDNA subtraction could be performed between genomic DNA (or cDNA) extracted from the revertant and from the parent hybrid. As a complementary approach to cloning this gene, candidates could be identified from the minimum common region of LOH found in tumours (our lab already has a contig spanning the region of LOH found in our panel of breast tumour samples). YAC clone DNA or interAlu PCR products generated from YAC clones could be used to perform direct cDNA selection, using pooled cDNA from various libraries as the starting material. In addition, as much of the interval has been sequenced, probes designed from sequences that either have significant homology to known genes or ESTs or have features of putative exons could be used to probe and extract the full length candidate from cDNA libraries. Candidate genes identified by any of the above methods would then be tested to determine whether they fulfil the properties expected of a tumour suppressor gene. The gene should be expressed in the tissues from which tumours showing 7q LOH are derived, testable by northern blot analysis of multi-tissue RNA preparations. Introduction of the gene into cell lines, for example the OVCAR5 single cell clone used previously, should suppress anchorage independent growth and tumourigenicity in the same way as did the whole chromosome. Detection of mutations or any alterations of expression of the gene in tumours could then be carried out as above.

When either gene has been identified further experiments will be necessary to elucidate its function. Any homology with known genes may give an initial indication. It would be important to ascertain the cellular distribution of the gene product, either by generating antibodies against it or by expressing the protein as an epitope-tagged molecule. In the case of the senescence gene, it would be interesting to determine whether it is actually upregulated in senescence, and whether any such upregulation is sustained or transient. Any effect of overexpression of the gene on telomerase activity or expression (in immortal cell lines) could also be analysed. For either gene product, the nature of its interactions would need to be investigated. Binding partners could be identified in a number of ways, including immunoaffinity chromatography, immunoprecipitation and two hybrid screening in yeast, insect or mammalian cells. It could then be determined whether any interacting proteins were also targets for mutation during tumourigenesis or immortalisation. The issue of whether the senescence gene

product is involved in transducing signals from shortened telomeres could also be addressed, by seeing whether mutant and wild-type cells differ in their ability to undergo senescence after deletion of their telomeres by homologous recombination. It would also be interesting to see whether mutant cells have an altered response to DNA damage. Assuming that mouse homologues of the senescence and tumour suppressor genes exist, knock-out mice could eventually be generated. These would allow investigation of the role of the genes in development and differentiation and assessment of the effect of gene disruption on tumour susceptibility and the replicative potential of mutant cells. However, because of the differences in the regulation of senescence in mice and human cells it is possible that the biological role of the chromosome 7 senescence gene is not identical in these species.

Immortality gives cells the opportunity to acquire the often multiple mutations needed for metastasis, therapeutic resistance and recurrence; it may thus ultimately be responsible for cancer lethality. Cloning of the chromosome 7 senescence gene would doubtless enhance understanding of the mechanisms that curb the proliferative potential of normal cells. It may also provide novel pathways to manipulate in cancer therapy. Identifying and characterising an additional chromosome 7 tumour suppressor gene might similarly provide new therapeutic targets, and would further increase our understanding of the multi-step process of tumourigenesis.

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APPENDIX

Table A1

Table A1
Human chromosome 7 polymorphic STSs used in allele loss analysis

STSs are listed in map order based upon the best composite of genetic, radiation hybrid, and STS-content mapping
[a] Accession numbers for sequences of listed markers deposited in GenEMBL. [b] Genetic distances from the top of chromosome 7 are given in Kosambi centiMorgans (cM; for chromosome 7, 1 cM is approximately 1.1 Mbp) and are based on a sex-averaged recombination map (J. Weber, Genome Database). Physical distances are given in centiRays (cR; for chromosome 7 1 cR is approximately 270 Kbp) and based on radiation hybrid mapping of the Genebridge 4 RH mapping panel (Gyapay *et al.*, 1996; Hudson *et al.*, 1996). [c] Size of cloned allele.
Listed markers derived by the CHLC (alias beginning CHLC.) are exclusively tetranucleotide repeats (Murray *et al.*, 1994), whereas those derived by Genethon (alias beginning AFM) are exclusively CA.GT dinucleotide repeats (Weissenbach *et al.*, 1992; Gyapay *et al.*, 1994). The dinucleotide repeat markers 727CA, 778CA, 724CA, 740CA and 786CA were derived by Adam Hurlstone, BICR. The dinucleotide repeat marker 17B-TA5/17B-RE3 was derived by Zielenski et al., 1991. The amplimer GH220/GH324 allows detection of an RFLP when restricted with *Msp* I and was derived by Horn et al., 1990. The marker D7S471 was derived by the WICGR.
CHLC, Co-operative Human Linkage Centre; WICGR, Whitehead Institute Centre for Genome Research.

Locus	Alias	GenEMBL identifier[a]	Map position cM/cR[b]	Size (bp)[c]	Primers
D7S1819	CHLC.GATA24F03	G09380	30.98 cR	178	L: CAATAGCCCTGACCTTATGC R: TACCTACCTACCTACCTCTATGGC
GATAP34087	CHLC.GATA84A08	G10238	44.53 cR	147	L: CTTGTTTCCAGTATACAATTCACC R: TGGCCTTAAGTCTACCGAAA
GATAP19270	CHLC.GATA85B06	G10071	64.57 cR	149	L: AAGCTCATCTGGAAGCTTGT R: TTTATCTGGATGAGATAGAGATGG
GATAP28070	CHLC.GATA91C01	G10090	80.22 cR	208	L: GGAGGATGGCATTTAGTAACC R: GCTGGAACTGTGGGTATCAG
D7S1831	CHLC.GATA4E06	G09131	190.49 cR	186	L: GATACATACTGCCAATAAATCACA R: TCCACATATAGACCCCATGG
D7S1797	CHLC.GATA21D08	G08599	98.4 cM 429.55 cR	229	L: TTCAAGAGCTAATCCATGCC R: AAATTGAGATCGCAGCTGAC
D7S820	CHLC.GATA3F01.511	G08616	98.4 cM	222	L: TGTCATAGTTTAGAACGAACTAACG R: CTGAGGTATCAAAAACTCAGAGG
D7S821	CHLC.GATA5D08.584	G08626	109.1 cM 465.72 cR	264	L: ACAAAACCCCAAGTACGTGA R: TATGACAGGCATCTGGGAGT
D7S1841	CHLC.GGAA6A12	G09451	483.91 cR	206	L: TCAAGTTTGATCATGAGATTGC R: GAAAAAAAGAAAGTTGGGGG
D7S501	AFM199VB2	Z16867	112.8 cM	170	L: CACCGTTGTGATGGCAGAG R: ATTTCTTACCAGGCAGACTGCT
D7S796	CHLC.GATA4E02.122	G08623	113.4 cM 491.10 cR	178	L: TTTTGGTATTGGCCATCCTA R: GAAAGGAACAGAGAGACAGGG
D7S818	CHLC.GATA6G06.207		114 cM 491 cR	154	L: GTCTCTTCCATTTATGCTTTTG R: GAAAGGAACAGAGAGACAGGG
D7S1799	CHLC.GATA23F05	G09385		181	L: ATGGTATTAGGAGATGGGGC R: TTGCATAAGCCAATTTCCAT
D7S692	AFM357TE1	Z24606	121.5 cM	166	L: CTGATGATTGCTATAGATATTCATC R: TGTAAACACTTTTGTAGAAGAACCT

D7S1817	CHLC.GATA21H01	G08603	494.67 cR	121	L: CAAATTAATGGCAAAAACTGC R: CCCCCCATTGAGGTTATTAC
D7S525	AFM248TC5	Z17106	122.3 cM 484.11 cR	222	L: GTTAGCCGAGATTGCCC R: CTTGCTGTTTAAGTACCACAAGTTC
D7S471			107.2 cM	185	L: AGCAGCTATTATGGAATTGC R: CAACATATGCAAGGTGCCTA
D7S523	AFM242YE3	Z17102	123.9 cM	223	L: CTGATTCATAGCAGCACTTG R: AAAACATTTCCATTACCACTG
D7S687	AFM323YG5	Z24392	123.4 cM 496.89 cR	237	L: AAAATATTACACATGCCTGAGTG R: ACAGTGAAGCGACACCATC
D7S816	CHLC.ATC2H06	G09255	497.66 cR	163	L: TCCTTGGCTTATACATTGTGC R: CTGAAAACACTGGATGTGACC
D7S1811	CHLC.ATA17F10	G08583		187	L: TGCCATATTTGGGGATTCTA R: ACTTACCTGCAATGTGCACA
D7S486	AFM098XG9	Z16567	125.3 cM 497.83 cR	145	L: AAAGGCCAATGGTATATCCC R: GCCCAGGTGATTGATAGTGC
D7S522	AFM242YC3	Z17100	125.1 cM	220	L: GCCAAACTGCCACTTCTC R: ACGTGTTATGCCACTCCC
D7S2460	AFM197XF10	Z51113	125.1 cM	193	L: CACATCCACTGTGTCTCATTT R: TATCTGGGACTTNACGCTTC
GH220/GH324	MET			203	L: CCATGTAGGAGAGCCTTAGTC R: GTCTAAGGACACACCTTGC
727CA					L: GATTTTGGGTTCAGTAACAGC R: CCAGGAAATAGAAACAGCAC
778CA					L: CTGTAGGATAGATAGGGAGC R: TACAGGAGATTGCATGGG
724CA					L: GCTTTGTTAGGGTTCTCCAG R: CATGTTTTCAGTCCTTCAGC
740CA					L: TCCTGACTGGCTGAATTG R: GAGCGACAGCAAAATCAG
D7S633	AFM200WE1	Z23505	125.2 cM	175	L: TGAGCCTCGCATCACTG R: TCTGGGGAGTCCTTTAACAGTA
D7S677	AFM303VH9	Z24230	125.2 cM 501.19 cR	278	L: ATCATTCACTATGGGATAGC R: GAATTACAAGTCACTCTATACAAAA

17TA-5/17B-RE3	CFTR			350	L: ATAATTTCCTTGAAATCGGA R: GCTGCATTCTATAGGTTATC
786CA					L: CATAACCGGCTGGCATCATG R: ACACATTCCTTTGGGGCCTC
AFMA073ZB9		Z67224	501.89 cR	145	L: GGTAATCTGTCCAAAAGCATGTGAG R: ACCTGATAGGATTGTGTGTGTCCG
D7S2847	CHLC.GATA44F09	G08621	125.2 cM	195	L: TCACCTTCAGAAAGTATTGCC R: TGAGGTGTTTCTCCAAGCTC
D7S643	AFM224ZF10	Z23691	126.3 cM	267	L: AGCTAATATTGCTGCCTTTT R: CAATCTCTTGCTAGATGCCA
D7S650	AFM240ZH10	Z23782	128.4 cM	274	L: AGGCTGCTTAGCCATAATC R: CCACTGGTATAAGTACATCAGAAA
D7S1835	CHLC.GATA64H06	G08630	510.47 cR	260	L: GAGCCAATTGTACTGGATTACC R: CAGGACATGTCAATTGGACA
D7S685	AFM317YC5	Z24334	129.4 cM 506.31 cR	181	L: AAGACCTGGCAACAGTTCTTACTA R: CGCTCATCAAGGATATTGG
D7S1809	CHLC.GGAA9C07	G08644	127.8 cM	202	L: AGGCAAGAGCAGTAGCAAGA R: TCCACTTTAAATCAGCAGCC
D7S1801	CHLC.GATA31D01	G08612		227	L: TTAGGGGGATCTTGCTCTCT R: AGCAGGGAATAGGCTGAGTT
D7S1822	CHLC.GATA28G04	G08609	132 cM	265	L: GTCAGGGGCGTAGTTGAATA R: TTAGGGGGATCTTGCTCTCT
D7S530	AFM249XF9	Z17136	136.4 cM 554.78 cR	111	L: TGCATTTTAGTGGAGCACAG R: CAGGCATTGGGAACTTTG
D7S2203	CHLC.GATA67A05	G08633	528.30 cR	232	L: GAGCCAATTGTACTGGATTACC R: TACCTTGTTCAGAAGCCCTG
D7S2197	CHLC.GATA113H05			244	L: TTAGATCATTGACACAAGGGC R: CAGGAAACCCGTGATTGTAC
D7S1804	CHLC.GATA43C11	G08619	137.0 cM 584.31 cR	258	L: TTCAAGTGGTTGGGTTCACT R: TGGGTCTAGTCCAGTGGTGT
D7S649	AFM240XE9	Z23771	138.0 cM 570.21 cR	276	L: ATTTTGATCCCCAGCA R: GCTTTATTATGTCTGTTGTATGA
D7S2202	CHLC.GATA63F08	G08628	149.9 cM 613.95 cR	155	L: TCTCTTACCCTTTGGGACCT R: CTTGCAGATGGCCTAATTGT

D7S794	CHLC.GATA2C04.755	G08607	630.79 cR	168	L: GCCAATTCTCCTAACAAATCC R: TATGCCCATGTGTTAGGGTT
D7S1805	CHLC.GATA4H10		647.70cR	197	L: CCTGCTTTGGCTTACCTGTA R: CCCACTTCTCTGCTATTACATAT
D7S1807	CHLC.GGAA2B12	G08640	657.00 cR	236	L: TCCTTTTCCTTTTCCCTTTC R: ATTAATAGGTTTGTCACGATTAACC