Cloning of a multi-tissue tumour suppressor/replicative senescence gene on human chromosome 7q31

Adam Felix Lloyd Hurlstone, MA

A thesis submitted to the University of Glasgow in part fulfilment for the degree of Doctor of Philosophy

July, 1998

CRC Beatson Laboratories

Beatson Institute for Cancer Research

Glasgow

&

Faculty of Medicine

University of Glasgow

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Forsan et haec olim meminisse iuvabit.

Aeneid, i.203

Virgil

Contents

List of Tables and Figures

Chapter 5

Chapter 6

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

APC	adenomatous polyposis coli
AT	ataxia telangiectasia
BAC	B1 artificial chromosome
BCC	basal cell carcinoma
BCNS	basal cell nevus syndrome
BICR	Beatson Institute for Cancer Research
BS	Bloom's syndrome
BZS	Bannayan-Zonana syndrome
c-	cellular
CD	Cowden disease
CEPH	Centre d'Étude Polymorphisme Humaine
CHLC	Co-operative Human Linkage Centre
DDS	Denys-Drash syndrome
EST	expressed sequence tag
G3PDH	glyceraldehyde 3 phosphate dehydrogenase
GAP	GTPase activating protein
GDB	Genome Database
GMS	genome mismatch scanning
GSE	gene suppressor element
HIV	human immunodeficiency virus
HNPCC	hereditary non-polyposis colon cancer
HPRC	hereditary papillary renal carcinoma
HPV	human papilloma virus
HTLV	human T-cell leukaemia virus
IMAGE	Integrated Molecular Analysis of Genomes and their Expression
JPS	juvenile polyposis syndrome
LDD	Lhermitte-Duclos disease
LOH	loss of heterozygosity
MEN	multiple endocrine neoplasia
NER	nucleotide excision repair
NCBI	National Center for Biotechnology Information
NHGRI	National Human Genome Research Institute

NIH	National Institue of Health
p	protein
PAC	P1 artificial chromosome
PAGE	polyacrylamide gel electophoresis
PCR	polymerase chain reaction
PJS	Peutz-Jeghers syndrome
RB	retinoblastoma
RDA	representational difference analysis
RH	radiation hybrid
RHMC	radiation-hybrid mapping consortium
RT	reverse transcription
SCLC	small cell lung cancer
SHGC	Stanford Human Genome Centre
SSCP	single strand conformation polymorphism
SSR	simple sequence repeat
STS	sequence-tagged site
TSG	tumour suppressor gene
UV	ultra violet
v-	viral
VHL	von Hippel-Lindau
VNTR	variable number of tandem repeats
WAGR	Wilm's tumour, aniridia, genito-urinary abnormalities, and mental retardation
WICGR	Whitehead Institute Centre for Genome Research
WS	Werner's syndrome
wt-	wild type
WT	Wilms'tumour
XP	xeroderma pigmentosum
YAC	yeast artificial chromosome

Chemicals

A	adenine,
ATP	adenosine 5′-triphosphate

APS	ammonium persulphate
C	cytosine
cDNA	complementary deoxyribonucleic acid
DEPC	diethylpyrocarbonate
dH_2O	de-ionised water
DMSO	dimethylsulphoxide
DNA	deoxyribonulceic acid
DNA'ase	deoxyribonuclease
dNTP	3′ deoxyribonucleoside 5′-triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
G	guanine
HEPES	N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulphonic acid)
mRNA	messenger ribonucleic acid
MOPS	3-(N-morpholino) propanesulphonic acid
OAc	acetate
PBS	phosphate buffered saline
PMSF	phenymethylsuphonyl fluoride
RNA	ribonucleic acid
RNA'ase	ribonuclease
SDS	sodium dodecyl sulphate
SSC	sodium chloride, sodium citrate
SSPE	sodium chloride, sodium phosphate, ethylenediaminetetra-acetic acid
T	thymine
TAE	tris, acetic acid, ethylenediaminetetra-acetic acid
TBE	tris, boric acid, ethylenediaminetetra-acetic acid
TEMED	tetramethylenediamine
Tris	2-amino-2-(hydroxymethyl) propane-1,3-diol

Units

A	Ampere
A_x	absorbance/ x = wavelength
bp	base pair

Ci	Curie
Da	dalton
g	gram
g	gravity
hr	hour
k	kilo
l	litre
M	mega
μ	micro
m	milli
M	molar
min	minute
n	nano
°C	degree Celsius
rpm	revolutions per minute
s	second
V	Voltare
v/v	volume for volume
w/v	weight for volume

Single letter amino acid code

A	alanine
R	arginine
N	asparagine
D	aspartic acid
C	cysteine
E	glutamic acid
Q	glutamine
G	glycine
H	histidine
I	isoleucine
L	leucine
K	lysine

M	methionine
F	phenylalanine
P	proline
S	serine
T	threonine
W	tryptophan
Y	tyrosine
V	valine

Acknowledgements

Donny, for showing me the error of my ways. Robert, for his humane blend of smut and inanity. Vincent, for respite from the plebeians. Ed, for being one of the good guys. Mary, for reminding me that there's always someone else with a more exasperating project. George, for giving me his job. David Gillespie and Brad Ozanne, for offering sane alternatives. Ken Parkinson, for long, long, long discussions. Glasgow, for showing me that weather counts, but football doesn't, and last but first, Angeliki, for teaching me Greek salads.

Abstract

The q31 region of human chromosome 7 is frequently deleted in a broad spectrum of human cancers, and is believed to harbour a multi-tissue tumour suppressor gene. I found loss of heterozygosity at one or more microsatellite markers from 7q31 in 36% of breast carcinomas. The smallest common deleted region was between two CA.GT microsatellite markers, D7S522 and 17TA-5/17B-RE3 in the *CFTR* locus. Loss of 7q31 markers in two non-tumourigenic, human fibroblast cell lines, SUSM-1 and KMST-6 (both immortality complementation group D), has been associated with the emergence of an immortal phenotype. This phenotype can be suppressed by re-introduction of an intact copy of chromosome 7. We conjectured that the multi-tissue tumour suppressor and immortality complementation group D gene, which we named *SEND*, are one and the same.

A physical and functional cloning strategy was adopted to isolate *SEND*. Intact copies of a hygromycin-resistance tagged human chromosome 7 were introduced into SUSM-1 cells by microcell-mediated monochromosome transfer. This induced replicative senescence in a significant proportion of the hygromycin-resistant colonies recovered. Occasional immortal segregants also arose, most likely as a result of inactivating *SEND* on the introduced chromosome. The sites of inactivation were mapped by analysing polymorphic microsatellite markers that differed between donor and recipient chromosomes. This also entailed my generating novel polymorphic markers. Using this strategy, I defined three 'hot spots' of allele loss on chromosome 7 in immortal segregants. One, an approximately 500Kbp interval between 724CA and 786CA, two novel CA.GT dinucleotide repeats, was nested within the smallest common region of allele loss determined for breast cancers. The putative tumour suppressor gene/*SEND* may reside in this interval.

I assembled a YAC, cosmid, and PAC clone contig for the smallest region of allele loss in breast tumours and mapped a number of genes to it by a combination of approaches, including exon-trapping, sequencing, and EST-content mapping. Certain candidate genes were further characterised. This analysis included cloning of full length cDNAs, determining the genomic (exon-intron) structure, determining expression in tumour cell lines by northern and western blot analysis, and looking for mutations by SSCP analysis. Perhaps the most interesting candidate, *CAVEOLIN-1*,

although not mutated in human cancers, was found to be transcriptionally silenced in a number of tumour-derived cell lines by methylation of 5′-sequences.

CHAPTER 1

INTRODUCTION

1. Introduction

1.1 Oncogenes and tumour suppressor genes

Cancer arises through the progressive expansion of clones of cells which have acquired multiple mutations in two distinct classes of nuclear genes: proto-oncogenes, which are positive regulators of cell growth, survival, invasion, and metastasis, and tumour suppressor genes (TSGs), which are negative regulators. The majority of mutations are somatically acquired, while some can be inherited. This dogma is the foundation of molecular oncology, embodying over two hundred years of scientific enquiry into this disease. Like all good dogma, it unifies a great many disparate observations, and provides a theoretical framework in which to fit new ones, but while it is correct in the broad brush strokes, the fine detail contains many caveats. For instance, epigenetic effects—events resulting in altered gene expression without mutation of DNA sequence—also contribute to tumourigenesis. Alternatively, the findings that under certain conditions oncoproteins, including E1A (White *et al.*, 1991), E2F1 (Qin *et al.*, 1994; Shan and Lee, 1994; Wu and Levine, 1994), JUN (Bossy-Wetzel, 1997), and MYC (Evan *et al.*, 1992), can potently induce apoptosis; that the tumour suppressor pRb can provide protection against apoptosis (Morgenbesser, 1994); and more recently that oncogenic *ras* can induce senescent-like cell growth arrest (Serrano *et al.*, 1996), appear paradoxical given the known roles of these genes in cellular proliferation, and strongly imply that the function of oncogenes and TSGs is context-driven. In addition, gene products whose purpose is to monitor and repair DNA damage are also frequently inactivated during tumourigenesis, but do not directly confer a selective growth, survival, or invasion advantage. Nonetheless, the existence of two classes of regulators of cell behaviour whose functions are diametrically opposed is a tacit requirement for tissue homeostasis; cancer reflects a breakdown in this fundamental symmetry.

An obvious distinction can be drawn between the types of mutations that occur in proto-oncogenes and those found in TSGs. Point mutations in proto-oncogenes are frequently missense mutations that activate the gene product, or lead to its gaining new functions. In contrast, mutations in TSGs are nonsense mutations, missense mutations, or splice-site mutations that lead either to a truncated gene product and loss of function, or loss of function alone, or give rise to a protein with a dominant-negative effect. This distinction extends to chromosomal aberrations found in cancer cells—deletions,

translocations, amplifications, aneusomy, *etc.*—which result in activation of oncogenes and inactivation of TSGs. The contrasting mutational profiles of these opposed molecules account for the dominant transforming ability of oncogenes (oncogenic alleles transform cells even in the presence of wild-type alleles) and the recessiveness at the cellular level of TSGs (both alleles of a TSG generally require to be inactivated for tumourigenesis). The precise locations and types of mutations found in genes associated with cancer often gives the first indication of the important functional domains of their encoded products. This is especially true when mutations are clustered at 'hot spots', as is the case, for instance, for *HRAS1* and *TP53* (McKay *et al.*, 1986; Nigro *et al.*, 1989).

1.1.1 The identification of oncogenes

The revolution in our understanding of the molecular events underlying tumourigenesis depended upon the identification and cloning of the genes crucial to these processes. Historically, this began with the identification of oncogenes. Over twenty distinct oncogenes were identified through the analysis of the genomes of acutely transforming retroviruses (Table 1.1A), starting with the ground-breaking discovery that v-*src*, the transforming gene of the Rous sarcoma virus, is homologous to the cellular proto-oncogene c-*src* (Stehelin *et al.*, 1976). Indeed, it transpires that nearly all retroviral oncogenes are transduced versions of cellular proto-oncogenes (the human immunodeficiency virus, HIV, and the human T-cell leukaemia virus, HTLV, provide two exceptions to this statement, since virally derived sequences from both can transform subsets of human cells). During the process of transduction the expression and activity of these genes becomes altered. In the first instance, viral oncogenes are regulated by strong promoters and enhancers in the viral long terminal repeat (LTR), which often results in high level transcription, whereas transcription of the corresponding proto-oncogene is physiologically regulated by its normal promoter sequences. Second, viral oncogenes lack introns and are usually truncated having lost one or both of the 5′ and 3′ untranslated regions. This can affect both the stability and translational efficiency of the viral mRNA when compared to the cellular mRNA. Third, viral oncoproteins are often expressed as fusion products with viral gag sequences at their amino termini. In many cases carboxy-terminal amino acids have also been deleted from viral oncoproteins. Truncated viral oncoproteins are potentially different in function from their proto-oncoprotein precursors. These structural and functional

differences are further heightened by the multiple point mutations observed in viral oncoproteins relative to their proto-oncoprotein homologues. The changes observed in viral oncogenes often affect important catalytic or regulatory domains of the protein, and are sometimes mirrored by naturally occurring mutations found in spontaneously arising tumours.

In addition to acutely transforming retroviruses, which induce tumours after a short latency period (days to weeks), chronic transforming retroviruses, including avian leukosis virus (ALV), mouse mammary tumour virus (MMTV), and leukaemia viruses of mice, rats, cats, and primates (MuLV, FeLV, and GaLV), have been identified which induce tumours only after a long latency period (months to years). Viruses of this type have normal virion genomes and possess no additional sequences. Work in the '70s by Temin and Baltimore had established that the life-cycle of retroviruses entails a reverse transcription step in which the virion RNA genome is transcribed into a provirion DNA genome by an RNA-dependent DNA polymerase—reverse transcriptase (Baltimore, 1970; Temin and Mizutani, 1970); proviral DNA then integrates into the host genome (Varmus *et al.*, 1972).

It was proposed that proviral integration in or near specific host cell target genes might result in neoplastic transformation. In the case of oncogenes, proviral insertion could activate the gene by subjugating it to the viral LTR, and through production of fusion products that contain viral sequences. Hayward *et al.* (1981) provided the first evidence to substantiate this mechanism of *insertional mutagenesis*. They showed that the *myc* proto-oncogene was activated in over 80% of ALV-induced chicken bursal lymphomas, and that proviral DNAs, in particular functional LTRs, were integrated upstream of the lymphoma *myc* genes. Insertional mutagenesis has been used prospectively to identify novel proto-oncogenes that are reproducible targets for proviral insertion (Nusse *et al.*, 1982). Table 1.1B lists proto-oncogenes that were first identified following their activation by insertional mutagenesis. Clearly proviral insertion in a proto-oncogene precedes its transduction and the formation of an acutely transforming retrovirus.

If viruses could induce tumours by misappropriating proto-oncogenes, then it seemed a reasonable proposition that spontaneously occurring or carcinogen-induced mutations in the same set of genes, or similarly acting ones, might account for sporadically occurring tumours. Evidence for this hypothesis was forthcoming from gene transfer experiments which indicated that about 20% of individual tumours possess

DNA sequences with transforming ability (Shi *et al.*, 1981). Moreover, oncogenes isolated in this fashion like H-*RAS*, K-*RAS*, *ROS*, and *RAF* were already known from their homology with various retroviral oncogenes, while other novel oncogenes often belonged to the *src* and *ras* superfamilies. It only remained to demonstrate the existence of activating mutations in these cellular oncogenes in order to substantiate the theory; again the proof was close at hand (Tabin *et al.*, 1982; Taparowsky *et al.*, 1982; Santos *et al.*, 1982). These findings confirmed that spontaneously arising neoplasms, including those occurring in man, are also due to mutations in proto-oncogenes, and suggested that these genes constitute a relatively small set. Occasionally oncogenes identified through the gene transfer technique possessed no homology with other known genes. Novel oncogenes identified this way can be divided into two groups: those activated in the tumours from which they were derived, and those activated by re-arrangements during the process of gene transfer itself (Table 1.1C).

Oncogenes have also been identified through their association with chromosomal abnormalities (Table 1.1D). Activation of oncogenes by translocation is akin to insertional mutagenesis in that oncogene sequences are juxtaposed to unrelated sequences which were previously separated. Again, this can result in inappropriate expression of the oncogene through the effect of nearby enhancer sequences or through the production of fusion products. This mode of oncogene activation is characteristic of cancers of the haematopoietic system, and in particular of cell types in which illegitimate end-joining occurs during the production of variability in antigen receptors, *i.e.* B- and T-lymphocytes. Translocations between the c-*MYC* gene, which encodes a transcription factor of the basic helix-loop-helix/ leucine zipper family, on the q arm of human chromosome 8 and either chromosome 14, chromosome 2, or chromosome 22 are frequently observed in Burkitt's lymphoma, a B lymphocyte neoplasm (Adams *et al.*, 1986). The sites of translocations on chromosomes 14, 2, and 22 correspond to the immunoglobulin heavy-chain, κ light-chain, and λ light-chain genes respectively, which ordinarily are expected to be actively expressed in B cells. Translocation results in constitutive expression of c-*MYC*, which is sufficient to activate *MYC* as an oncogene.

On the other hand, the 9;22 t(q34;q11) translocation giving rise to the Philadelphia (Ph^1) chromosome in over 90% of cases of chronic myelogenous leukaemia (Nowell and Hungerford, 1960), creates a fusion gene in which the 5′ half of the breakpoint cluster region (*BCR*) gene on chromosome 22 is joined upstream of the c-*ABL* proto-oncogene on chromosome 9 (De Klein *et al.*, 1982; Groffen *et al.*, 1986).

Transcription of this fusion gene results in a chimeric BCR-ABL fusion protein (Gale and Canaani, 1984), in which the tyrosine kinase activity of c-ABL is enhanced and its substrate specificity altered (Lugo *et al.*, 1990). A similar translocation activates the *ABL* proto-oncogene in some acute lymphocytic leukaemias, except that the breakpoint falls further upstream in the *BCR* gene (Chan *et al.*, 1987; Kuzrock *et al.*, 1987).

Amplifications of genomic DNA in the form of double minute chromosomes and homogeneously staining regions are frequent anomalies observed in the karyotypes of cells cultured from tumours. Amplification of the copy number of a proto-oncogene would be expected to increase its expression and possibly activate it as an oncogene. Insertional mutagenesis and translocations have already revealed how over-expression of wild-type c-*MYC* is sufficient to activate it. Similarly, c-*MYC* was the first oncogene to be shown to be activated following its amplification in human neoplasms (Collins and Groudine, 1982; Dalla-Favera *et al.*, 1982). New members of the *MYC* gene family, L- and N-*MYC*, have also been found to be amplified in human tumours (Schwab *et al.*, 1983; Nau *et al.*, 1985; Wong *et al.*, 1986).

Finally, the identification of oncogenes by these classic means facilitated the identification of further oncogenes through homology at the sequence level. Both cross hybridisation with DNA probes derived from existing oncogenes and amplification by the polymerase chain reaction (PCR) using degenerate oligonucleotide primers have been used to isolate homologous gene family members from genomic and cDNA libraries (Table 1.1E).

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, HST, 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, ERB-B2, AIB1/SCR1*

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

*ELK1, ELK2, EPH, ERB-B3, ERB-B4, ERG, FOS*B, *FRA1, FRA2, HCK, JUNB, JUND, LYN, MAX*

Table 1.1. The identification of novel oncogenes. (See Hesketh, 1995 for the majority of these entries)

1.1.2 The function of oncogenes

Both proto-oncogenes and TSGs exist as evolutionarily conserved homologues in a number of different eukaryotes from yeast, worms, flies, frogs, and mice through to man. Consequently, classical and molecular genetic studies have employed organisms such as *S. cerevisiae, S. pombe, C. elegans, D. melanogaster, X. Laevis, M. musculus,* in addition to *H. sapiens* to study the functions of these genes *in vivo*. Findings from these model organisms have cross-fertilised with other lines of cancer research to establish critical roles for proto-oncogenes and TSGs in many aspects of cell behaviour *viz.* proliferation, differentiation, apoptosis, replicative senescence, motility, and invasion. A distillation of this research suggests that the majority of proto-oncogenes are elements in signal transduction pathways (Table 1.2). Many of these pathways initiate at the cell surface via specific receptor–ligand interactions and are relayed through the cytoplasm to the nucleus, where transcription factors elicit a response by modulating gene expression. Cumulative mutations in proto-oncogenes result in the deregulation of such pathways and consequently to cell growth, survival, differentiation, *etc.* becoming autonomous. This transformation in cell behaviour is immensely deleterious in multicellular organisms, where there is an absolute requirement for these processes to be co-ordinately regulated both during embryonal development and for maintenance of the adult body.

A) Ligands

FGF5, INT2, HST1, SHH, SIS/PDGFB, WNT1, 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, TAN1/NOTCH1

D) Non-receptor tyrosine kinases

membrane-associated:
SRC, FGR, FYN, HCK, LCK, LYN, 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, MYC, MYB, REL, TAL1, SKI

J) Transcription co-activator (acetyl transferase)

AIB1/SCR1

Table 1.2. Classification of oncoproteins according to their biochemical role in signal transduction.

The mitogenic signal transduction pathway (Figure 1.1) serves to goad cells out of a non-dividing G0 state into a dividing state, and stimulates progression through G1 phase of the cell cycle (Pardee, 1989; Sherr, 1994). Progression through the cell division cycle is catalysed by a number of holoenzymes comprising cyclins (the regulatory subunits) and cyclin-dependent kinases (CDKs, the catalytic subunits), which are sequentially assembled and activated (Figure 1.2). Specifically, mitogens induce the expression of D-type cyclins and then promote the formation and activation of cyclin/CDK complexes which regulate the transition between G1 and S phases of the cell cycle (Sherr, 1993). Passage through a restriction point in late G1 of the cell cycle, beyond which progression is independent of mitogenic stimulation, commits a cell to complete at least one round of DNA replication and division. The genes which encode cyclin D1 and CDK4, namely *CCND1* and *CDK4*, two positive regulators of the G1 checkpoint, have also been found oncogenically activated in human tumours (Motokura *et al.*, 1991; Hunter and Pines, 1994; Hall and Peters, 1996). Constitutive expression and activation of these two gene products would be expected to substitute for mitogenic stimulation in terms of their ability to force cell cycle progression. One possible consequence of oncogenic activation, therefore, is the uncoupling of cell growth from regulation by extrinsic factors either through constitutive stimulation of the mitogenic signalling pathway or through direct stimulation of cell cycle progression. As a result, cancer cells tend to remain in cycle, and because cell cycle exit promotes maturation and terminal differentiation, these processes are subverted as well.

Proliferation is only one term in the equation that determines the rate of cell growth. An equally important factor is cell death. First established by Kerr *et al.* (1972), apoptosis (genetically programmed self-destruction) is an essential physiological process for removing cells during embryonic development, tissue involution, differentiation of thymocytes, and in the homeostasis of self-renewing tissues. Resistance to apoptosis is a major attribute of the malignant phenotype. Obversely, apoptosis possibly serves as an anti-tumour adaptation in removing cells which have sustained genetic lesions. Members of the *Bcl*-2 family of genes have been identified as regulators of the apoptotic process. The oncogene *Bcl*-2, which is the prototype for this family, was first identified at the breakpoint of translocations commonly occurring in follicular lymphoma (Bakhshi *et al.*, 1985; Tsujimoto *et al.*, 1985; Cleary and Sklar, 1985), and was shown to extend cell survival following growth factor withdrawal (Vaux *et al.*, 1988; Nunez *et al.*, 1990) and to inhibit apoptosis (White, 1996).

Figure 1.1. Schematic representation of the mitogenic signal transduction pathway. Signalling initiates through specific receptor/ligand interactions at the cell surface. Ligand binding induces dimerization and autophosphorylation of the receptor tyrosine kinase (RTK). The Grb2 adaptor molecule, which is localised in the cytoplasm, is bound through its SH3 domains to the carboxyl terminus of Sos. Upon ligand stimulation, Grb2 interacts through its SH2 domain with the phosphorylated tyrosine residues of the activated receptor. Sos then activates Ras by catalysing GDP/GTP exchange. Alternatively, Shc binds to the phosphotyrosine residues of the activated receptor and becomes phosphorylated. The phosphotyrosine moiety of Shc can then function as an alternative binding site for the SH2 domain of the Grb2 molecule. The GTP-bound Ras finally activates the archetypal mitogen activated protein kinase (MAPK) cascade. c-Raf phsophorylates MAPK/ERK kinase (MEK) on serine residues. MEK, a dual specificity kinase, then phosphorylates ERKs 1 and 2 on threonine and tyrosine residues. ERKs phosphorylate and activate other downstream kinases and transcription factors, modulating protein synthesis and gene expression. The connection between heterotrimeric G-protein coupled receptors (GPCR) and the mitogen signalling pathway is still tentative, with inputs being made at various levels. The activation of Ras and ERK2 is probably mediated by the $\beta\gamma$ complex, possibly through an intermediary tyrosine kinase, PYK2. White text against a black background indicates that these molecules have been found mutated in cancers. Arrows indicate positive interactions. A dashed arrow indicates an indirect activation. Lines ending in bars represent inhibitory interactions.

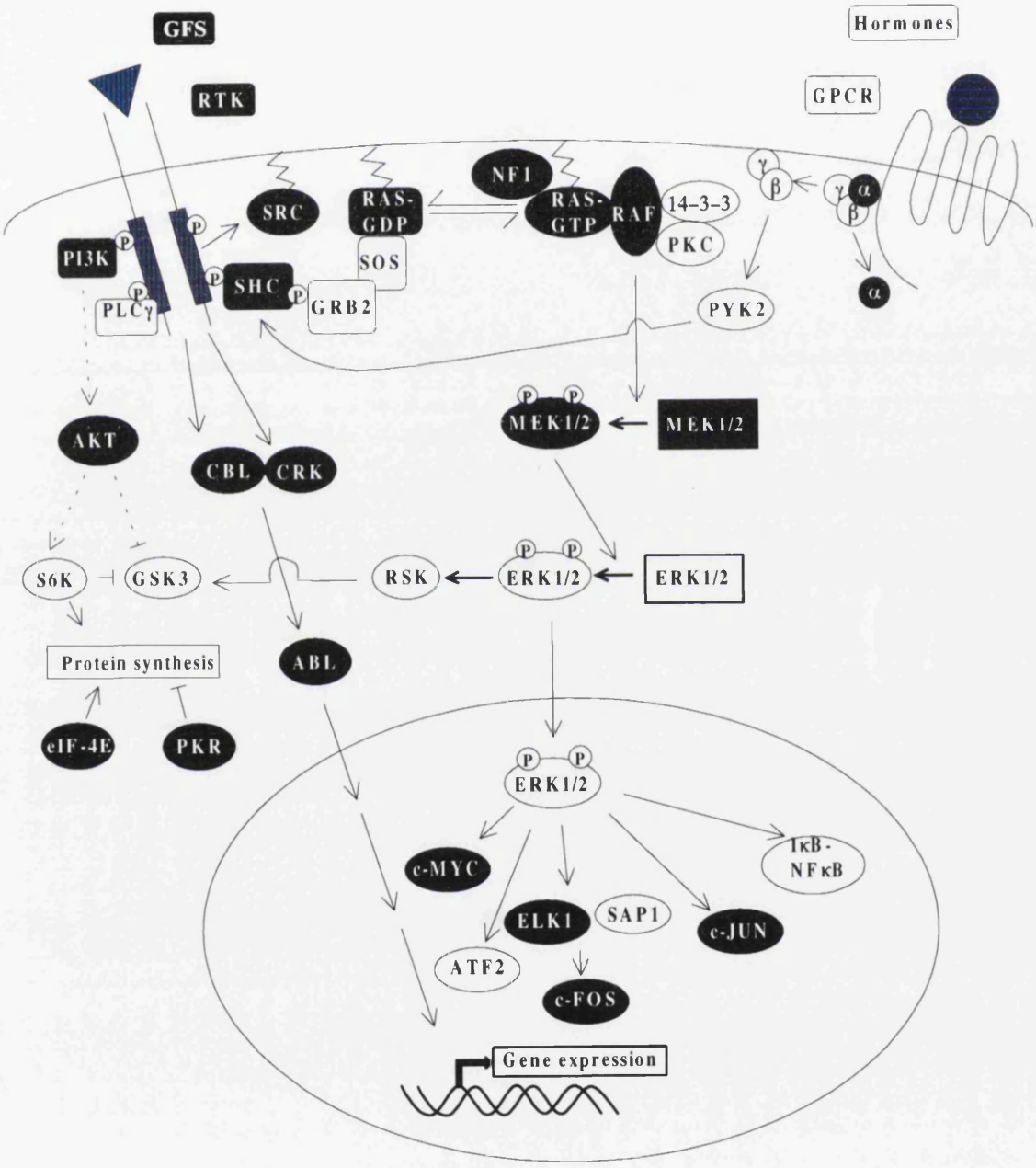
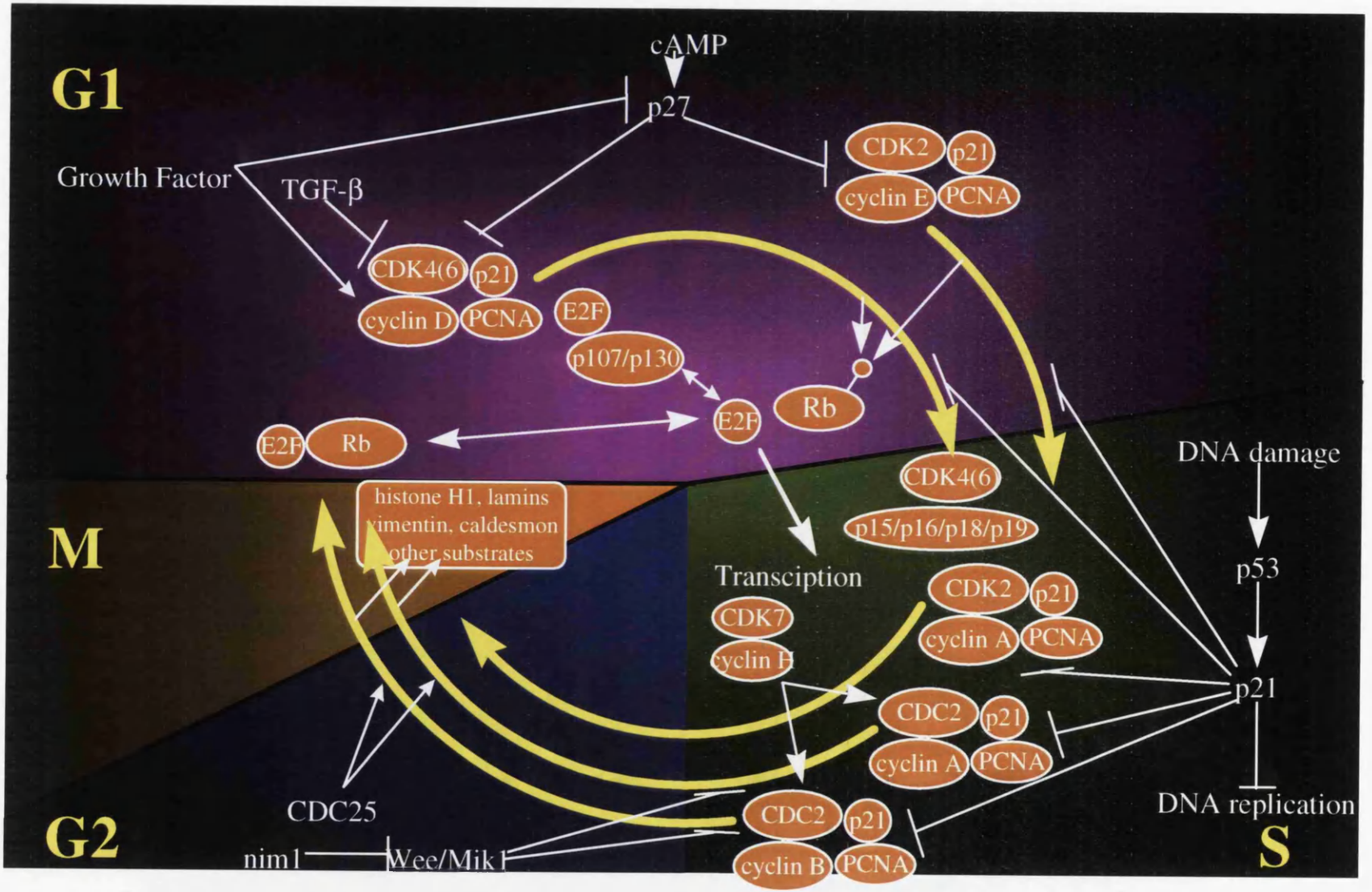

GFS
RTK
Hormones
GPCR
NF1
RAS-GDP
RAS-GTP
RAF
14-3-3
PKC
SRC
SOS
PI3K
SHC
GRB2
PLCγ
PYK2
AKT
CBL
CRK
MEK1/2
MEK1/2
S6K
GSK3
RSK
ERK1/2
ERK1/2
Protein synthesis
ABL
eIF-4E
PKR
ERK1/2
c-MYC
IκB-NFκB
ELK1
SAP1
c-JUN
ATF2
c-FOS
Gene expression

Figure 1.2. Regulation of the cell division cycle by cyclin/CDK complexes. Entry and progression through the cell division cycle are catalysed by complexes of cyclin dependent kinases (CDKs) and cyclins. For orderly cell division, CDKs have to be activated and inactivated at specific time points during the cell cycle. The regulation is best understood for CDK1(cdc2)/cyclin B. This CDK/cyclin complex is activated by phosphorylation by cyclin activating kinase (CAK), which is itself a complex of CDK7 and cyclin H, and also by dephosphorylation by the cdc25 phosphatase, which opposes the action of Wee1 and Mik1. Activated cdc2/cyclin B then phosphorylates a number of substrates required for progression of M phase of the cell cycle. On completion of metaphase, cyclin B is destroyed by the ubiquitin-proteasome pathway, which abolishes cdc2 kinase activity. Entry into the cell cycle and transit through the restriction point in late G1 is catalysed by a different set of CDK/cyclin complexes. Mammalian cyclins D and E interact with CDK4 and 6, and CDK2 respectively during the G1/S phase transition, and are responsible for phosphorylating Rb-like proteins, resulting in release and activation of E2F transcription factors, which are required for entry into S phase. A number of molecules falling into two families: the WAF1/CIP/KIP family and the INK4 family, have been identified which can inhibit cell cycle progression. These molecules bind and inhibit CDK/cyclin complexes. A number of the proteins shown in this scheme have been found to be mutated in human cancer and are described elsewhere in the text. Small arrows indicate stimulation. Bars with a T-shaped head indicate inhibition. Double-headed arrows indicate associations between protein complexes. Short bars with a filled circle indicate phosphate groups.


G1
cAMP
p27
Growth Factor
TGF-β
CDK2
p21
cyclin E
PCNA
CDK4(6)
p21
cyclin D
PCNA
E2F
p107/p130
E2F
Rb
E2F
Rb
DNA damage
CDK4(6)
p15/p16/p18/p19
M
histone H1, lamins
vimentin, caldesmon
other substrates
Transciption
p53
CDK2
p21
CDK7
cyclin H
cyclin A
PCNA
p21
CDC2
p21
cyclin A
PCNA
CDC25
nim1
Wee/Mik1
CDC2
p21
cyclin B
PCNA
DNA replication
G2
S

The Bcl-2 family of proteins comprises two categories: inhibitors of apoptosis like Bcl-2, and accelerators of apoptosis like the Bcl-2 binding protein Bax. Physical interactions between family members are mediated through Bcl-2 homology regions BH1, BH2, and BH3. Induction of or resistance to apoptosis could then be determined by the ratio of agonists to antagonists (*e.g.* Bax *versus* Bcl-2) in a cell (Oltavi and Korsmeyer, 1994). The crystal structure of Bcl-X_L, an apoptosis inhibitor, has been solved and reveals structural similarities with bacterial toxins such as diptheria toxin and the colicins, particularly in their membrane insertion domains (Muchmore *et al.*, 1996). By functional analogy, the Bcl-2 family of proteins may form membrane pores and function by regulating processes dependent on pH, voltage, or ionic strength. However, whether any of the members of this family can function as ion channels remains to be demonstrated.

Oncogenes have also been implicated in the regulation of the actin cytoskeleton and of cell adhesion during cell movement, aspects of cell behaviour which are altered in cancer cells. Work in the past six years has converged on three Rho-related GTPases, Rho, Rac, and Cdc42, as being of central importance in both these processes (Ridley *et al.*, 1992; Ridley and Hall, 1992; Kozma *et al.*, 1995; Nobes and Hall, 1995). In particular, Rac and Cdc42 control actin polymerisation at the leading edges of migrating or spreading cells, leading to the formation of lamellipodia, membrane ruffles, and filopodia. Rho on the other hand regulates the formation of stress fibres, contractile cables of bundled actin filaments and actomyosin which traverse the cell and terminate in the plasma membrane at focal adhesion complexes, whose assembly is also dependent upon the activity of Rho-related GTPases. Focal adhesions are multi protein assemblies which link the cytoskeleton to the extracellular matrix via cell surface integrin adhesion receptors. Besides their role in attaching cells to the extracellular substrate, focal adhesions also act as signalling complexes. Ligand occupation of integrins can activate the ERK 1/2 MAP kinase cascade (Guan *et al.*, 1991, Kornberg *et al.*, 1991), and is an obligatory requirement for cell cycle progression (Guadagno *et al.*, 1993). Growth factor stimulation of benign cells in the absence of integrin-dependent signalling leads to apoptosis, or anœkis (Ruoslahti and Reed, 1994). Tumour cells often display anchorage independent growth and survival, suggesting that transformation by oncogenes such as *src* or *ras* can stimulate integrin signalling in the absence of matrix, again obviating any requirement for extrinsic growth control.

Rho-related GTPases themselves have not so far been found to be mutated in human cancers, although ectopic expression of *rac* can transform cells (Qiu *et al.*, 1995), or confer an invasive phenotype (Habets *et al.*, 1994). Like all members of the RAS superfamily of small GTPases, these molecules are molecular switches cycling between an inactive GDP-bound conformation and an active GTP-bound form. Exchange of GDP is facilitated by guanine nucleotide exchange factors (GEFs), a large number of which have now been identified for Rho-related GTPases, including Dbl, Ect2, Lbc, Tiam1, and Vav. Significantly, these molecules have been implicated in tumourigenesis (Eva and Aaronson, 1985; Miki *et al.*, 1993; Habets *et al.*, 1994, Toksoz and Williams, 1994; Katzav *et al.*, 1989).

1.2 TSGs and hereditary Cancer

The products of TSGs are envisaged to directly oppose the action of oncoproteins, that is to constrain cellular proliferation and survival either by inhibiting mitogenic signalling, or by impeding cell cycle progression, or by promoting terminal differentiation and maturation arrest, or by inducing apoptosis. Enforcing a limit on the replicative life-span of a cell is also a potential mechanism of tumour suppression, and a role for three TSGs, namely *RB1*, *TP53,* and *CDKN2A/p16INK4A*, in conferring replicative senescence has emerged. (This topic is dealt with in detail below.) In addition, genes have been identified which suppress the invasive and metastatic potential of cancer cells, and which are inactivated during tumourigenesis; these too qualify as TSGs.

In comparison to the plethora of oncogenes that have now been identified, only a handful of *bona fide* TSGs have been cloned, although the rate of discovery is increasing exponentially. TSGs are relative latecomers in the field of molecular oncology; many of the genes mentioned below were discovered only in the past five years. This is perhaps ironic since the concept of tumour suppression grew out of early experiments involving cell fusions between normal somatic cells and tumour cells (Harris *et al.*, 1969; Stanbridge, 1976). Almost invariably the *normal x tumour* (or *less malignant x more malignant*) hybrids exhibited a normal phenotype demonstrating that malignancy was a recessive trait, a finding that was more consistent with the idea that tumour cells had lost critical functions rather than gained dominant transforming ones (a result anticipated more than fifty years before by the renowned cytogeneticist, Thomas

Boveri [Boveri, 1914]). These *normal x tumour* hybrids also provided evidence for a genetic basis to tumour suppression: hybrid cells frequently shed chromosomes, apparently at random, but the segregation of certain chromosomes was consistently associated with re-emergence of the malignant phenotype (Jonasson *et al.*, 1977; Klinger, 1980; Evans *et al.*, 1982). This allowed early mapping of chromosomes that could suppress the malignant phenotype, chromosomes which harboured presumptive TSGs.

It was not through mapping chromosomal losses in revertant hybrid tumours, however, that the first representative of this class of genes was identified, but rather through the study of hereditary cancer (the actual mechanics of TSG cloning are dealt with in more detail in section 1.4). Many forms of cancer have a higher incidence in relatives of cancer patients than in the general population, suggestive of an inherited component in their aetiology. Some familial cancers even show Mendelian inheritance, the commonest mode of transmission being autosomal dominant. These cancer predisposition syndromes are believed to be due to segregation of highly penetrant mutated alleles of susceptibility genes (Mulligan *et al.*, 1993; Hofstra *et al.*, 1994; Zuo *et al.*, 1996; Schmidt *et al.*, 1997), a number of which have now been identified (Table 1.3). The majority of cancer susceptibility genes so far identified appear to be TSGs.

Individual cancer predisposition syndromes although rare may together account for 10% of the total incidence of cancer. However, less penetrant cancer susceptibility alleles may account for a much higher proportion of the total incidence, and are probably responsible for the majority of small cancer clusterings within families. These alleles, whose effect is necessarily much harder to demonstrate by linkage analysis, would each contribute quantitatively to cancer susceptibility. One such possible candidate is the ataxia telangiectasia gene, *ATM*. Individuals homozygous for a mutation in *ATM* develop cancer at a rate 100 times that of the general population and have an increased risk of breast cancer. It has also been proposed that females heterozygous for *ATM* have a modest increased risk of breast cancer. In one study by Athma *et al.* (1996), heterozygosity at this gene was calculated to confer a 3.8 relative risk of developing breast cancer.

Another candidate for non-Mendelian (multigenic) cancer susceptibility is a familiar player in oncogenesis, *HRAS1*, but in a new guise. Krontiris *et al.* (1985) first demonstrated the presence of a rare RFLP associated with the *HRAS1* locus in the germ-line DNA of cancer patients. In this case, the variation in length of the restriction

Gene	Disease/Syndrome[a]	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. Genes involved in hereditary predisposition to cancer. [a] 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.

fragments is due to the presence of a VNTR (variable number of tandem repeats) minisatellite in the promoter region of *HRAS1*. Carriers of rare, larger repeat number alleles of this minisatellite have been shown to have an approximately 2-fold increasedrisk of developing cancer of the breast, colorectum, and urinary bladder (Krontiris *et al.*, 1993), and the *HRAS1* VNTR locus has also been shown to modify the penetrance of other cancer predisposition genes like *BRCA1* (Phelan *et al.* 1996). Clues to the mechanism underlying this phenomenon are beginning to emerge. Transcription factors of the rel/NF-κB family have been shown to bind the minisatellite region of the *HRAS1* promoter (Trepicchio and Krontiris, 1992), and large repeat number alleles appear to be more active in a reporter gene assay (Green and Krontiris, 1993). In one possible scenario the level of expression of RAS protein could be subtly modified by the presence of rare *HRAS1* VNTR alleles. Increased expression of RAS could cause cells to proliferate, increasing the target population available for further mutational events leading to malignant transformation. Low penetrance cancer susceptibility has also been attributed to polymorphisms in drug metabolising enzymes—cytochrome P450 enzymes, glutathione S-transferases, and N-acetyl transferases (reviewed in Smith *et al.*, 1995).

1.2.1 *RB1* and hereditary retinoblastoma—the 'two hit' hypothesis

The prototypical familial cancer syndrome is retinoblastoma, a rare ocular tumour that affects 1 in 20,000 children. It was through studying the inherited form of this cancer that the whole notion of TSGs acting as recessive oncogenes at the cellular level became ensconced, and that the *RB1* gene—the first acknowledged TSG—was cloned (Friend *et al.*, 1986; Lee *et al.*, 1987). Germ-line mutations in this gene account for about 40% of retinoblastoma cases; the majority of carriers develop bilateral lesions, whereas only a single eye is affected in children without the germ-line mutation. Most carriers have no previous family history, a fact that is explained by new mutations occurring at a rate of 8×10^{-6} per locus per generation. However, approximately 50% of all the offspring of newly mutant cases of either sex will develop the tumour, *i.e.* susceptibility to retinoblastoma is transmitted as an autosomal dominant trait, with a penetrance near 100% (Knudson, 1978).

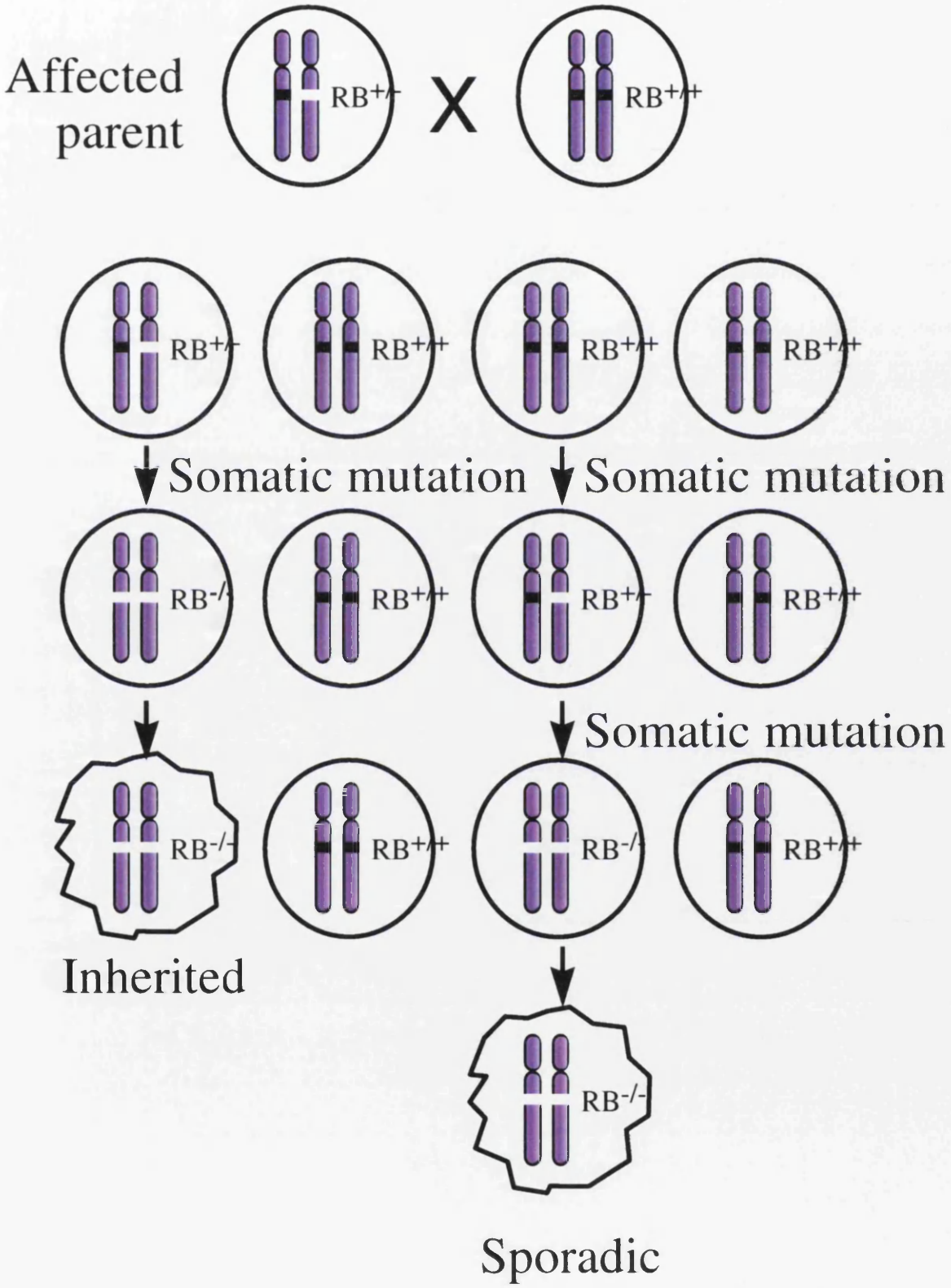
It was Alfred Knudson who hypothesised that *RB1*, and by extrapolation other familial cancer susceptibility genes, acted recessively at the cellular level (Knudson,

1971). He reasoned that since a mutated *RB1* allele would be present in every cell of a carrier, yet only a single cell became initiated to give rise to a retinoblastoma, that the rate limiting step in the familial form of the disease was inactivation of the remaining wild type allele by somatic mutation. In a wild type homozygote, two somatic mutations in *RB1* would be required to give rise to a retinoblastoma (Figure 1.3), an unlikely set of events that would account for the absence of bilateral lesions in sporadic cases and the comparatively later age of onset (typically, familial cancers occur in affected individuals at an earlier age of onset than is common for the sporadic form of the disease).

Knudson's now famous 'two-hit' model has become engraved in the annals of cancer genetics. The model applies not only to retinoblastoma but also fits with the epidemiological data for other familial cancers like neuroblastoma, pheochromocytoma and Wilms' tumour, and it requires simply to be enlarged upon in order to encompass cancers which require the accumulation of several more mutations before expression of the fully malignant phenotype. Colorectal carcinoma occurs in both familial and sporadic forms, but the epidemiological data suggest that accumulation of approximately six independent mutational events is required (Armitage and Doll, 1954). The earlier age of onset for the familial disease implies that one or two fewer mutations are necessary in patients who carry an inherited mutation (Ashley, 1969). Fearon and Vogelstein (1990) have demonstrated that an early, probably initial, requirement for the development of colon cancer in both familial and sporadic cases is inactivation of both copies of the *APC* gene; germ-line mutations in *APC* have been shown to predispose carriers to colon cancer (Groden *et al.*, 1991; Kinzler *et al.*, 1991[a]; Nishisho *et al.*, 1991). Despite the requirement for further somatic mutations to occur stochastically before the emergence of frank colon carcinoma, inheritance of a single inactivated copy of a TSG is again sufficient to confer an increased risk of developing the disease, by initiating a chain of tumourigenic events.

The 'two-hit' hypothesis was based purely upon statistical modelling; experimental evidence in its support was furnished by cytogenetic and molecular studies. These experiments paved the way to finally isolating the *RB1* gene, and also established a mould for future forays into TSG cloning. About 5-10% of individuals who inherit retinoblastoma possess a constitutional deletion of part or all of chromosome 13q14 (Yunis and Ramsay, 1978). These findings originally mapped the *RB1* locus; they also provided visible evidence of Knudson's hypothesised first hit. The analysis of syntenic (linked) polymorphic genetic markers provided evidence for second

Figure 1.3. The two-hit model of cancer susceptibility. The figure shows cells containing a homologous pair of chromosomes in which a marker gene (*RB*) is either wild-type (black box) or a loss-of-function mutant (white box). The homozygous wild-type and heterozygous cells have the same normal phenotype, demonstrating the recessive nature of the mutant allele. Tumourigenesis will only ensue if both copies of the gene are mutated. In an individual who inherits a mutant copy from one parent, only a single somatic mutation is needed for tumourigenesis. In individuals carrying two wild-type alleles, both copies must sustain independent somatic mutations.


Affected parent
RB+/-
X
RB+/+
Somatic mutation
Somatic mutation
RB-/-
Somatic mutation
Inherited
Sporadic

hits occurring within tumours, both validating the hypothesis and permitting demonstration of at least some of the mechanisms predicted for second events (Knudson, 1978), namely: focal mutation, deletion, chromosomal nondisjunction, somatic (mitotic) recombination, and gene conversion (we can also add gene silencing by methylation of promoter regions).

The technique employed by Cavenee *et al.* (1983) exploited the existence of DNA sequence polymorphisms which can be detected by restriction digestion—*restriction fragment length polymorphisms* (RFLPs)—to follow the fate of individual chromosome segments in tumours. Loss of heterozygosity (LOH) of a given RFLP, or indeed any polymorphism, within a clone of tumour cells is taken to provide evidence for genetic inactivation of a TSG allele at or near that locus; the other allele is presumed to be inactivated by the first hit. This approach can be used to define an interval that is consistently deleted within tumours, and in which the targeted TSG should lie. In the case of *RB1*, probes for certain of these polymorphisms failed to hybridise at all to some tumour DNA samples indicating the presence of homozygous deletions within these samples. In these instances, both copies of the *RB1* gene had been inactivated through deletion, with some deletions appearing to be constrained within the gene itself. DNA probes derived from the smallest deleted region detected a 4.5 Kbp transcript on a Northern blot that was absent from retinoblastoma tumours; this finding together with the observed deletions clinched the identification of the gene by Friend *et al.* (1986). The normal cDNA of *RB1* was then shown to cause reversion of the tumourigenic properties of cultivated tumour cells that were mutant for *RB1* (Huang *et al.*, 1988), truly substantiating its position as the first recognised TSG.

Not surprisingly, the *RB1* gene itself has been the subject of intense investigation since its discovery. Mutations within the gene occur at high frequency in a variety of tumour types (Harbour *et al.*, 1988; Lee *et al.*, 1988; Toguchida *et al.*, 1988; Chen *et al.*, 1990); the gene product, a 105 kDa nuclear phosphoprotein termed pRb, is ubiquitously distributed throughout the body. Together these observations suggest that pRb has an important role in the maintenance of a broad range of tissues. From biochemical studies (reviewed in Weinberg, 1995), pRb appears to function as a regulator of the cell cycle, with the capacity to block the G1/S transition, and it is through this mechanism that pRb is supposed to mediate tumour suppression. In G0 and G1 phases of the cell cycle, pRb is found in a hypophosphorylated form, but becomes increasingly hyperphosphorlyated from mid/late G1. This phosphorylation of pRb, which inactivates the protein, is

mediated by complexes of cyclins and cyclin-dependent kinases (CDKs), cyclin D/CDK4-6 and cyclin E/CDK2 complexes being most strongly implicated. pRb and D-type cyclins have been shown to physically interact (Ewen *et al.*, 1993; Dowdy *et al.*, 1993; Kato *et al.*, 1993), disruption of this interaction enhances, rather than eliminates, the ability of cyclin D1 to antagonise the growth suppressive effects of pRb in cultured cells (Dowdy *et al.*, 1993), suggesting that one physiological role of pRb is to limit the amount of cyclin D available to form complexes with CDKs.

A second mechanism by which pRb might impede cell cycle progression and mediate tumour suppression is through its direct interaction with the transcription factor E2F. E2F binding sites are present in the promoters of a number of genes required for entry into S phase, including thymidine kinase, dihydrofolate reductase, c-*MYC*, and cyclins E and A (Weinberg, 1996). Over-expression of E2F has been shown to induce expression of cyclin E and A (DeGregori *et al.*, 1995), and to induce S phase entry and transformation (Johnson *et al.*, 1993). Therefore, the simplest model by which pRb blocks entry into S phase is by binding to and inhibiting the transcriptional activity of E2F. Phosphorylation of pRb by cyclin/CDK complexes is known to interrupt this interaction (Ewen *et al.*, 1993; Dowdy *et al.*, 1993), which would leave E2F free to induce expression of G1/S-regulatory genes. However, a number of observations may require that this model be revised. A chimeric GAL4-pRb fusion protein is capable of repressing transcription of promoters with GAL4 binding sites (Weintraub *et al.*, 1995; Bremner *et al.*, 1995), implying that pRb mediates transcriptional repression directly. E2F may be recruiting pRb to E2F sites in order to silence transcription. Rather than antagonising E2F, the two proteins may synergize to repress target gene expression and inhibit cell cycle progression. In support of this, if E2F sites are mutated in a reporter gene construct such that the sites no longer bind E2F, then pRb mediated repression is relieved (Weintraub *et al.*, 1992). Further, it has been shown *in vivo* by DNA-footprinting studies that contrary to expectation the E2F site in the promoter of the G1/S regulated oncoprotein b-*myb* is occupied in G1, but becomes vacant in S phase when transcription is turned on (Zwicker *et al.*, 1996). Perhaps the most convincing evidence in support of the view that E2F and pRb co-operate to inhibit cell growth is the finding that 'knockout' mice nullizygous for *E2F-1* develop a wide range of tumours between 8-18 months, consistent with *E2F* being a growth inhibitory gene and TSG (Field *et al.*, 1996; Yamasaki *et al.*, 1996).

RB-knockout mice (Lee *et al.*, 1992; Jacks *et al.*, 1992; Clark *et al.*, 1992), on the other hand, revealed a previously unknown cellular function for pRb that is seemingly at odds with its role as a tumour suppressor. $Rb^{-/-}$ mice die at the 15th day of gestation largely because of defects in hepatic erythropoiesis. This supports the growth suppression function of pRb, but in addition widespread apoptosis is observed in certain neuronal layers in the brain and in the developing lens, suggesting that pRb functions to protect cells against apoptosis (Morgenbesser, 1994). pRb has been identified as a substrate for caspases (cysteinyl-aspartate specific proteases) during apoptosis, and is degraded following TNF- or CD95(Fas)-induced apoptosis (Janicke *et al.*, 1996; Chen *et al.*, 1997). p53 inactivation rescues $Rb^{-/-}$ cells from apoptosis (Weinberg, 1995), perhaps explaining why mice defective for both TSGs are especially cancer prone (Williams *et al.*, 1994). This more recently described effect of pRb may again involve its interaction with E2F, since overproduction of E2F-1 is capable of inducing p53-dependent apoptosis (Weinberg, 1995), and an E2F-1 mutant defective in binding pRb but active in transactivation is a more potent activator of apoptosis (Shan *et al.*, 1996).

1.2.2 *p53* and Li-Fraumeni syndrome

The *p53* gene (formally designated *TP53*) was initially believed to be an oncogene since it could apparently co-operate in transforming cells when co-transfected with *ras* (Eliyahu *et al.*, 1984). However, this transforming ability of *p53* results from mutations (Hinds *et al.*, 1989) which allow the protein product to behave in a dominant negative fashion by forming inactive oligomers which include both mutant and wild-type proteins (Herskowitz, 1987; Green, 1989). Transfection of the wild-type gene suppresses the malignant phenotype (Finlay *et al.*, 1989), so the status of the *p53* gene had to change from oncogene to TSG. Additionally, 17p13 the chromosomal region which harbours the *p53* gene (Isobe *et al.*, 1986) is frequently deleted in a wide range of human tumours, while remaining (non-deleted) *p53* alleles often contain inactivating point mutations (Baker *et al.*, 1989; Nigro *et al.*, 1989), a hallmark of TSGs. Mutations in *p53* remain the most common mutation across human tumour types (Hollstein *et al.*, 1996). The role of *p53* as a TSG has been confirmed by other *in vivo* findings. Germ-line mutations in the gene confer an autosomal dominant predisposition to cancer of the breast, brain, bone, soft tissues, haemopoietic system, and adrenal cortex, termed the Li-Fraumeni syndrome (Malkin *et al.*, 1990; Srivastava *et al.*, 1990). Importantly, loss of

the remaining wild-type *p53* allele could be demonstrated by LOH in DNA samples from tumours arising in carriers. In addition, mice nullizygous for *p53* develop a diverse array of tumours following birth (Donehower *et al.*, 1992).

p53 has been implicated in a diverse range of cellular functions and responses, including both activation and repression of transcription, regulation of protein translation, inhibition of DNA and RNA helicase activity, DNA repair, cell cycle arrest, and apoptosis (reviewed in Gottlieb and Oren, 1996, and Ko and Prives, 1996). It is perhaps surprising to note then that development is apparently normal in $p53^{-/-}$ mice; as with mice nullizygous for *RB1*, the vast majority of cell divisions are completed correctly during development, implying that neither p53 nor pRb are essential requirements for cell cycle progression. This is at first difficult to reconcile with the obvious importance of these two genes in suppressing unrestrained cellular proliferation. The likely resolution of this paradox rests on the observation that both molecules are inactivated during normal cell growth, pRb through phosphorylation and p53 through carboxy-terminal mediated repression (Bayle *et al.*, 1995), and possibly also cytoplasmic sequestration (Moll *et al.*, 1996), and are probably only pressed into service under exceptional circumstances. Such circumstances have been identified for p53 which can mediate growth arrest or apoptosis in response to DNA damage (Kastan *et al.*, 1991; Lowe *et al.*, 1993), ribonucleotide depletion (Linke *et al.*, 1996), hypoxia (Graeber *et al.*, 1996), and viral oncoproteins (Debbas and White, 1993; Lowe and Ruley, 1993), collectively termed 'stress'. These factors stimulate p53 activity while modulating p53 protein levels at both the transcriptional (Sun *et al.*, 1995), translational (Fu *et al.*, 1996; Mosner *et al.*, 1995), and post-translational levels (Kastan *et al.*, 1991; Tishler *et al.*, 1993). Most recently p300/CBP-mediated acetylation of the carboxy-terminus of p53 has been shown to activate its latent sequence-specific DNA-binding activity, both *in vitro* and *in vivo* (Gu and Roeder, 1997). The choice of the appropriate cellular response—apoptosis or growth arrest—is influenced by a number of variables including cell type, availability of survival or growth factors, the presence of other oncogenes, and the extent of DNA damage.

A number of biochemical properties have been attributed to p53 that allow it to mediate its diverse cellular functions. Thus p53 can itself bind to the ends of single-stranded DNA (Bakalkin *et al.*, 1994) and to sites with sequence mismatches (Lee *et al.*, 1995). This in turn activates the sequence-specific DNA binding function of p53 and its transactivating ability (Jayaraman and Prives, 1995). By binding to specific sites within

their promoters, p53 induces the transcription of various genes including *p21*$^{WAF1/CIP1}$, *GADD45*, *mdm2*, *bax*, *thrombospondin 1*, *IGF-BP3*, and *E1-2A*. The products of these genes actuate individual aspects of p53-dependent cellular responses. For instance, p21$^{WAF1/CIP1}$ promotes cell cycle arrest in G1 by inhibiting cyclin/cdk complexes, while bax and IGF-BP3 stimulate apoptosis. Mutations in p53 can selectively alter its ability to transactivate various of these target genes, implying that the particular gene induced by p53 is determined by its interaction with other transcription factors (Ludwig *et al.*, 1996; Friedlander *et al.*, 1996).

p53 has been dubbed the 'Guardian of the genome' because of its central role in maintaining genomic integrity. Cellular stress factors cause genetic lesions which in the absence of p53-mediated growth arrest and DNA repair or p53-mediated apoptosis more readily lead to neoplastic transformation (Kemp *et al.*, 1994). The ability of p53 to bind damaged DNA might allow it to monitor and signal the presence of such damage directly. Through its interactions with other proteins, p53 could then determine the way in which the damage is to be repaired, and direct the cell to growth-arrest or self-destruct. p53 has been demonstrated to interact with and inhibit RAD51 protein (Sturzbecher *et al.*, 1996) which is required for the homologous recombination repair pathway, while loss of p53 reduces the rate and efficiency of nucleotide excision repair (NER) (Ford and Hanawalt, 1995; Wang *et al.*, 1995). Moreover, p53 possesses an intrinsic 3′→5′ exonuclease activity that is possibly important in DNA recombination, replication, and repair (Mummenbrauer *et al.*, 1996). It can also be inferred that p53 provides an interface between cell cycle checkpoint molecules and down-stream effector mechanisms. For instance, it has been noted that chromosomal defects such as gene or centrosome amplification, which arise through repeated rounds of unimpeded endoreduplication, occur at much higher rates in the absence of p53 (Yin *et al.*, 1992; Livingstone *et al.*, 1992; Cross *et al.*, 1995; Fukusawa *et al.*, 1996), suggesting that an important checkpoint which licenses DNA replication to once per cell division cycle has been abrogated.

1.2.3 Wilms' tumour and *WT1*

Wilms' tumour (WT) or nephroblastoma is the commonest solid paediatric tumour, affecting 1 in 10,000 children usually during the first five years of life. The disease occurs in both sporadic and inherited forms. Inherited cases occur either in

isolation or as one manifestation of three distinct growth abnormality syndromes: WAGR, Denys-Drash syndrome (DDS), or Beckwith-Wiedemann syndrome (BWS). The *WT1* gene on 11p13 is implicated in both WAGR and DDS (Bonetta *et al.*, 1990; Call *et al.*, 1990; Gessler *et al.*, 1990). Whereas WAGR is associated with constitutional deletion of the 11p13 region that includes *WT1*, DDS is associated with point mutations of *WT1*. A second WT locus on chromosome 11p15.5 has been identified based upon deletions in WTs that involve this region but not 11p13, as well as through linkage with BWS (Koufos *et al.*, 1989; Reeve *et al.*, 1989). In addition, allelic loss studies suggest the presence of a third WT suppressor gene locus on 16q (Maw *et al.*, 1992), while more recently, linkage studies have implicated the involvement of a gene on 17q12-21 in simple forms of familial WT (Rahman *et al.*, 1996), but this may not be the site of a TSG (Rahman *et al.*, 1997).

WT1 encodes a nuclear protein with four zinc fingers, most closely homologous to those in the early growth response (EGR) and KROX families of transcription factors. Indeed, WT1 binds to the same DNA sequence as EGR1 (Rauscher *et al.*, 1990) repressing gene expression (Madden *et al.*, 1991), and therefore appears itself to be a transcription factor. Moreover, WT1 binds p53 modulating some of its functions. Thus the interaction, which stabilises p53, inhibits its apoptotic function but allows growth arrest (Maheswaran *et al.*, 1995). Alternatively spliced variants of WT1 appear to co-localise with the spliceosomal complex, suggesting that besides its potential role as a transcription factor, WT1 may also be involved with splicing (Larsson *et al.*, 1995).

WT1 is a classical TSG in that both copies are functionally inactivated in malignancy. However, it is unusual in that heterozygous constitutional mutations are associated with developmental abnormalities, and as with p53 a dysfunctional WT1 protein, as is sometimes found in DDS patients, can produce more severe symptoms than complete loss of expression of the gene product, as in WAGR patients. This suggests that mutant WT1 protein in DDS patients may act in a dominant negative fashion sequestering other cellular factors, possibly even p53, into inactive complexes, or may gain functions, perhaps leading to inappropriate gene expression or splicing.

Two candidate genes from 11p15 have been suggested to give rise to WT in the context of BWS: that encoding insulin-like growth factor 2 (*IGFII*), and the other encoding the cyclin/cdk inhibitor $p57^{KIP2}$. Ordinarily, both genes undergo genomic imprinting, so that only the paternal allele of *IGFII* is expressed (DeChiara *et al.*, 1991), while only the maternal allele of the $p57^{KIP2}$ gene is expressed (Hatada *et al.*, 1995;

Matsuoka *et al.*, 1996). Paternal trisomy and paternal duplication of the 11p15 region observed in BWS patients (Ping *et al.*, 1989) and the relaxation of genomic imprinting of the *IGFII* gene in BWS (Weksberg *et al.*, 1993) and in WTs that do not show LOH at 11p15 (Ogawa *et al.*, 1993) support the role for a paternally-derived allele in the aetiology of BWS and associated tumours, with *IGFII* being the strongest candidate. On the other hand, paternal uniparental disomy of chromosome 11 (Henry *et al.*, 1991) and translocations involving maternal 11p15 also found in certain BWS patients and the specific loss of 11p15 maternal alleles observed in WTs and other childhood tumours associated with BWS (Hastie, 1994) all suggest that loss of expression of a maternally-derived allele is entailed, consistent with a role for $p57^{KIP2}$ in this disease. This link is further strengthened by the finding of a maternally inherited mutation in $p57^{KIP2}$ resulting in a null allele in an individual with BWS (Hatada and Mukai, 1996), and by a recent study showing that mice nullizygous for the $p57^{KIP2}$ gene or mice who have inherited a null allele from their mother have growth abnormalities strongly reminiscent of those found in BWS patients (Zhang *et al.*, 1997). However, due to the post-natal lethality resulting from this mutation in mice, it was not possible to assess the cancer predisposition in these animals. Mechanistically, IGF-2 and $p57^{KIP2}$ could be two halves of the same growth balancing act, increased expression of the former and decreased expression of the latter having the same effect, and therefore both genes could contribute to WT development.

1.2.4 *VHL*

Germ-line mutations in *VHL* give rise to von Hippel-Lindau disease, a multiple tumour predisposition syndrome (Latif *et al.*, 1993). The protein encoded by this gene interacts with elongin proteins B and C (Duan *et al.*, 1995), which are part of the transcription elongation factor elongin (SIII). VHL may inhibit the expression of genes such as *MYC* family members or c-*FOS* that are subject to transcription attenuation control (Krumm and Groudine, 1996), by sequestering elongins B and C. Alternatively, a complex of VHL and elongins B and C may itself function as a transcription factor, whose activity or specificity is affected by mutations in the VHL protein.

The development of well-vascularized tumours, such as renal cell carcinoma and pheochromocytoma, is a characteristic of von Hippel-Lindau disease. Recently it was shown that ectopic expression of wild-type *VHL* in human renal carcinoma cells lines

lacking functional VHL suppressed deregulated expression of vascular endothelial growth factor VEGF mRNA (Siemeister *et al.*, 1996). VEGF is an endothelial specific mitogen that induces angiogenesis and vascular permeability, and its role in tumour angiogenesis appears to be pivotal. Ectopic expression of VHL has no effect on the growth characteristics of these cancer cells, but it does dramatically reduce their tumourigenicity in nude mice (Iliopoulos *et al.*, 1995). Taken together these findings suggest that VHL is of central importance in repressing the expression of target genes required for neovascularization; the deregulated expression of such genes and consequent angiogenesis which results from mutational inactivation of VHL promotes tumour development.

1.2.5 *NF1, NF2, and TSC2*

Germ-line mutations in *NF1* give rise to benign and malignant tumours of neural crest derived cells, and in *NF2* to schwanommas (Cawthon *et al.*, 1990; Viskochil *et al.*, 1990; Wallace *et al.*, 1990; Rouleau *et al.*, 1993; Troffater *et al.*, 1993). Neurofibromin, the *NF1* gene product, has a region of considerable homology with the catalytic domain of $p120^{GAP}$, which when expressed in *Saccharomyces cerevisiae* acts as a $p21^{ras}$-specific GTPase activating protein (GAP) (Xu *et al.*, 1990; Ballester *et al.*, 1990). GAPs play two roles in the cell: they negatively regulate the levels of active RAS-GTP and thus inhibit RAS dependent signalling; they also perform other discrete effector functions, although no such functions have yet been ascribed to neurofibromin. Tuberin, the product of *TSC2*, a TSG inactivated in tuberous sclerosis, also appears to be a GAP (The European chromosome 16 tuberous sclerosis consortium, 1993).

The protein encoded by *NF2*, merlin or schwannomin, is a member of the band 4.1 superfamily, and shares considerable homology in its amino-terminal half with ERM (ezrin/radixin/moesin) proteins. These proteins are actin filament binding proteins associated with the plasma membrane through their interaction with the cytoplasmic portions of transmembrane adhesion molecules like CD44 and membrane phosphatidylinositides. Thus ERM proteins cross-link the actin cytoskeleton with the plasma membrane. Schwannomin also localises at the cell membrane, preferentially in ruffles (Gonzales-Agosti *et al.*, 1996), but whether it binds actin is yet to be demonstrated and the proteins with which it specifically associates (Takeshima *et al.*, 1994) remain uncharacterised. Since ablation of ezrin has been shown to inhibit cell

movement (Lamb *et al.*, 1997), it is possible that the *NF2* product too may play a role in cell motility; certainly reduction in the level of expression of *NF2* by antisense oligonucleotides has been shown to impair cell adhesion (Huynh and Pulst, 1996).

1.2.6 *APC*, *DCC*, *DPC4*, and colon cancer

The cloning of *APC* was preceded by findings of LOH at 5q21, where the gene resides, in the majority of colon carcinomas and even adenomas (Vogelstein *et al.*, 1989), suggesting that mutations in the *APC* gene are acquired early on in tumour progression perhaps even being required for tumour initiation. This appears to be the case in both sporadic forms of the disease and in familial adenomatous polyposis (FAP), an inherited predisposition to colon carcinoma characterised by the occurrence from a young age of numerous benign adenomatous polyps of the colon, a small fraction of which progress to carcinoma. Germ-line mutations in *APC* were identified in individuals with this condition, and since the gene was constitutionally deleted in a fraction of cases and LOH could be observed in tumours, it was established as a TSG. Another gene, *MCC* for mutated in colon cancer, which maps 150 Kbp proximal to *APC* had previously been identified as being mutated in sporadic colon carcinoma (Kinzler *et al.*, 1991), however germ-line mutations have not been identified for this gene suggesting it is not responsible for FAP.

APC encodes a 300 kDa cytoplasmic protein which has homology to myosins and keratins in regions that are predicted to form coiled-coil structures. The APC protein can form homo-oligomers via interactions of these amino terminal sequences. In addition, APC interacts through other peptide motifs with at least six other proteins: β-catenin, γ-catenin (plakoglobin), tubulin, EB1, hDLG a homologue of the *Drosophila* Discs Large tumour suppressor protein, and glycogen synthase kinase-3β (GSK-3β), a mammalian homologue of the Drosophila Zeste-White-3 kinase or Shaggy (Polakis, 1997). β-Catenin originally discovered as a cadherin binding protein has recently been shown to function as a transcriptional activator when complexed with members of the Tcf/Lef family of DNA binding proteins (Molenaar *et al.*, 1996; Behrens *et al.*, 1996); it is also known to play an essential role in Wingless-Wnt signalling (Gumbiner, 1995). In the absence of Wnt signals, APC simultaneously interacts with GSK-3β and with β-Catenin. Phosphorylation of APC by GSK-3β appears to increase its interaction with β-catenin, which in turn destabilises β-catenin perhaps through phosphorylation by GSK-

3β. Wnt signalling appears to antagonise GSK-3β activity, resulting in β-catenin stabilisation. Stabilised β-catenin exists primarily as cytoplasmic monomers that are free to form complexes with Tcf/Lef factors. Significantly, transcriptional activation by complexes of β-catenin and Tcf/Lef is constitutively present in cancer cell lines where APC is defective (Korinek *et al.*, 1997). Mutations in β-catenin, which have also been observed in cancer cells, that may remove its ability to be phosphorylated by GSK-3β and simultaneously stabilise it, also achieve the same effect as inactivating APC (Morin *et al.*, 1997; Rubinfeld *et al.*, 1997). Genes whose expression are regulated by β-catenin/Tcf complexes have yet to be identified in mammals, but include *engrailed* and *Ubx* in *Drosophila* and *siamois* in *Xenopus*, which are clearly involved in the development of these organisms. It is possible, therefore, that APC is involved in determining cell fate within the colonic epithelium, but whether this is correct awaits the identification of the target genes whose expression it helps to regulate. APC may also exert an influence on malignancy through its role in adherens junctions, where again it is associated with β- and also γ-catenin. The functional significance of its interactions with the other proteins mentioned above is less well understood and requires further analysis.

The genetic events that accompany the progression of colon cancer have been established (Fearon *et al.*, 1990), making it perhaps the best characterised model of tumour progression known. Inactivation of *APC* is the earliest detectable event; later hits include activation of *KRAS*, inactivation of a TSG on 18q, and loss of p53 (Figure 1.4). *DCC* is a candidate for the TSG on 18q that is inactivated in late stage colon tumours (Fearon *et al.*, 1990). This gene encodes a protein which has homology with other members of the Ig superfamily of adhesion proteins. The definitive evidence that *DCC* is in fact a TSG has not been forthcoming. As its name implies (DCC stands for deleted in colon cancer) the gene has been shown to be completely or partially deleted in a fraction of colon cancers, and its expression is either lost or reduced in 70% of colon tumours, but due to the large size of *DCC* it has been difficult to identify somatic point mutations in tumours that inactivate its encoded protein. Further, there is no evidence for germ-line mutations in *DCC* that predispose to colon cancer, and perhaps most significantly mice in which this gene has been inactivated through homologous recombination fail to support a tumour suppressor function for Dcc (Fazeli *et al.*, 1997). What is known about the function of DCC has largely been inferred from studying mutations in homologues of the gene in *Drosophila* (*Frazzled*) and *C. elegans* (*UNC-40*). DCC like proteins (DCCPs) are most strongly expressed on the surface of

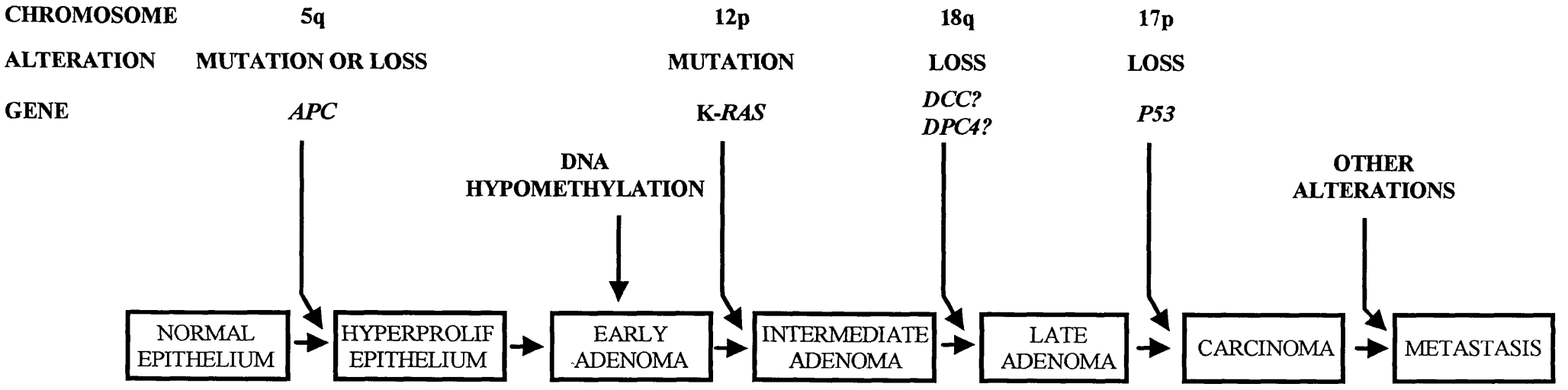


Figure 1.4. A genetic model for multistep colorectal tumourigenesis. Tumourigenesis proceeds through a series of genetic alterations involving oncogenes (*RAS*) and TSGs (particularly those on chromosomes 5q, 17p, and 18q). In patients with FAP, a mutation in APC is inherited. This alteration may be responsible for the hyperproliferative epithelium present in these patients. In tumours arising in patients without polyposis, the same gene may also be lost and/or mutated at a relatively early stage of tumourigenesis. Hypomethylation is present in very small adenomas in patients with or without polyposis, and this alteration may lead to aneuploidy, resulting in the loss of suppressor gene alleles. ras gene mutations (usually K-*RAS*) appear to occur in a single cell of a pre-existing small adenoma and through clonal expansion produces a larger and more dysplastic tumour. The chromosomes most frequently deleted include 5q, 17p, and 18q; the putative target of the loss event (i.e the TSG) on each chromosome is indicated as well as the relative timing of the chromosome loss event. However, the order of these changes is not invariant and accumulation of these changes rather than their order with respect to one another is more important.

commissural axons in the central nervous system where they bind netrin (Kolodziej *et al.*, 1996; Chan *et al.*, 1996; Keinu-Masu *et al.*, 1997), a laminin like chemoattractant secreted by midline cells in *C.elegans*, *Drosophila* and vertebrates. Moreover, loss of either DCCP or netrin expression disrupts axon guidance towards the midline in *C.elegans* and *Drosophila*. Both netrin and DCCPs are expressed in the epithelium of the gut in many organisms. In this context the molecules may maintain tissue integrity; however, no gut abnormalities were reported for either *Frazzled* or *UNC-40* mutants.

DPC4, which maps to chromosome 18q21.1 proximal to *DCC*, is also a candidate for the TSG inactivated in advanced colon cancers. Evidence from recombinant mice which are compound heterozygotes for null mutants of *apc* and *dpc4* directly support a role for inactivation of *DPC4* in the malignant progression of colorectal tumours. Such mice show an increased frequency of conversion of benign intestinal polyps to adenocarcinoma compared to simple $apc^{+/-}$ mice (Takaku *et al.*, 1998). Although mice heterozygous for an inactivating mutation of *dpc4* alone did not show increased susceptibility to developing intestinal polyps or malignancies, germ-line loss of function mutations of *DPC4* in man are believed to be responsible for a subset of familial juvenile polyposis (Howe *et al.*, 1998). This autosomal dominant disease is characterised by a predisposition to hamartomatous polyps and gastrointestinal cancer.

DPC4 was first identified as a candidate TSG deleted in a large proportion of pancreatic carcinomas, and subsequently shown to possess frequent loss of function mutations in tumour DNA samples (Hahn *et al.*, 1996[a]). The gene encodes SMAD4 a member of a family of homologous proteins that include Mothers against decapentaplegic (Mad) in *Drosophila*, the products of the *Sma* genes in *C. elegans*, and their homologues in vertebrates (SMADs). These factors are believed to transduce signals from members of the transforming growth factor-β (TGF-β) superfamily of growth factors, that includes TGF-βI-III, activin, bone morphogenetic proteins (BMPs), and decapentaplegic, which regulate cell growth and differentiation, and tissue morphogenesis in many organisms (Kingsley, 1994). TGF-β-like molecules signal through heterodimeric receptor complexes of type I and type II serine/threonine kinase receptors. Heteromers of SMAD proteins which become activated upon receptor ligation directly translocate to the nucleus where they activate transcription. DPC4/SMAD4 is an essential component in the signal transduction pathway of all TGF-β-like peptides (Zhang *et al.*, 1997), including TGF-β1 which is a potent anti-proliferative agent for

many epithelial cells. Blockade of TGF-β1 induced growth arrest by mutations which inactivate *DPC4* is compatible with its role as a tumour suppressor.

1.2.7 Hereditary non-polyposis colon cancer and DNA mismatch repair

The observation that simple sequence repeats (microsatellites) are erroneously replicated in hereditary non-polyposis colon cancer (HNPCC) and also in a subset of sporadic tumours (Ionov *et al.*, 1993; Thibodeau *et al.*, 1993; Aaltonen *et al.*, 1993) first established a link between the mismatch repair (MMR) pathway and cancer, and provides the most direct support for the 'Mutator Hypothesis' of Carcinogenesis, which posits that defects in maintaining genomic integrity result in the multiple mutations required for malignancy. Linkage in HNPCC families established that one locus responsible for the condition maps to chromosome 2p16. A positional candidate approach unmasked the perpetrator as *hMSH2* (Fishel *et al.*, 1993), a human homologue of the *E. coli mutS* gene (part of the DNA adenine methylase instructed MutHLS pathway of DNA repair) responsible for the initial recognition and binding of mismatched nucleotides in DNA that result from polymerase misincorporation errors.

E.coli which are mutant for *mutS* have an approximately 1000-fold enhanced mutation rate (Siegel and Bryson, 1967). In addition, they are resistant to the cytotoxic effects of DNA alkylating agents, and are deficient in transcription-coupled repair (Karran and Marinus, 1982; Mellon and Champe, 1996). These features are shared by eukaryote cells with defective MMR components (Mellon *et al.*, 1996). The *hMSH3* and *-6* genes encode two further human mutS homologues, while *hMLH1*, *hPMS1*, and *hPMS2* encode mutL homologues. HNPCC and microsatellite instability observed in familial and sporadic tumours have also been attributed to mutations in these genes (Liu *et al.*, 1995; Liu *et al.*, 1996). Mutations in *hMSH2* and *hMLH1* account for over 90% of HNPCC families (Moslein *et al.*, 1996). Human cell lines which contain mutations in *hMSH2* and *hMLH1* are resistant to the cytotoxic effect of the DNA alkylating agent *cis*-diaminedichloroplatinum (II) (cisplatin), a commonly used anti-cancer therapeutic. Moreover, cisplatin treatment of an ovarian cancer cell line resulted in selection of resistant variants (Aebi *et al.*, 1996) which had mutated an MMR gene, demonstrating that the acquisition of defects in MMR promote clonal evolution of cancer cells and that such abrogation is of great clinical consequence.

1.2.8 Familial melanoma and *CDKN2A/MTS1/MLM*

Two genes have been identified which when inherited as mutated copies confer an autosomal dominant susceptibility to developing malignant melanoma: the *CDKN2A* gene (also known as *MTS1* or *MLM*) on 9p21, which encodes the $p16^{INK4A}$ inhibitor of CDK4 and CDK6 (Hussussian *et al.*, 1994; Kamb *et al.*, 1994; Sherr and Roberts, 1995), and more rarely the *CDK4* gene itself on chromosome 12q13 (Zuo *et al.*, 1996). Mutations in *CDKN2A* both in familial melanoma and other sporadic tumours result in loss of function or the complete absence of the gene product and therefore place *CDKN2A* within the TSG category, whereas the one susceptibility mutation so far identified in *CDK4* was a missense mutation which negated the protein product's ability to interact with $p16^{INK4A}$. As mentioned above, complexes of cyclins and CDKs facilitate cell cycle progression; $p16^{INK4A}$ inhibits complexes which phosphorylate pRb and promote S phase entry. It now appears that the restriction point in G1 phase of the cell cycle can be abrogated by mutational modification of any one of the factors involved in its maintenance; correspondingly, mutations in them are mutually exclusive. Thus amplification or activation of cyclins or CDKs, or loss of function of pRB, including inactivation by viral oncoproteins, or $p16^{INK4A}$ achieve the same end result, and modification in at least one of these components occurs in the majority of cancers (Sherr, 1996).

The first mutations to be characterised in *CDKN2A* were deletions and point mutations, largely identified from the analysis of tumour cell line material. Controversy raged over whether this gene was also inactivated in tumours *in vivo* or merely represented an artefact of selection during *in vitro* culture. Subsequently, loss of expression has been established to occur frequently in late stage tumours and most commonly as a result of gene silencing by promoter methylation (Merlo *et al.*, 1995; Herman *et al.*, 1995; Reed *et al.*, 1996).

1.2.9 *PATCHED* and basal cell nevus syndrome

Basal cell nevus syndrome (BCNS), also known as Gorlin's syndrome, is an autosomal dominant disease characterised by developmental defects and a predisposition to basal cell carcinomas (BCCs), medulloblastomas, and meningiomas. The defective gene in BCNS which maps to chromosome 9q is the human homologue of

patched (*ptc*) (Johnson *et al.*, 1996; Hahn *et al.*, 1996[b]), first identified in *Drosophila* as a regulator of embryonic pattern formation. *PTC* is also inactivated in numerous sporadic BCCs (Gailani *et al.*, 1996).

ptc encodes a transmembrane receptor protein that represses transcription of genes encoding TGF-β and Wnt class signalling proteins and Ptc itself (Ingham, 1995; Dean, 1996; Perrimon, 1996; Hammerschmidt *et al.*, 1997). Ptc is a receptor for hedgehog (Hh), or sonic hedgehog (Shh) in vertebrates (Chen *et al.*, 1996; Stone *et al.*, 1996); receptor ligation inactivates Ptc relieving transcriptional repression. Rarely, *SHH* itself is somatically mutated in tumour types associated with BCNS (Oro *et al.*, 1997). Normally, Shh/Ptc signalling plays a role in determining cell fate in embryonal germ layers. Ptc and Shh expression in the mouse embryo is related to hair follicle formation (Bitgood and McMahon, 1995; Iseki *et al.*, 1996); BCC are thought to arise from basal keratinocytes in hair follicles. *PTC* joins *APC* and *WT1* in a class of TSGs which are entailed in controlling early epithelial proliferation and differentiation. In all three cases, haploinsufficiency results in developmental abnormalities, while loss of the remaining wild-type copy contributes to malignancy. It is plausible that escaping a commitment to terminally differentiate through inactivation of such genes allows an initiated cell to avoid the maturation arrest or apoptosis that would otherwise ensue, and hence constitutes transformation.

1.2.10 Breast cancer susceptibility and the *BRCA* genes

The cloning of *BRCA1* followed shortly by *BRCA2* (Miki *et al.*, 1994; Wooster *et al.*, 1995; Tavtigian *et al.*, 1996) created almost equal excitement among both the public and the research community. This excitement was based largely upon the not unprecedented expectation that these genes would be mutated in sporadic breast and ovarian cancers, as well as conferring an inherited susceptibility to these cancers, and hence increase our scientific understanding of two major fatal diseases of women. Germ-line mutations in these two genes account about equally for 90% of high penetrance susceptibility to breast cancer, *BRCA1* also confers a sizeable relative risk of developing ovarian cancer, while *BRCA2* accounts for a proportion of male familial breast cancer (Couch *et al.*, 1996; Thorlacius *et al.*, 1996). Germ-line mutations in the oestrogen and androgen receptor genes, in the ataxia telangiectasia gene, in *TP53*, and in the recently identified Cowden disease gene (*PTEN/MMAC1*), partially account for the

remainder of high penetrance susceptibility to breast cancer (Zuppan *et al.*, 1991; Wooster *et al.*, 1992; Liaw *et al.*, 1997). In addition, linkage and LOH studies support the existence of a third breast cancer susceptibility locus, *BRCA3*, on the short arm of chromosome 8 (Kerangueven, 1995[a]; Seitz *et al.*, 1997). Certainly, the identification of the *BRCA* genes will allow individuals within affected kindreds to know whether they have inherited mutant copies, but how exactly this will contribute to prevention of disease has not yet been thoroughly evaluated. The only measures as yet on offer are prophylactic bilateral radical mastectomy and oophorectomy, and even then protection from the development of breast cancer is not necessarily guaranteed.

The expectation generated by cloning the *BRCA* genes has also fallen short against the other mark. Neither gene shows substantial involvement in sporadic cases of breast or ovarian cancer (Futreal *et al.*, 1994; Merajver *et al.*, 1995;Lancaster *et al.*, 1996; Teng *et al.*, 1996), despite frequent findings of LOH near to where these two genes map. (The reasons why this might be so are explored below.) When the *BRCA* genes were identified initial homology searches against other known proteins revealed little to throw light on their possible function. BRCA1 possesses a RING-finger motif near its amino terminus which might mediate protein–protein or protein–DNA interactions. Indeed, two proteins BARD1 and BAP1 have been identified from two hybrid screens which interact with this RING-finger domain (Wu *et al.*, 1996; Jensen *et al.*, 1998). Another region, known as the BRCA1 carboxy-terminal (BRCT) domain, has homology with domains in a number of other molecules, including yeast RAD9, BARD1, DNA ligases III and IV, XRCC1 and a cloned p53 binding protein (Koonin *et al.*, 1996; Callebaut and Mornon, 1997). Its function is unknown, but at least some of the proteins bearing this motif are involved in repairing DNA double-strand breaks and in homologous recombination; the motif may allow these proteins to interact concertively. At one time, it was mooted that BRCA1 might be secreted (Jensen *et al.*, 1996) owing to its possessing a motif found in granins, a family of neuroendocrine secretory peptides, that has subsequently been identified in BRCA2; this idea appears to have since fallen from favour. BRCA1 and BRCA2 are otherwise unrelated at the protein level.

Both BRCA1 and -2 have been shown to interact stably with hRAD51 protein (Scully *et al.*, 1997[a]; Sharan *et al.*, 1997), one of a growing family of eukaryotic homologues of the *E. Coli RecA* gene which are known to be involved in recombination-linked repair of DNA damage, suggesting that both genes may lie on the

same DNA-repair pathway. Supporting such a role for BRCA proteins in DNA repair is the finding that embryonic mouse cells nullizygous for *brca2* are hypersensitive to γ-irradiation (Sharan *et al.*, 1997), reminiscent of cells derived from *rad51*-knockout mice, and that the subnuclear location and cell-cycle dependent phosphorylation of BRCA1 is altered following treatment of cells *in vitro* with DNA damaging agents (Scully *et al.*, 1997[b]). Disruption of the BRCA/RAD51 pathway may result in genomic instability; certainly *rad51*-knockout mice show frequent abnormal mitoses with a markedly reduced chromosome number. In turn this Mutator phenotype could promote neoplastic transformation, explaining the role of *BRCA* mutations in cancer.

The similarities in function between BRCA1 and BRCA2 are further extended by the finding that when negatively charged carboxy-terminal residues from BRCA1 and amino-terminal residues from BRCA2 with homology to c-Jun are linked to the DNA-binding domain of the yeast GAL4 transcription factor, they confer potent transcriptional activation capacity (Chapman and Verma, 1996; Milner *et al.*, 1997). Thus both proteins either singly or in combination with other co-activators may regulate expression of a number of target genes important in suppressing the malignant phenotype. One such possible target is the cell-cycle inhibitor $p21^{WAF1/CIP1}$, which was recently shown to be induced independently of p53 by ectopic expression of BRCA1. This property of BRCA1 is abolished by mutations resulting in loss of the nuclear localisation signal, the C-terminal transactivation domain, or the RAD51-interacting domain (Somasundaram *et al.*, 1997). These findings were extended to show that BRCA1 fails to induce cell-cycle arrest in cells lacking wild-type $p21^{WAF1/CIP1}$.

1.2.11 Xeroderma pigmentosum, Bloom's syndrome, Werner's syndrome, and Ataxia telangiectasia

These four autosomal recessive multiple tumour type predisposition syndromes again result from germ-line loss of function mutations in genes which normally maintain genomic stability, giving rise to a Mutator phenotype. Individuals with xeroderma pigmentosum demonstrate heightened sensitivity to UV-irradiation which translates as an increased risk of developing skin cancer. The disease is characterised at the biochemical level by an inability to repair UV-induced cyclobutane pyrimidine dimers and pyrimidine-pyrimidone (6-4) photoproducts, implicating defects in the nucleotide excision repair (NER) pathway. Cells from XP individuals define seven genetic

complementation groups, which result from mutations in each of seven NER genes, *XPA-XPG* (Hoeijmakers, 1993). XPA and XPE are responsible for recognising DNA lesions, *XPB* and *XPD* encode DNA helicases with opposite polarities required for unwinding the DNA duplex at damaged sites, *XPF* and *XPG* encode endonucleases responsible for excising the lesions, and XPC is a single-stranded DNA binding protein. XPB and XPD are integral components of a six-protein complex TFIIH, which is an essential part of the basal transcription machinery (Schaeffer *et al.*, 1993; Feaver *et al.*, 1993); TFIIH is therefore implicated in transcription coupled repair. In addition, cells deficient in XPB and XPD are defective in p53-mediated apoptosis (Wang *et al.*, 1996), providing a direct link between the DNA repair pathway and apoptosis.

Two more DNA helicases encoded by the *BLM* and *WRN* genes are implicated in Bloom's syndrome (BS) and Werner's syndrome (WS) respectively (Ellis *et al.*, 1995; Yu *et al.*, 1996). Genomic instability is again apparent, manifested in BS as an increased frequency of chromosome breakage, and chromatid exchange both between homologous chromosomes and sister chromatids. Indeed, intragenic homologous recombination resulting in correction of the elevated levels of sister chromatid exchange within clones of BS cells allowed the gene responsible for BS to be localised precisely. In WS, chromosome instability is exhibited by variegated inversion, translocation, and deletion mosaicism among different clonal populations of WS cells. In both syndromes the rate of spontaneous mutations is increased despite DNA repair pathways appearing to function normally (Warren *et al.*, 1981; Fukuchi *et al.*, 1989). The physiological role of the BLM and WRN helicases is at present unknown; mutational analysis reveals that neither gene is essential. The increased rate of DNA recombination observed in BS and WS may reflect activation of recombination-mediated repair mechanisms by default pathways which are otherwise inhibited in the presence of the functional helicases.

Loss of the wild-type product of the *ATM* gene gives rise to the human disease ataxia telangiectasia (AT) (Savitsky *et al.*, 1995), characterised by progressive cerebellar degeneration, the appearance of damaged blood vessels in the skin and conjunctiva of the eye, gonadal atrophy, and a predisposition to malignancies. At the cellular level, AT individuals are extremely sensitive to ionizing radiation (Friedberg *et al.*, 1995), exposure to which results in a greatly increased number of chromosomal breaks observed against an already elevated background level of spontaneous breaks. AT cells also appear to be defective in a number of DNA-damage responsive cell cycle checkpoints (Friedberg *et al.*, 1995). These features of AT, which imply a role for ATM

in the repair and processing of DNA double-strand breaks, have been reproduced in knockout mouse models of the disease (Barlow *et al.*, 1996, Xu *et al.*, 1996[a and b]). Both human and mouse cells carrying *ATM* defects are defective in up-regulating p53 on induction of DNA damage, suggesting that p53 is downstream of ATM in a signalling pathway (Kastan *et al.*, 1992; Xu *et al.*, 1996[b]; Westphal *et al.*, 1997). Recombinant mouse models also provide evidence for ATM functions in meiosis and lymphocyte differentiation.

The protein encoded by *ATM* is a member of the PI3-kinase-related protein kinase superfamily, whose members are distinguished by shared homology in their carboxy-terminal protein kinase domains, and their sequence similarity to the p110 lipid kinase domain of PI3-kinase. In mammals, this family comprises the FKBP12 and rapamycin-binding protein kinase FRAP, DNA-dependent protein kinase DNA-PK, implicated in non-homologous end-joining of double-strand DNA breaks, ATM, and ATR. ATM is also related to the MEC1 and TEL1 proteins of *S. cerevisiae*, Rad3 of *S. pombe*, and MEI-41 of *D. melanogaster*, all of which are required for DNA-damage sensitive checkpoint controls and produce repair deficient phenotypes when mutated. TEL1 has also been implicated in the maintenance of telomere length in yeast (Greenwell *et al.*, 1995). Since telomeric attrition has been shown to underlie cellular ageing (more below), this suggests that ATM may be involved in replicative senescence and that loss of ATM could contribute to cellular immortalisation.

1.2.12. More tumour suppressors

Past successes in identifying TSGs have established a positive trend, and the rate of TSG gene discovery appears to be increasing exponentially with time. 1997 saw three more keenly awaited candidate TSGs ushered into the molecular oncology arena, namely *MEN1, PTEN/MMAC1* and *TSC1* (Chandrasekharappa *et al.*, 1997; Li *et al.*, 1997; Steck *et al.*, 1997; van Slegtenhorst, 1997). In keeping with most other TSGs, germ-line mutations in these three genes are associated with cancer predisposition syndromes: multiple endocrine neoplasia type I, BZS/CD/LDD, and tuberous sclerosis respectively; while *MEN1* and *PTEN/MMAC1* have also been implicated in sporadic tumours. Indeed, inactivation of *PTEN/MMAC1*, which maps to chromosome 10q23, appears to be an obligatory requirement for the progression of advanced stage glioblastoma (glioblastoma multiforme), and is a frequent finding in advanced prostatic

adenocarcinoma. Little is known about the functions of the products of these most recently identified TSGs. Homology searches against other known proteins revealed no significant similarity in the case of *MEN1* or *TSC1*. However, PTEN/MMAC1 possesses two regions of highly significant homology: the amino terminus is very similar to members of a family of dual-specificity phosphatases, including CDC14, PRL-1 and BVP, that remove phosphate groups from tyrosine residues as well as serine and threonine; a more extensive region of the protein resembles tensin, a protein found in focal adhesions which binds actin filaments. Myers *et al.* (1997) have demonstrated that PTEN/MMAC1 possesses dual-specific phosphatase catalytic activity as predicted, and that this activity is ablated in mutant proteins occurring in tumours. Clearly, it will be interesting to demonstrate whether or not PTEN/MMAC1 binds actin filaments and localises to focal adhesions. Since focal adhesions act as signalling complexes that are involved in cell cycle progression, adhesion, and cell motility, should PTEN/MMAC1 be located in these structures, it would be well placed to regulate one or more of these processes. Moreover, it is known that upon focal adhesion formation a number of substrates found there, including vinculin and paxillin, become phosphorylated; the $pp60^{src}$ family of non-receptor associated tyrosine kinases and the focal adhesion kinase (FAK) are believed to be responsible for this. PTEN/MMAC1 may contribute to the down-regulation of focal adhesion signalling through the dephosphorylation of its constituent components. The association of *PTEN/MMAC1* mutations and LOH of chromosome 10q23-24 polymorphic markers with predominantly advanced cancers (Ittmann, 1996; Cappellen *et al.*, 1997; Li *et al.*, 1997; Steck *et al.*, 1997) supports a role for PTEN/MMAC1 in invasion and metastasis.

Over fifty familial cancer syndromes have been described suggesting the existence of many more TSGs whose inactivation through germ-line mutations predispose to cancer. Known TSGs fulfil a great many cellular and biochemical functions and as for proto-oncogenes their identification promises to illuminate a much wider area of biology than cancer alone. Cytogenetic, allelic loss, and linkage studies point to the likely location of these other as yet unidentified TSGs. In this respect, a putative TSG, *NB1*, implicated in neuroblastoma has been mapped to chromosome 1p36 (Brodeur *et al.*, 1977; Weith *et al.*, 1989; Laureys *et al.*, 1990; Biegel *et al.*, 1993, White *et al.*, 1995), a region that is frequently lost in melanoma and colon cancer as well. *p73*, a recently cloned homologue of *p53*, maps to this region and is a strong candidate for *NB1* (Kaghad *et al.*, 1997). Although not yet found to be mutated in human cancer, one

allele of this gene is constitutively silenced in the germ-line by genomic imprinting, while loss of expression of the other allele through LOH is a frequent finding in neuroblastoma cell lines, but also occurs in other cancer cell lines as well (Kaghad *et al.*, 1997). Significantly, p73 shares the ability with p53 to induce apoptosis (Jost *et al.*, 1997) and to suppress the transformed phenotype when ectopically expressed, which is concomitant with the induction of $p21^{WAF1/CIP1}$ (Kaghad *et al.*, 1997); however, unlike p53, p73 protein does not appear to be produced in response to DNA damage (Kaghad *et al.*, 1997). Together these observations strongly support a role for *p73* as a TSG.

Loss of genetic material and rearrangements on the short arm of chromosome 3 are commonly observed in a broad range of human tumours, but especially in lung cancer and renal cell carcinoma (Druck *et al.*, 1995; Buchhagen, 1996; Hughson *et al.*, 1996; Todd *et al.*, 1997). The 3p14.2 cytogenetic band is most often involved, and is the site of a constitutional translocation segregating with hereditary renal cell carcinoma (Wang and Perkins, 1984) and of an aphidicolin inducible chromosome fragile site, *FRA3B* (Glover *et al.*, 1988). The *FHIT* gene which maps to *FRA3B* and is disrupted by this translocation has been proposed as a TSG candidate on the basis of deletions in the gene observed in a number of cancer cell lines, and also due to the finding of aberrant transcripts in these cell lines and uncultured tumours (Ohta *et al.*, 1996) and in other tumour samples (Luan *et al.*, 1997; Zou *et al.*, 1997). However, the accompanying presence of wild-type transcripts in many of the same tumour samples, as well as the observation of aberrant splicing in benign tissue (Luan *et al.*, 1997; Panagopoulos *et al.*, 1997) has called in to question the candidacy of the *FHIT* gene as a tumour suppressor, which is further mired by the apparent absence of loss of function point mutations. Finally, two more loci have been implicated in prostate cancer: one which maps to chromosome 8p22 is associated with the sporadic form of the disease (Bova *et al.*, 1993, Suzuki *et al.*, 1995; Kagan *et al.*, 1995; Macoska *et al.*, 1995; Vocke *et al.*, 1996), while the other, which was shown by linkage to map to chromosome 1q24-25, is implicated in familial prostate cancer (Smith *et al.*, 1996).

1.3 'Gatekeepers' *vs.* 'caretakers'

A number of interesting issues can be raised about the role of TSGs in cancer, particularly inherited forms of the disease. One perplexing problem is why genes like *RB1*, *BRCA1*, *BRCA2*, and *APC*, which are widely expressed, predispose most strongly

to only a limited range of tumour types. Why for instance is small-cell carcinoma of the lung (SCLC), a cancer type in which both copies of *RB1* are frequently found to be mutated, rarely observed in survivors of familial retinoblastoma. A small target-cell pool or a low tissue-specific mutation rate cannot easily be invoked as an explanation since sporadic SCCL is found much more commonly than sporadic retinoblastoma.

One possible solution to this conundrum is supplied by the multiple hit model of carcinogenesis. Retinoblastoma may require many fewer mutational events to arise than for instance SCCL, and would therefore develop after a much shorter latency period. This explanation is not entirely satisfactory though, since the majority of common tumours have fairly comparable associations between incidence and age suggesting that a similar number of mutations need to accumulate in each type, yet *APC* predisposes strongly to colon carcinoma, but not to lung or breast carcinoma.

Redundancy in the number of back up pathways is an alternative explanation: *APC* may perform a unique function in colon cells that is essential for tumour suppression, whereas a number of stand-ins could be waiting off-stage in other cell types. Redundancy certainly exists among cell cycle regulatory molecules; thus, there are two families of cyclin-CDK inhibitors, the INK4 family comprising $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$ and $p19^{INK4D}$, and the CIP/KIP family consisting of $p21^{WAF1/CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$. There is further redundancy among the Rb-like pocket proteins of which pRb is the prototype, while p107 and p130 were discovered subsequently. We would anticipate that it is the non-degenerate growth-regulatory pathways that will be modified in cancer cells, and these may differ from one cell type to another. However, against this idea is the absence or very low frequency of mutations in tumours in any of these redundant molecules mentioned other than *CDKN2A* and *RB1*. Likewise, with the exception of $p27^{KIP1}$ (Fero *et al.*, 1996; Kiyokawa *et al.*, 1996; Nakayama *et al.*, 1996), mice in which these genes have been inactivated by homologous recombination do not show an inherited predisposition to developing cancer (Deng *et al.*, 1995; Lee *et al.*, 1996; Crobinik *et al.*, 1996), whereas $p16INK4A^{-/-}$ and $Rb^{+/-}$ mice do (Serrano *et al.*, 1996; Lee *et al.*, 1992; Jacks *et al.*, 1992; Clark *et al.*, 1992).

Another unanswered intrigue concerns the breast cancer susceptibility genes. Despite germ-line mutations in *BRCA1* and *BRCA2* conferring an increased life-time risk of developing breast and ovarian cancers, as mentioned somatic mutations in these genes are rarely if ever observed in sporadic cases of these cancers. A TSG need not necessarily be inactivated by mutation; promoter silencing by methylation—an

epigenetic mechanism—is emerging as a common way of inhibiting TSG expression, an example has already been given for *CDKN2A*. This mechanism is perhaps a more facile way for cancer cells to inactivate genes rather than waiting for chance mutations. *BRCA1* does appear to be dramatically down-regulated in breast carcinomas (Thompson *et al.*, 1995); further, this reduction in expression may be mediated by methylation since the gene possesses a CpG island in its promoter region (Rodenhiser *et al.*, 1996). An alternative mechanism whereby TSGs which are wild-type in sequence may still be functionally inactivated is by the subcellular mislocalisation of their encoded product, that is through the inability of the gene product to reach the cellular locale in which it performs its function; again, evidence for this mode of inactivation has been supplied for the BRCA1 protein (Chen *et al.*, 1995).

An alternative explanation for these observations is that the aetiology of familial breast and ovarian cancers differs fundamentally from that for sporadic cancers in these tissues, the two types of cancer progressing via distinct series of genetic mishaps. Certainly, it has been reported that breast and ovarian tumours resulting in carriers of *BRCA1* mutations have a better prognosis than sporadic cases (Marcus *et al.*, 1996; Rubin *et al.* 1996), suggesting inherent differences in the tumours' genetic make-up, although a number of extrinsic factors could also account for this such as the younger age of cancer patients with germ-line mutations. Experimental evidence which directly supports the existence of distinct genetic pathways in hereditary and sporadic breast tumours has been provided by Tirkkonen *et al.* (1997), who used comparative genomic hybridisation to identify regions of genomic gains and losses which differed disproportionately between tumours from carriers of *BRCA1* and *BRCA2* mutations and control cases. Perhaps the nature of the initiating lesion in different tumours influences the targets for subsequent genetic modification, recalling how different combinations of oncogenes complement the transforming ability of one another.

Kinzler and Vogelstein (1997) have furnished us with a timely paradigm that is likely to shape thinking in the field of TSGs as much as Knudson's 'two-hit' hypothesis. Their model has the virtue of explaining a great deal of the phenomenology. Its point of departure is the classification of TSGs as either 'gatekeepers' or 'caretakers'. Gatekeepers control cellular proliferation directly by inhibiting growth or promoting death and from the description of the functions of TSGs given above would obviously include *RB1*, *TP53*, and *CDKN2A* among others. A gatekeeping modality was the original way in which the products of TSGs were construed to behave; however, it is

becoming increasingly apparent that caretakers, genes whose products maintain the integrity of the genome, may be even more frequent causes of inherited predispositions to cancer. Caretakers include the mismatch repair genes mutated in HNPCC, the nucleotide excision repair genes implicated in Xeroderma pigmentosum, and genes like *ATM*, *BLM* and *WRN* that maintain chromosome integrity after DNA damage and recombination.

This functional categorisation is perhaps not so cut and dry though for the following reasons: both BRCA proteins possess domains with transactivation potential, a property associated with transcription factors, and BRCA1 as mentioned can induce the expression of the cell-cycle inhibitor $p21^{WAF1/CIP1}$; the expression of both *BRCA1* and *BRCA2* is co-ordinated with cell-cycle progression (Rajan *et al.*, 1996; Vaughn *et al.*, 1996; Ruffiner *et al.*, 1997); further, decreased expression of *BRCA1* has been shown to increase the rate of cell growth in normal breast epithelial cells (Thompson *et al.*, 1995); while ectopic expression retards the growth of both breast and ovarian carcinoma cells, and inhibits tumourigenicity (Holt *et al.*, 1996). These features are more in keeping with a gatekeeper role for these genes. Their association with RAD51 and the DNA damage signalling pathway in contrast argues for a role as caretakers. p53 might also be said to share in this ambivalence. Kinzler and Vogelstein argue, however, that the absence of mutations in *BRCA* genes in sporadic forms of breast and ovarian cancer places them within the caretaker camp.

They reason that inactivation of a gatekeeper gene manifests itself directly as a growth advantage to the affected cell. In contrast, inactivation of a caretaker gene does not result directly in tumour initiation, but rather increases the mutation rate of all genes, including gatekeepers, through promoting genomic instability. It is this Mutator phenotype that confers the increased risk of developing cancer. Because additional mutations in gatekeeper genes are still required for tumour initiation in individuals with germ-line mutations in caretaker genes, the risk of cancer is generally only 5–50-fold greater than in the general population—much less than the several thousand-fold relative risks to carriers of mutations in gatekeeper genes. Importantly, mutations in caretaker genes would not be expected to occur in sporadic tumours since a single cell would need to acquire four independent mutations (two caretaker alleles plus two gatekeeper alleles) to become initiated. The odds of acquiring even three somatic mutations before a cell undergoes maturation arrest, apoptosis, or replicative senescence are slight, which

probably explains why most cancer susceptibility syndromes due to inherited mutations in caretaker genes are recessive disorders.

1.4 The identification of TSGs

It is no historical accident that the identification of TSGs has lagged behind that of proto-oncogenes. The problems are due both to the inherent nature of these genes as much as to technical obstacles. Thus, while functional screens greatly facilitated the identification of dominantly transforming oncogenes, it has proven considerably more difficult to isolate TSGs by functional methods alone. First, because TSGs act recessively at the cellular level, both copies need to be inactivated before a transformed phenotype can be observed. Although it is possible to achieve targeted homozygous inactivation of a gene by homologous recombination in cultured cells (Detloff *et al.*, 1994; te Riele *et al.*, 1990; Wu *et al.*, 1994; Hanson and Sedivy, 1995), and in recombinant animals (Capecchi, 1989), it cannot be applied prospectively when knowledge of DNA sequence is not available. Second, TSGs that play a caretaker role in the cell and that when mutated give rise to a Mutator phenotype, would not necessarily be expected to suppress tumourigenicity on re-introduction because the genetic lesions underlying the altered growth in cells in which these genes are mutated have presumably already occurred and cannot be reversed. For this reason alone, complementation would not prove to be wholly reliable when screening for genes which can suppress tumourigenicity, even before size limitations and efficiency of gene transfer are considered. Also, where complementation would result in clones of cells whose growth and survival are diminished, recovery of sufficient material to analyse would require that the phenotype be reversible or its induction tightly regulated, or require sensitive cloning methods. Ultimately, it may be difficult to distinguish between non-specific cytostatic or cytotoxic effects and physiological growth- or tumour-suppression.

Despite these concerns, if the particular biochemical defect underlying the malignant phenotype can be clearly established and an appropriate assay devised, then complementation can be extremely powerful in identifying novel genes that are responsible. This was demonstrated in the cloning of the *XP* genes involved in nucleotide excision repair (Hoeijmakers, 1993). Further, the scope and efficiency of gene transfer techniques are continually improving. The transfer of large genomic inserts in bacterial cloning vectors (cosmids, BACs, P1 clones, and PACs) and in yeast

artificial chromosomes (YACs), and of entire mammalian chromosomes into a range of host cell types has been achieved, allowing the study of gene expression under appropriate physiological control. The use of microcell mediated monochromosome transfer, in particular, is proving very effective in the mapping of TSG loci, and in providing direct functional evidence of tumour suppression attributed to a locus (Stanbridge, 1988; Chen *et al.*, 1995; England *et al.*, 1996; Gustafson *et al.*, 1996; Karlsson *et al.*, 1996).

However due to the difficulties inherent in complementation, it is positional cloning, or 'reverse genetics', that has been the mainstay of investigators trying to identify novel TSGs: *APC*, *ATM*, *BLM*, *BRCA1*, *BRCA2*, *DCC*, *DPC4*, *FHIT*, *LKB1*, *NF1*, *NF2*, *MEN1*, *MMAC1*, *RB1*, *TSC1*, *TSC2*, *VHL*, *WRN*, and *WT1* were all identified by this approach. (Occasionally, in certain extremely fortunate instances, serendipity can intervene, as with the cloning of *p73*.) In the case of TSGs associated with a familial cancer predisposition syndrome, linkage was often used to first map the gene to a chromosomal region. Linkage disequilibrium where there is a strong founder effect (Ellis *et al.*, 1994; Goddard *et al.*, 1996, Matsumoto *et al.*, 1997) and homozygosity mapping of recessive disorders in consanguineous families (German *et al.*, 1994) can then sometimes be used to localise the gene more precisely. Cytogenetic abnormalities including deletions, translocations and chromosome fragile sites can further substantiate and refine the location of a TSG, as can molecular genetic analysis of LOH and homozygous deletions, which can be used independently of linkage to identify TSG loci involved in sporadic tumours (Fearon *et al.*, 1990; Hahn *et al.*, 1996[a]).

Positional information can be combined with knowledge about candidate genes in the region, referred to as a positional candidate approach, to identify TSGs which also predispose to cancer. This approach implicated *TP53* in Li-Fraumeni syndrome, *CDKN2A* in familial melanoma, *DPC4* in JPS, *E-CADHERIN* in familial gastric carcinoma, *hMSH2* in HNPCC, $p57^{KIP2}$ in BWS, and *PTEN/MMAC1* in BZS, CD, JPS, LDD. The assembly of a transcript map for the entire human genome (Schuler *et al.*, 1996) will no doubt greatly facilitate positional-based means of identifying disease-associated genes in the future, as ultimately will completion of the human genome sequencing project. With this information in hand, the positional candidate approach will probably become the predominant method for identifying cancer susceptibility genes in the future (Collins, 1995). A purely candidate approach, based only on

knowledge of the function of genes, led to the identification of mutations in *hMLH1*, *hPMS1*, and *hPMS2* in cases of HNPCC not linked to chromosome 2p16.

Establishing the chromosomal sub-region that harbours a TSG locus is often the rate limiting step in its positional cloning. Linkage, LOH, and deletions can resolve intervals of several hundred Kbp at best. The implicated region then has to be cloned and genes included in the interval identified (reviewed in Monaco, 1994) and screened for the presence of mutations (reviewed in Grompe, 1993). Again the human genome project has provided information, resources, and reagents that accelerate this process, principal among these being various physical *sequence tagged site* (STS) maps of the genome (Chumakov *et al.*, 1995; Hudson *et al.*, 1995; Gyapay *et al.*, 1996) and reference libraries of DNA clones (Dausset *et al.*, 1992; Ioannou *et al.*, 1994; Nizetic *et al.*, 1994). The advantage of positional cloning is that it is targeted and exhaustive, while the demonstration of inactivating mutations offers incontrovertible evidence that the correct gene has been identified. The disadvantage is that many of the steps entailed are laborious and time-consuming. Various recent scientific advances have resulted in gene cloning strategies that do not require positional information, and which exploit other features of TSGs such as their altered expression in tumours to aid in their isolation.

Representational difference analysis (RDA) and genome mismatch scanning (GMS) are two techniques which allow an entire genome to be scanned at one time for sequences linked to trait loci. RDA combines both subtraction and DNA re-association kinetics to enrich for sequences that are present within a 'tester' population of DNA molecules but that are absent from a 'driver' population (Lisitsyn *et al.*, 1993). The tester population can for instance be derived from normal genomic DNA and hybridised to a driver population derived from tumour DNA. Sequences which are common to both populations are depleted, while sequences present only in the tester, due say to deletions or rearrangements in the driver, are selectively amplified. This technique was successfully employed in the cloning of *PTEN* (Li *et al.*, 1997), and contributed to the cloning of both *BRCA2* and *FHIT* (Schutte *et al.*, 1995; Ohta *et al.*, 1996). RDA can also be combined with genetic linkage (genetically directed RDA) to clone DNA from a locus linked to an inherited trait (Lisitsyn *et al.*, 1994).

In contrast to RDA, GMS seeks to identify large regions of sequence identity between two individuals, with the assumption that in an outbred population these must represent regions of 'identity by descent' (Nelson *et al.*, 1993). This technique exploits

the ability of restriction enzymes to distinguish between methylated and non-methylated DNA, and of the bacterial mismatch repair enzymes, MutS, MutH, and MutL, to recognise and cleave heteroduplexes of mismatched DNA, to leave only sequences which are identical between individuals intact. To search for TSGs which predispose to cancer, regions of identity by descent could be sought in pairs of affected cousins or in a collection of individuals from an isolated subpopulation, although this has yet to be attempted.

The expression of TSGs is often decreased in cancer cells, and transcripts can be alternatively spliced or otherwise re-arranged by insertions and deletions, features that can be exploited in TSG identification. Differential hybridisation, subtractive hybridisation, and differential display are three methods which allow differentially expressed genes to be cloned. Differential hybridisation (or differential screening) was the first of these three techniques to be developed and has been employed successfully in the cloning of TSGs as for instance in the isolation of the metastasis suppressor *nm23* (Steegs *et al.*, 1988). While it is also the least sensitive of the three techniques, it has lately been revamped. Hybridisation of labelled cDNA molecules from cancers and normal tissue to replica filters containing densely gridded cDNA arrays detected autoradiographically or even by using low-light sensitive cameras and video enhancement has been used to screen whole populations of expressed sequences for differentially expressed genes (Gress *et al.*, 1996). It is possible to anticipate a time when such hybridisations will be performed using DNA microchip technology. The technique of subtractive hybridisation has also evolved since its conception. Latest versions of the technique no longer rely on chromatography to remove subtracted sequences but use primer modifications such as biotinylation, or exploit other primer features that allow selective amplification by PCR (Diatchenko *et al.*, 1996; Lavery *et al.*, 1997). Differential display (Liang and Pardee, 1992) employs random primers and anchored oligo dT primers to reverse transcribe and amplify by PCR representations of expressed sequences which can be resolved by gel electrophoresis to produce a fingerprint or expression profile. Differences in fingerprints between sample RNAs represent differentially expressed or processed genes. These techniques are rapid and allow high throughput screening and are greatly increasing the pool of candidate TSGs.

Two further technical innovations may also increase our ability to detect TSGs by functional means. Li and Cohen (1996) have devised a novel method of identifying genes whose inactivation results in malignant transformation which does not depend

upon prior knowledge of DNA sequence. The method employs a retroviral-based gene search vector to inhibit expression of genes into which the provirus integrates by production of an antisense RNA complementary to the sense transcript of the interrupted gene. Using this technique, these workers identified *tsg101* a mouse gene whose product shares features with many transcription factors and which interacts with stathmin, a putative oncoprotein and integrator of diverse signalling pathways, through a coiled-coil domain. However, controversy exists over whether this gene is actually involved in human cancers. Although original studies using PCR-based techniques detected altered transcripts of *TSG101* in breast tumours that apparently arose through large intragenic deletions (Li *et al.*, 1996), this observation has failed to be confirmed by Southern blot analysis of breast tumour DNA samples (Steiner *et al.*, 1997; Lee and Feinberg, 1997), or by western blotting (Zhong *et al.*, 1997).

The second technique again allows a number of potential TSGs genes to be functionally assayed simultaneously and has parallels to the first technique. It relies upon the production of gene suppressor elements (GSEs), fragments of genes which can act as dominant negative peptides or give rise to inhibitory antisense molecules that retard the action of the cognate gene from which they are derived (Holzmayer *et al.*, 1992; Gudkov *et al.*, 1994). GSEs derived from TSGs might be capable of transforming cells, extending their replicative life span, or allowing them to acquire resistance to apoptosis. Indeed, this approach has been used to identify GSEs from the *TP53* gene which confer resistance to cisplatin treatment (Gallagher *et al.*, 1997), and to identify a novel tumour suppressor gene *ING1* which may be involved in replicative senescence (Garkavtsev *et al.*, 1996; Garkavtsev and Riabowol, 1997). Loss of function mutations have subsequently been identified in *ING1* in sporadic tumours, and the encoded protein product p33 has been shown to interact with p53 and to be essential for p53-dependent growth arrest and transcription of $p21^{WAF1/CIP1}$ (Garkavtsev et al., 1998).

Animal models have been used extensively to characterise the function of oncogenes and TSGs in an *in vivo* setting, but their full potential for the identification of novel TSGs has not been realised. Clearly, many organisms lend themselves well to genetic analysis, and our ability to induce mutations efficiently should make animal models of cancer a rich hunting ground for TSGs. In particular, animal models may well prove invaluable in the identification of low penetrance tumour susceptibility loci. Towards this end, Nagase *et al.* (1995) have used crosses between strains of mice resistant to skin tumour development and strains of mice that are susceptible, to map

quantitative trait loci involved in tumour susceptibility by meiotic recombinations and LOH in resultant tumours. These loci appear to contribute both to tumour initiation and progression. Mutagenic screens in Drosophila have also identified over 50 loci which when mutated give rise to altered cell growth (Watson *et al.*, 1994). The genes *discs large* (*dlg*), *fat*, *lethal(2) giant larvae* (*l(2)gl*), and *warts* are perhaps the best characterised of those that have been identified so far. Since, as has been stressed, these genes are likely to be conserved between various species, including man, using animal models like *Mus musculus* and *Drosophila* could greatly enrich our knowledge of cancer genes mutated in man.

With the rapid flux in methods of detecting genes inactivated in cancer and our changing awareness of their functions, it is becoming increasingly difficult to provide an all-embracing definition of TSGs. Haber and Harlow (1997) urge us to return to first principles for guidance in this matter; they suggest the definition: '*genes that sustain loss-of-function mutations in the development of cancer*' as '*the simplest, most inclusive and cleanest*'. This they argue still leaves room for epigenetic events, dominant negative mutations, and heterozygous mutations in genes where dosage is critical. Merely demonstrating loss of expression in tumours or the ability to suppress cellular proliferation does not qualify a gene as belonging to the TSG class in the absence of definitive evidence of inactivating mutations. Such a definition may help to resolve several recent controversies over the role of novel genes identified by some of these latest gene cloning methods in the aetiology of human cancer.

1.5 Replicative senescence

With the possible exception of stem cells and primitive embryonic lineages, normal mammalian somatic cell populations have a limited proliferative potential *in vitro* and *in vivo*. For many cell types, including the majority of epithelial cells, the ability to proliferate is lost upon acquisition of the mature differentiated state (referred to as terminal differentiation or maturation arrest), which is an irreversible quiescent state. Activation of proto-oncogenes and loss of function of TSGs has been demonstrated to interfere or completely obviate terminal differentiation, allowing cells to remain in cycle (Yoakum *et al.*, 1985; Yuspa *et al.*, 1985; Dmitrovsky *et al.*, 1986; Prochownik *et al.*, 1986; Klein, 1987; Pritchard-Jones *et al.*, 1990; Hedrick *et al.*, 1994; Zacksenhaus *et al.*, 1996). Terminal differentiation can also be abrogated during *in vitro*

culture, but primary cultures of epithelial cells, and indeed of fibroblasts which demonstrate conditional renewal *in vivo*, still possess only limited proliferative potential (Hayflick, 1965; Rheinwald and Green, 1975). Upon achieving a particular number of population doublings (eponymously named the Hayflick limit), which depends upon the species, age and genetic background of the donor, as well as cell type, cells again become irreversibly growth-arrested, a state that has been termed replicative senescence, or more recently mortality stage 1 (M1). A limited number of studies suggest that replicative senescence does occur *in vivo* also, and is not merely an artefact of *in vitro* culture (reviewed in Campisi, 1996).

The biochemistry of senescence has best been characterised for primary cultures of fibroblasts, and clearly distinguishes senescence from other forms of irreversible growth arrest. A distinction should also be drawn between senescence and cell death: senescent cells are viable and can be maintained in culture for months to years; indeed, senescent cells are remarkably resistant to apoptosis (Wang, 1995). During senescence cells arrest with a G1 DNA content and cannot be stimulated to enter S phase by any combination of mitogens. However, treatment with mitogens still induces the expression of a number of genes, among them the oncogenes *MYC*, *JUN*, and *RAS* (Rittling *et al.*, 1986; Seshadri and Campisi, 1990), suggesting that senescence does not result from a general failure in signal transduction. Rather, senescence appears to require the selective repression of a few key growth regulatory genes such as the c-*FOS* proto-oncogene (Seshadri and Campisi, 1990), the helix-loop-helix *ID-1* and *ID-2* genes (Hara *et al.*, 1994), and *E2F-1* and *E2F-5* (Dimri *et al.*, 1994; Good *et al.*, 1996).

In addition to these deficiencies in positive growth regulators, the arrested state in senescent cells is achieved and maintained by the contribution of negative growth regulators as well. This was first implied by the phenotype of hybrids obtained when normal cells are fused with cells capable of indefinite division, *i.e.* immortal cells; these hybrids exhibit limited division potential (Bunn and Tarrant, 1980; Muggleton-Harris and DeSimone, 1980; Pereira-Smith and Smith, 1981; Pereira-Smith and Smith, 1983). Further, fusions between immortal cells from different cell lines result in hybrids in which replicative senescence has been restored (Pereira-Smith and Smith, 1983; Pereira-Smith and Smith, 1988), indicating, in a fashion analogous to the demonstration of the existence of TSGs, that the replicative senescence phenotype is dominant and that immortality results from the recessive inactivation of senescence inducing genes. Subsequently, certain of these have been identified in senescing cells. In this regard the

two tumour suppressors p53 and pRb, which is found in its growth suppressive hypophosphorylated state in senescent cells (Stein *et al.*, 1990), have been shown to be required for the timely initiation of growth arrest during replicative senescence. Loss of function of either gene product either through mutation or inactivation by viral oncoproteins such as E6 or E7 of the HPV virus, delays the onset of M1 by up to twenty population doublings (Shay *et al.*, 1993; Bond *et al.*, 1994; Rogan *et al.*, 1995). The effect is even more marked if both TSGs are inactivated together, suggesting that the biochemical pathways initiated by these two regulators are parallel and synergistic. This double hit occurs for instance when cells express the SV40 large T antigen, and greatly increases the frequency with which recipient cells become immortalised, that is fail to senesce altogether (Bryan and Reddel, 1994). Remaining cells enter a second stationary growth phase, crisis or mortality stage 2 (M2), where cell production is counterbalanced by cell death (Figure 1.5). Unlike M1, crisis cannot be viewed as a regulated growth control mechanism, being essentially a breakdown of normal cell function.

While it is true that senescence in fibroblasts and many epithelial cell types including keratinocytes (Loughran *et al.*, 1996[a]) is regulated both by a p53-dependent pathway and a pRb-dependent pathway, in certain other cell types only one major pathway is apparently playing a role. Thus, distinct populations of mammary glandular epithelial cells can escape senescence through inactivation of p53 alone (Band *et al.*, 1991; Wazer *et al.*, 1995; Gollahon and Shay, 1996), or pRb alone (Wazer *et al.*, 1995; Foster and Galloway, 1996). Moreover, in thyroid epithelial cells a state of viable growth arrest resembling M1 in fibroblasts can be induced in the absence of both functional p53 and pRB (Bond *et al.*, 1996). The mechanism of regulation of M1 is, therefore, dependent on cell type.

Senescent human fibroblasts over-express at least two more growth inhibitors: $p21^{WAF1/CIP1}$ and $p16^{INK4A}$ (Alcorta *et al.*, 1996; Hara *et al.*, 1996). Indeed, $p21^{WAF1/CIP1}$ was originally cloned as a transdominantly acting cDNA (*sdi1*) isolated from senescent fibroblasts capable of mediating growth arrest in young cycling cells (Noda *et al.*, 1994); $p16^{INK4A}$, as mentioned already, has been implicated in tumour suppression. Both these molecules interact with and inactivate complexes of cyclins and CDKs which are required for the G1-S phase transition of the cell cycle through the phosphorylation and attendant inhibition of pRb. While $p16^{INK4A}$ is a specific inhibitor of cyclin D, $p21^{WAF1/CIP1}$ has a broad range of CDK inhibitory activity; $p21^{WAF1/CIP1}$ is also able to antagonise E2F directly in a pRb independent manner (Dimri *et al.*, 1996). The

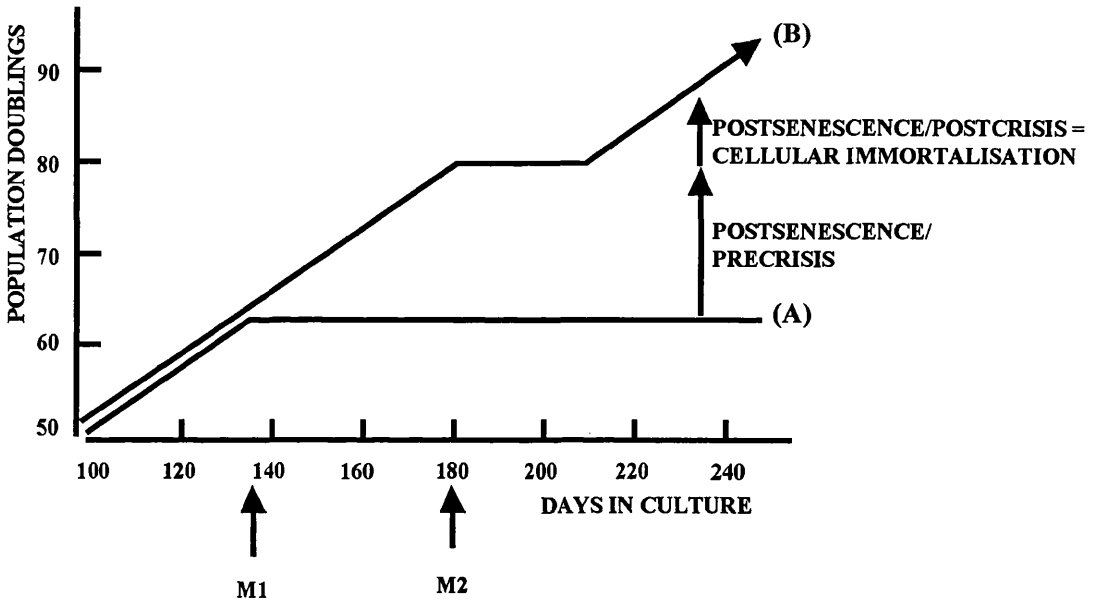


Figure 1.5. Two stage model of cellular immortalisation. A) Mortality stage 1 (M1) involves a loss of mitogen responsiveness, the production of DNA synthesis inhibitors, and arrest in G1 phase of the cell cycle and is the process commonly viewed as *in vitro* replicative senescence. B) Bypass of M1 (through, for example, expression of SV40 T antigen) extends the proliferative lifespan of cells until they reach a second stationary growth phase, mortality stage 2 (M2), corresponding to the classical description of postsenescent crisis. Inactivation of the M2 mechanism results in the final immortalisation event; this inactivation is a rare event, suggesting a mutational origin.

induction of these two cell cycle inhibitors probably accounts for the accumulation of inactive cyclin/CDK complexes during senescence (Dulic *et al.*, 1993) and for the lack of E2F activity in senescent cells (Dimri *et al.*, 1994; Good *et al.*, 1996). Induction of $p21^{WAF1/CIP1}$, which is transient, precedes that of $p16^{INK4A}$, which is prolonged (Alcorta *et al.*, 1996). $p21^{WAF1/CIP1}$, with its wider inhibitory range, may be responsible for the acute phase of growth arrest, while the prolonged induction of $p16^{INK4A}$ may underlie the irreversible nature of replicative senescence. The means by which $p21^{WAF1/CIP1}$ and $p16^{INK4A}$ are induced during senescence are not known. The induction of $p21^{WAF1/CIP1}$ in this instance is not dependent upon transactivation by p53 (Bond *et al.*, 1995).

Loss of $p21^{WAF1/CIP1}$ expression in human fibroblasts, brought about by homologous recombination, has been shown to bypass replicative senescence (Brown *et al.*, 1997), establishing an essential role for this protein during senescence. Brown *et al.* also showed that $p16^{INK4A}$ levels continued to increase in $p21^{WAF1/CIP1-/-}$ cells with passage number, indicating that this event is insufficient to induce senescence. However, other observations support an essential role for $p16^{INK4A}$ as well in replicative senescence. Fibroblasts from recombinant mice nullizygous for *CDKN2A* are more readily immortalised than normal counterparts (Serrano *et al.*, 1996), and this locus on mouse chromosome 4 is also frequently inactivated during spontaneous immortalisation of mouse embryo fibroblasts (Obata *et al.*, 1997), but caution needs to be exercised when extrapolating from rodents to humans because of interspecies differences in the mechanisms of senescence. Independent evidence for the involvement of $p16^{INK4A}$ in the senescence of human cells has been supplied both by somatic cell genetics and expression studies which show that inactivation of the $p16^{INK4A}$ locus or loss of expression of $p16^{INK4A}$ accompanies the emergence of the immortal phentotype (Rogan *et al.*, 1995; England *et al.*, 1996; Loughran *et al.*, 1996[a]; Noble *et al.*, 1996; Reznikoff, 1996), while restoration of functional $p16^{INK4A}$ to deficient immortal cells confers replicative senescence (Uhrbom *et al.*, 1997).

p53 and the pRb-pathway are the most common targets for mutational inactivation in human cancer to be recognised. While undoubtedly this inactivation confers a selective growth advantage to tumour cells in allowing them to bypass critical cell cycle check-points, a further expectation formulated upon the significance of these proteins in senescence is that such mutational events will promote indefinite division potential in cancer cells. In keeping with this, most common human carcinomas contain immortal variants. This is a more frequent finding among advanced cancers than among

early stage tumours and pre-malignant lesions (Edington *et al.*, 1995), suggesting that there is selection for increased replicative potential during cancer development. The corollary of this proposal is that annulment of replicative senescence is advantageous to cancer cells. Certainly, immortality would permit indefinite clonal expansion and selection, increasing genetic diversity, and may ultimately be responsible for cancer lethality by promoting metastasis, therapeutic resistance, and recurrence. The physiological relevance of replicative senescence, therefore, is as a tumour suppressive adaptation, and this is re-inforced by the essential role of several tumour suppressor genes in its upkeep.

Since the onset of replicative senescence is determined by the number of cell divisions and not chronological time, cells must possess a means of detecting the number of divisions they have passed; current wisdom favours a 'telomeric clock' model. Telomeres are specialised structures present at the ends of chromosomes, found in apparently all eukaryotes, that allow cells to overcome the problem of replicating the ends of linear DNA molecules—the 'end replication problem' (Watson, 1972; Olovnikov, 1973)—and that protect chromosomes from exonuclease degradation and prevent chromosome fusions and recombination (McClintock, 1941; Muller and Herskowitz, 1954). Telomeres comprise both DNA and associated binding proteins. Telomeric DNA is of two types: simple direct DNA repeats with a characteristically G-rich strand that forms the extreme 3′-end of the chromosome and its complementary C-rich strand, and telomere associated sequences proximal to these repeats which are relatively complex, middle-repetitive elements and shorter, tandemly repeated, satellite-like sequences (reviewed in Wellinger and Sen, 1997). Progress has been made into the identification and function of telomere binding proteins in lower eukaryotes, but they remain more elusive in higher eukaryotes (Chong *et al.*, 1995; de Lange, 1996).

Telomeric repeat units are synthesised by telomerase (Greider and Blackburn, 1985), a multimeric ribonucleoprotein comprising an RNA molecule that serves as a template for the addition of repeats, and various peptides with regulatory and catalytic functions, that again have been isolated and most widely studied in lower eukaryotes, but that have also recently been identified in humans (Feng *et al.*, 1995; Harrington *et al.*, 1997; Nakamura *et al.*, 1997; Meyerson *et al.*, 1997). In the absence of telomerase activity, telomeres shorten in length by about 30-150bp per cell division, as a direct consequence of the end replication problem. In humans, telomerase activity has been detected in embryonic tissues, germ-line cells, and in certain stem cells and highly

proliferative cells, but is lost at some time during development in the majority of somatic cells (Wright *et al.*, 1996[a]). Chromosome ends would, therefore, be expected to recede in most cells with each division. Such decay in telomere length has been demonstrated for cells cultured *in vitro* (Harley *et al.*, 1990; Hastie *et al.*, 1990; Vaziri *et al.*, 1994) and also *in vivo* for cells from donors of increasing age (Hastie *et al.*, 1990; Wainwright *et al.*, 1995). Telomere shortening, then, provides a measure of cell division, and it has been proposed that replicative senescence ensues after a requisite amount of telomeric attrition. Abrogation of M1 control mechanisms allows additional rounds of cell division until M2 occurs, at which time few, if any, telomeric repeats remain at the ends of chromosomes (Counter *et al.*, 1992).

Further associative evidence in support of the 'telomere clock hypothesis' to that cited above comes from the study of the classic premature ageing illness, Werner's syndrome, Down's syndrome, and AT. In all three instances, telomeres of cultured cells and cells *in vivo* shorten at an accelerated rate, and cells senesce after a correspondingly shorter replicative lifespan (Kruk *et al.*, 1995; Yu *et al.*, 1996; Metcalfe *et al.*, 1996; Cossarizza *et al.*, 1991; Vaziri, *et al.*, 1993). These findings support roles for the *ATM* and *WRN* genes, both associated with inherited cancer predisposition syndromes in man, in the maintenance of telomeres. This role of *ATM* is supported by the finding in budding yeast that inactivation of the *ATM*-homologue, *TEL1*, also results in telomeric attrition (Greenwell *et al.*, 1995). Cogent indirect evidence in support of the telomeric clock model has been supplied by Wright *et al.*, (1996[b]), who used oligonucleotides to experimentally lengthen telomeres in immortal cells expressing telomerase. Cells manipulated in this way were then fused with ordinary mortal cells and gave rise to hybrids with a greater division potential than hybrids between normal and untreated immortal cells. Thus in this instance, the onset of senescence was delayed in a manner that precisely correlated with telomere length. The recent cloning of the catalytic protein subunit of the telomerase enzyme (Lingner *et al.*, 1997[a and b]; Meyerson *et al.*, 1997), which revealed homologies between telomerase and other known reverse transcriptases, has afforded an opportunity to rigorously test the telomere clock hypothesis. Ectopic expression of this protein in human mortal cells fully reconstituted telomerase activity and resulted in indefinite clonal expansion, apparently by bypassing the senescence program altogether (Bodnar *et al.*, 1998; Vaziri and Benchimol, 1998); telomeric attrition was not found to occur in these clones. Hence, cells manipulated in this way

behave like embryonic stem cells or germ-line cells. To date this work is the most direct evidence in support of the telomere clock model for cellular ageing.

Still missing from the telomere clock model is a plausible mechanism whereby shortened telomeres signal to the cell-cycle machinery to bring around growth arrest and to maintain the quiescent state irreversibly. Ultimately, a complete explanation will have to account for the known biochemical features of the senescent state, in particular how certain positive growth regulators are selectively suppressed, while other cell-cycle inhibitors are induced or activated. There are currently three hypothetical mechanisms which have been proposed, based largely upon experiments in lower eukaryotes. The first suggests that a short telomere may signal a DNA-damage response. A denuded chromosome end may appear to a cell like a double-stranded DNA break and induce cell-cycle arrest through an ATM/p53 dependent pathway. This could account for the role of p53 in the regulation of M1, although the level of p53 does not rise in senescent cells the way it does following DNA damage (Afshari *et al.*, 1993), and it does not explain why loss of ATM function should accelerate senescence. In budding yeast it was shown that the loss of a single telomere could effect cell cycle arrest by a RAD9-dependent pathway (Sandell and Zakian, 1993), however the growth inhibition observed was only transient whereas senescence in mammalian cells is irreversible. The second alternative proposes that telomeres sequester transcription factors which are released as telomeres shorten and that co-ordinately regulate a program of senescent cell gene expression. Again, yeast have provided a strong precedent: Rap1, which binds yeast telomeric repeats, interacts with Sir3p and Sir4p, which are themselves entailed in gene silencing through their interaction with the histone proteins H3 and H4. Sequestration of Sir3p and Sir4p at the telomere adversely effects silencing at an internal chromosomal locus (Marcand *et al.*, 1996). Although a handsome proposition, there are currently no data to support this mechanism in mammalian cells, and no mammalian homologue of Rap1 has been identified. As a third possibility, the shortening of telomeres may influence the heterochromatic structure of subtelomeric DNA, converting it to euchromatin and activating the expression of genes which had been lying dormant within it (Wright and Shay, 1992); these could include transcriptional activators and repressors, as well as inhibitors of cell growth. Silencing of genes at loci near telomeres has been demonstrated in yeast (Thompson *et al.*, 1994), and is even relieved upon cellular ageing (Kim *et al.*, 1996), although in yeast this does not involve telomere shortening (Jazwinski, 1993).

The role of critically shortened telomeres in the onset of M2 appears even less assailable. Chromosomes in cells in crisis are perilously poised, and the loss of essential genetic material through deletions, promiscuous recombination and chromosome fusions is believed to account for the high rate of cell death in populations of these cells. Rarely, immortal variants arise in which telomeres have been stabilised, although they may still be shorter than telomeres in cultures of senescent cells (Counter *et al.*, 1992). These rare events are most often associated with up-regulation or reactivation of telomerase (Counter *et al.*, 1992; Kim *et al.*, 1994). An essential role for telomerase in immortalisation is further strengthened by the finding that inhibition of telomerase activity using an antisense transcript of human telomerase RNA induced telomere shortening and crisis in immortal cells (Feng *et al.*, 1995). However, it is apparent that telomerase independent mechanisms to immortalisation also exist (Murnane *et al.*, 1994; Bryan *et al.*, 1995), which may involve recombination or transposition between telomere associated sequences (Lundblad and Blackburn, 1993; Levis *et al.*, 1993). In keeping with the immortal nature of the majority of cancer cells, telomerase activity has now been detected in most human cancers (Kim *et al.*, 1994; Shay and Wright, 1996), making it the most widely expressed cancer marker presently known. Cloning of the catalytic subunit of telomerase in humans has allowed direct demonstration of its re-induction in human cancers (Meyerson *et al.*, 1997). It is also a highly specific marker, since as stated telomerase activity is absent from the majority of somatic cells, making it an extremely attractive anti-tumour target (Holt *et al.*, 1996; Kim, 1997).

Replicative senescence has been presented as a multigenic program, requiring the repression of particular cell cycle promoters and the induction of several growth inhibitory proteins. Likewise, the inverse process of immortalisation requires the mutational inactivation of multiple targets and the activation of a number of other genes. The induction of telomerase appears to be the single most common method for overcoming M2, but again a number of independent events are entailed in this one pathway. Evidence exists for the presence of a telomerase suppressor on chromosome 3 (Ohmura *et al.*, 1995), which could be a target for inactivation during the process of immortalisation; since the short arm of chromosome 3 displays a high frequency of LOH and deletions in a broad range of human tumours (reviewed in Kok *et al.*, 1997), the gene responsible for this effect may be present on 3p. More recently, Soder *et al.*, (1997) have demonstrated that over-representation and amplification of the human RNA component of telomerase, which maps to chromosome 3q, occurs in a number of tumour

types *in vivo*, showing that telomerase components themselves can function as oncogenes. Although not yet stated explicitly, continued abrogation of the pathways regulating M1 of replicative senescence is a pre-requisite for immortalisation. This has been demonstrated in experimental systems where inactivation of p53 and pRb was reversible (Radna *et al.*, 1989;Wright *et al.*, 1989). The obverse is not true, however, since inactivation of telomerase in immortal cells reverses immortality but does not restore senescence (Feng *et al.*, 1995).

Unlike M2, multiple pathways appear to be operative in the regulation of M1. As mentioned, fusions between different immortal human cells can result in hybrids which senesce, in which case the genomes of the fusion partners complement each other's genetic defects, restoring normal M1 control (hybrids undergo irreversible growth arrest rather than entering crisis). Occasionally, such fusions produce hybrids which retain their unlimited replicative potential, such cell lines are assumed to possess the same underlying M1 defect and can thus be designated to the same complementation group. In this way Pereira-Smith and Smith, (1988) were able to assign immortal cells to four complementation groups, A-D. This categorization does not appear to reflect cell type, embryonal layer of origin, or tumour type from which the immortal cells have been derived (Pereira-Smith and Smith, 1988), nor does it correlate with the p53, pRb-pathway or telomerase activity status of the cell lines (Whitaker *et al.*, 1995). However, the majority of group A immortal cell lines studied by Pereira-Smith and Smith had been immortalised by SV40 virus, indicating a bias for subsequent mutational inactivation in these cells. The existence of four genetic complementation groups for immortality finds a physical (if still enigmatic) correlate in the cellular distribution of mortalin, a member of the hsp70 family of heat shock proteins (Wadhwa *et al.*, 1995). This 66 KDa protein generates a diffuse granular pattern in the cytoplasm of mortal cells by immunocytochemical staining, while the staining in immortal cells is less diffuse, adopting a distinct distribution dependent on the immortality complementation group status. Thus staining for mortalin presents a facile way of assigning complementation group status to an uncharacterised cell line that appears wholly reliable.

The recessive genetic basis for M1 control has been further re-inforced by microcell-mediated monochromosome transfer studies, which have simultaneously allowed mapping of the complementation group genes. These studies show that the transfer of a single chromosome can restore senescent properties only to recipient cells which fall within a particular complementation group, and that this is a unique property

of the selected chromosome. In this way, the genes corresponding to complementation groups B, C, and D have been mapped to chromosomes 4, 1 and 7 respectively (Ning *et al.*, 1991; Hensler *et al.*, 1994; Ogata *et al.*, 1993; Ogata *et al.*, 1995). Complementation by re-introduction of chromosomes 1, 4, or 7 into the appropriate immortal cell line results in delayed growth arrest and induction of senescence associated markers (Dimri *et al.*, 1995; Nakabayshi *et al.*, 1997). Additional chromosomes have also been reported to confer delayed growth arrest to immortal cells when introduced by micro-cell mediated monochromosome transfer (Uejima, 1992; Koi *et al.*, 1993; Sandhu *et al.*, 1994; Ohmura *et al.*, 1995), and occasionally more than one chromosome has been shown to confer senescence on the same cell line (Yamada *et al.*, 1990). It is not clear in all these instances whether it was specifically an M1 program that was restored or whether cells may be losing their replicative potential due to other processes such as terminal differentiation. However, these observations raise the possibility that multiple TSGs interact to establish and maintain replicative senescence. The nature and function of these genes is unknown and can only be speculated on. Their products may provide the missing links between telomere shortening and induction of the senescence program or, like pRb, p53, $p16^{INK4A}$, and $p21^{WAF1/CIP1}$, may be cell-cycle inhibitory molecules.

1.6 Evidence for a tumour suppressor/replicative senescence gene on human chromosome 7

The senescence gene corresponding to complementation group D has been mapped to human chromosome 7 (Ogata *et al.*, 1993; Ogata *et al.*, 1995). Microcell-mediated transfer of chromosome 7 into two immortal, non-tumourigenic, fibroblast cell lines, SUSM-1 and KMST-6, obtained by treating normal human diploid fibroblasts with a genotoxic regimen, and a human hepatoma cell line, HepG2, all assigned to complementation group D, resulted in senescence of these cells within 10-30 population doublings following treatment. This effect was specific to chromosome 7: introduction of chromosomes 1 and 11, which have been shown to induce delayed growth arrest in other immortal cell lines (Hensler *et al.*, 1994; Koi *et al.*, 1993), had no effect upon the replicative potential of any of these three cell lines. In addition, no effect was observed upon introduction of chromosome 7 into three other tumour-derived immortal cell lines, HT-1080 (fibrosarcoma), HeLa (cervical carcinoma), and TE85 (osteosarcoma), which are representative of complementation groups A, B, and C, respectively. Re-introduction

of a functional replicative senescence gene on chromosome 7 was accompanied by telomeric attrition, and induction of senescence-associated (s.a) β-galactosidase activity and redistribution of mortalin (Nakabayashi *et al.*, 1997). Again by using microcell-mediated monochromosome transfer, it has been shown that human chromosome 7 can in addition suppress tumourigenicity when introduced into a murine squamous cell carcinoma derived cell line (Zenklusen *et al.*, 1994[a]), providing independent evidence for the existence of a tumour suppressor gene on this chromosome.

Independent lines of investigation implicate a single chromosomal region, 7q31, in both of these phenomena: tumour suppression and replicative senescence. Several reports have indicated that karyotypic alterations of chromosome 7 are common in many different types of human neoplasias. Monosomy 7 is a frequent finding in haematopoietic and other non-epithelial disorders, whereas trisomy 7 is often observed in various neoplasias, primarily of epithelial origin. The trisomic cases, however, frequently present limited interstitial deletions of the long arm of the chromosome as well, and in some cases these have been shown to correspond to the cytogenetic band 7q31 (reviewed in Zenklusen and Conti, 1996). A number of studies have revealed a high incidence of LOH on the long arm of chromosome 7 in a broad range of human tumours, including carcinomas of the breast (Bieche *et al.*, 1992; Zenklusen *et al.*, 1994[b]), colon (Zenklusen *et al.*, 1995[a]), kidney (Shridhar *et al.*, 1997), ovary (Zenklusen *et al.*, 1995[b], Kerr *et al.*, 1996; Koike *et al.*, 1997), pancreas (Achille *et al.*, 1996), prostate (Zenklusen *et al.*, 1994[c]; Takahashi *et al.*, 1995), and stomach (Kuniyasu *et al.*, 1994) as well as squamous cell carcinomas of the head and neck (Zenklusen *et al.*, 1995[a]; Loughran *et al.*, 1996[b]). The highest frequency of LOH was reported for the CA.GT repeat D7S522. Collectively, these studies implicate the chromosomal region 7q31 as harbouring a multi-tissue TSG. High frequency LOH was also observed for the syntenic region A2 of mouse chromosome 6 in chemically induced hepatomas, and squamous cell carcinomas (Zenklusen *et al.*, 1996[b]; Zenklusen *et al.*, 1997), suggesting, together with the ability of human chromosome 7 to suppress tumourigenicity in a mouse carcinoma cell line (Zenklusen *et al.*, 1994[a]), that the TSG on human chromosome 7 has been conserved through evolution. RFLP analysis of DNA from the two immortal fibroblast cell lines, SUSM-1 and KMST-6, also revealed loss of genetic material from the 7q31 region (Ogata *et al.*, 1993), suggesting that a gene from this area is involved in replicative senescence as well. Since replicative senescence can be viewed as an anti-tumour adaptation, then the convergence of these two groups of studies, loss

of heterozygosity in tumours and in the group D cell lines, on a common chromosomal region raises the possibility that a single gene is responsible for both phenomena.

1.8 Aims

The aim of my project is a subset of the overall aim of our research group, namely: to clone and characterise the tumour suppressor/ replicative senescence gene on the long arm of human chromosome 7. Towards this ultimate end, more immediate needs include mapping the region on 7q in which the gene resides through the analysis of LOH in tumour DNA samples and immortal SUSM-1/*Hytk7* segregants; developing additional polymorphic markers from this region to facilitate higher resolution mapping; establishing yeast and bacterial clone coverage of the implicated region and developing novel STS markers to facilitate their contiguation; and identifying genes from within the region. Finally, and in keeping with Haber and Harlow's rigorous demand, the chromosome 7 tumour suppressor/ replicative senescence gene will be identified through the demonstration of loss-of-function mutations in genes isolated from the critical region in tumours, complementation group D cell lines, or immortal SUSM-1/*Hytk7* segregants. I have endeavoured to dirty my hands with some of all this business.

CHAPTER 2

MATERIALS AND METHODS

2. Materials and Methods.

2.1 Materials.

2.1.1 Chemicals and reagents.

All chemicals not individually listed were obtained (AnalaR grade) from BDH Chemicals Ltd., Poole, Dorset, UK. Solutions and buffers were prepared using de-ionized water (dH_2O) obtained from a Millipore MilliRO 15 system.

Chemical	Source
Redivue [α-^{32}P] dCTP~3000Ci/mmol	Amersham International plc., Amersham, Buckinghamshire, UK
CsCl Hepes Mops	Boehringer Mannheim UK, Lewes, East Sussex, UK
Butan-1-ol Chloroform 38% (w/v) Formaldehyde Propan-1-ol	Fisher Scientific UK. Ltd., Loughborough, Leicestershire, UK
Dimethyl formamide	Fluka Chemika-Biochemika AG, Buchs, Switzerland
Ethanol	James Burrough Ltd., Witham, Essex, UK
Tris Trizol	Life Technologies Ltd., Paisley, UK
Deoxyribonucleotides	Promega, Southampton, UK
Water-saturated phenol	Rathburn Chemicals Ltd., Walkerburn, UK

Bicinchoninic acid solution	Sigma Chemical Co. Ltd.,
Bovine serum albumin (BSA)	Poole, Dorset, UK
Bromophnol blue	
$CuSO_4$	
DEPC	
Dithiothreitol	
Ethidium bromide	
MES	
NP40	
PMSF	
TEMED	
Tween 20	

2.1.2 Enzymes

All DNA modifying enzymes and their buffers, except those listed below, were obtained from Life Technologies Ltd., Paisley, UK.

Enzyme	Source
Bst XI Klenow polymerase Proteinase K	Boehringer Mannheim UK, Lewes, East Sussex, UK
NovoZyme	Novo BioLabs, Bagsvaerd, Denmark.
DNA'ase free RNA'ase A	Sigma Chemical Co. Ltd., Poole, Dorset, UK
Taq polymerase	Bioline, London, UK
T4 DNA ligase	Northumbria Biologicals Ltd., Cramlington, Northumberland, UK

2.1.3 Kits

Kit	Source
ECL western blotting detection kit	Amersham International plc., Amersham, Buckinghamshire, UK
HighPrime random-priming labelling mixture	Boehringer Mannheim UK, Lewes, East Sussex, UK
TA-cloning kit	Invitrogen, NV Leek, Netherlands.
First strand cDNA synthesis kit	Life Technologies, Paisley, UK
Geneclean II	BIO 101 Inc., Vista, CA, USA
ABIPRISM DNA sequencing kit	PE Applied Biosystems, Warrington, UK
Riboprobe Sp6/T7 combination system Wizard Genomic DNA isolation kit	Promega, Southampton, UK

2.1.4 General plasticware

	Source
Filter pipette tips	Greiner Labortechnik Ltd., Gloucestershire, UK
Falcon tubes	Becton-Dickinson Labware, Plymouth, UK
5 ml bijous 20 ml universals	Bibby-Sterilin Ltd., Staffordshire, UK
microcentrifuge tubes pipette tips	Elkay, Galway, Eire

2.1.5 Miscellany

	Source
MicroSpin S-200 and S-400 HR columns	Pharmacia Biotech. Inc., Herts., UK
Sonicated, denatured genomic DNA from human placenta Torula yeast RNA type VI	Sigma Chemical Co. Ltd., Poole, Dorset, UK

2.1.6 Electrophoresis gels

	Source
Agarose, ultrapure, electrophoresis grade	Life Technologies, Paisley, UK
Polyacrylamide	Severn Biotech Ltd., Kidderminter, UK
Sequagel	BS+S Edinburgh, UK

2.1.7 Molecular weight markers

Marker	Source
S. cerevisiae size standard	Bio-Rad Laboratories, Hercules, CA, USA
ϕX174 DNA/ *Hae* III fragments λ DNA/ *Hind* III fragments 0.249.5 Kbp RNA ladder	Life Technologies Ltd., Paisley, UK
Prestained 'rainbow' protein markers (2,350–46,000 Da)	Amersham International plc., Amersham, Buckinghamshire, UK

2.1.8 Membranes, paper, and X-ray film.

	Source
Hybond nylon membranes	Amersham International plc.,

	Amersham, Buckinghamshire, UK
Immobilon-P	Millipore (UK) Ltd., Watford, Hertfordshire, UK
3MM filter paper	Whatman International Ltd., Maidstone, Kent, UK
X-ray film (X-OMAT-AR)	Eastman Kodak Co., Rochester, New York, USA

2.1.9 Antibodies

Antibody	Source
anti-caveolin-1 polyclonal antibody (C13630)	Affiniti Research Products Ltd., Exeter, UK
horseradish peroxidase conjugated anti-rabbit Ig sary antibody	Amersham International plc., Amersham, Buckinghamshire, UK

2.1.10 Microbial host, media, and supplies.

Sterile glassware and Luria (L)-broth (Maniatis *et al.*, 1989) were prepared by Beatson Institute for Cancer Research, BICR, central services.

	Source
Petri dishes	Bibby-Sterilin Ltd., Staffordshire, UK
Cosmid and PAC clones	Central Resource/Primary Database of the German Human Genome Project, Berlin, Germany
Bacto-agar Bacto-peptone Bacto-yeast extract Tryptone Yeast nitrogen base without amino acids	Difco, Detroit, MI, USA
INVαF' competent *E. Coli*	Invitrogen,

	NV Leek, Netherlands.
DH5α competent *E. Coli* NZY broth	Life Technologies, Paisley, UK
CEPH megaYAC clones 746h5, 905g2, 921b4 and 976b5	Research Genetics Inc. Huntsville, AL, USA
Ampicillin Kanamycin	Sigma Chemical Co. Ltd., Poole, Dorset, UK
PAC clone 162-O21	UK Human Genome Mapping Project Resource, Hinxton Hall, Cambridge, UK

2.1.11 Plasmid vectors

Vector	Source
pBluescript SK (+/-)	Stratagene Ltd., Cambridge, UK.
pSPL3	Dr Melissa Brown Imperial Cancer Research Fund, Lincolns Inn Fields, London, UK

2.1.12 Libraries

Library	Source
Senescent human foreskin fibroblast cDNA library	Dr George Reid, BICR
Human chromosome 7 specific cosmid library filters	Central Resource/Primary Database of the German Human Genome Project, Berlin, Germany
HeLa cDNA library	Stratagene Ltd., Cambridge, UK
Human genomic DNA PAC library filters	UK Human Genome Mapping Project Resource, Hinxton Hall, Cambridge, UK

2.1.13 Breast carcinoma DNA samples

100 paired breast carcinoma/peripheral blood DNA samples (100 ng/μl) in TE buffer (10 mM Tris.HCl, 1 mM EDTA) were the kind gift of Prof. Ellen Solomon, Guy's Hospital, London, UK.

2.1.14 Cell culture media and supplies.

Sterile glassware, PE, PBS, and water were prepared by the (Beatson Institute for Cancer Research) BICR central services.

	Source
Freezing vials	A/S Nunc, Roskilde, Denmark
Cell culture plastic dishes	Becton-Dickinson Labware, Plymouth, UK
Electroporation cuvettes	Bio-Rad Laboratories, Hercules, CA, USA
FCS	Bioclear UK Ltd. Devizes, Wilts, UK
Hygromycin B	Calbiochem-Novabiochem UK Ltd., Beeston, Nottingham, UK
Dimethyl sulphoxide (DMSO)	Fisher Scientific UK. Ltd., Loughborough, Leicestershire, UK
L-glutamine Trypsin	Life Technologies, Paisley, UK
DMEM Penicillin Sodium bicarbonate Sodium pyruvate Streptomycin	Sigma Chemical Co. Ltd., Poole, Dorset, UK

2.1.15 Cell lines

The ovarian carcinoma cell lines listed below were kindly provided by Dr. R. Brown, CRC Beatson laboratories, Medical Oncology, Glasgow, UK, pancreatic cell lines by Dr. N. Lemoine, ICRF, London, UK, and prostatic carcinoma cell lines by Dr. R. Leake, Dept. of Biochemistry, University of Glasgow, Glasgow, UK. All other cell lines were provided by Dr. E. K. Parkinson, BICR.

Cell Line	Tumour of Origin
A1698	bladder
A1698OR	
J82	
MCF-7	breast
MDA-MB-231	
ZR-75-1	
KMST-6	fibroblast*
SUSM-1	
HT1080	fibrosarcoma
143 BTK	osteosarcoma
A2780	ovarian
CH1	
OVCAR3	
OVCAR4	
OVCAR5	
COLO357	pancreas
HS700T	
PACA3	
SUIT2	
TMSG	
DU145	prostate
LnCaP	
PC3	
HeLa	vulva

* Both are non-tumourigenic, immortalised fibroblast cell lines

2.1.16 Websites

Centre d'Etude du Polymorphisme Humain (CEPH):

http://www.cartagene.cephb.fr/bio/ceph-genethon-map.html

Cooperative Human Linkage Centre (CHLC):

http://www.chlc.org

Genethon:

http://www.genethon.fr/genethon_en.html

Genome Data Base:

http://gdbwww.gdb.org

National Center for Biotechnology Information (NCBI):

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

National Human Genome research Institute (NHGRI):

http://www.nhgri.nih.gov

Whitehead Institute/MIT Center for Genome Research:

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

2.2 Methods.

2.2.1 Cell culture

2.2.1.1 Carcinoma cell lines and SUSM-1/Hytk7 immortal segregants

A1698, A1698OR, J82 MDA-MB-231, SUSM-1, HT1080, 143-BTK, COLO357, SUIT2, TMSG, DU145, and PC3 cell lines were cultured in Dulbecco's MEM (DMEM) supplemented with 10% (v/v) FBS, 2 mM L-glutamine, and 1 mM sodium pyruvate. 100 units/ml hygromycin B was added to the medium for selection of SUSM-1/*Hytk7* segregants. MCF-7, ZR-75-1, A2780, OVCAR3, -4, and -5, and LnCaP

cell lines were cultured in RPMI supplemented with 10% (v/v) FBS, 2 mM L-glutamine, and 1 mM sodium pyruvate.

2.2.1.2 Culture and transfection of COS7 cells with pSPL3

For exon-amplification (Buckler *et al.*, 1991), recombinant pSPL3 plasmid (Church *et al.*, 1994) containing restricted cosmid DNA as insert (see section 2.2.4 for preparation of recombinant plasmid) was transfected by electroporation into COS7 cells. COS7 cells were propagated in DMEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine, and 1 mM sodium pyruvate. For transfection COS7 cells were grown to 75-85% confluence, trypsinised, collected by centrifugation, and washed in ice cold PBS. The washed cells (approximately 4 x 10^6) were then resuspended in 0.7 ml PBS and combined in a pre-cooled electroporation cuvette with 0.1 ml PBS containing 10 μg recombinant pSPL3 or non-recombinant pSPL3 as a control. After 10 min on ice the cells were gently resuspended, electroporated (1.2 kV, 25 mF) in a Gene Pulser (Bio-Rad Laboratories, Hercules, CA, USA), and placed on ice again. After 10 min the cells were transferred to a 100 mm tissue culture dish containing 10 ml pre-warmed culture medium.

2.2.2 Nucleic acid preparation and quantitation

2.2.2.1 Extraction and purification of mammalian genomic DNA

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 by trituration in 1 ml of lysis buffer (100 mM Tris.HCl pH 8.5, 5 mM EDTA, 0.2% (w/v) SDS, 200 mM NaCl, 100 μg Proteinase K/ml), followed by incubation for several hours at 37 °C with constant agitation. DNA was precipitated by addition of an equivalent volume of propan-2-ol with gentle mixing until viscosity was gone. The aggregated precipitate was then removed by lifting from the solution with a sterile plastic inoculation loop. Excess liquid was dabbed off and DNA was rinsed in 70% (v/v) ethanol. After being allowed briefly to air-dry, DNA was dispersed into 0.5 ml TE (10 mM Tris.HCl, 1 mM EDTA pH 8.0).

2.2.2.2 Preparation of yeast DNA

CEPH megaYACs are propagated in *S. cerevisiae* host strain AB1380 (*trp1 ura3 ade2*). The YAC vector, pYAC4, complements the AB1380 auxotrophic deficiencies allowing positive selection of YAC DNA in minimal medium lacking tryptophan and uracil, *e.g.* AHC medium (0.175 (w/v) yeast nitrogen base without amino acids, 38 mM $(NH_4)_2SO_4$, 1% (w/v) acid hydrolysed casein, 540 μM adenine hemisulphate), while disruption of the *sup4ochre* gene by insertion in the YAC vector cloning site allows identification of recombinant yeast, which develop a pink pigmentation when grown with limiting amounts of adenine. Agar stab cultures of YAC clones were used to inoculate both flasks containing YPD medium (an enriched all-purpose, complex growth medium comprising 1% (w/v) bacto-yeast extract, 2% (w/v) bacto-peptone, 2% (w/v) glucose) for the preparation of glycerol stocks and AHC plates (1.5% (w/v) agar in AHC medium). Plates were incubated over-night in an inverted position, while flasks were agitated in an orbital shaker. Liquid cultures were mixed with an equivalent volume of 50% (v/v) glycerol and aliquoted into cryotubes for preservation of yeast at -70 °C.

A single pink colony of YAC clone was picked from an AHC plate and used to inoculate a flask containing AHC medium. For STS content mapping of YACs, total yeast DNA was prepared from over-night cultures using a Wizard Genomic DNA kit according to the manufacturer's instructions. To prepare yeast DNA for isolation of YACs by preparative pulse-field gel electrophoresis (PFGE), it was necessary to lyse yeast in low melting point (LMP) agarose to prevent shearing of chromosomal DNA. Yeast from a 30 ml over-night culture were pelleted by centrifugation at 3000 rpm for 5-10 min at room temperature in a bench top centrifuge and then resuspended in 15 ml Tris.HCl pH 7.5 and 50 mM EDTA. Yeast were then pelleted again and this time resuspended in 15 ml SCE (1 M sorbitol, 0.1 M sodium citrate pH 5.8, 10 mM EDTA). Following centrifugation, yeast were resuspended in 600 μl of SCE containing 10 mM DTT. Novozyme was added at a concentration of 8 mg/ml and the yeast incubated at room temperature for 5 min before being transferred to a water bath at 50 °C. Yeast were then mixed with an equivalent volume 1% (w/v) molten LMP agarose in SCE also equilibrated to 50 °C. The yeast-agarose mixture was gently mixed by inversion before 200 μl was aliquoted into the wells of a pre-chilled block former on ice. Blocks were

allowed to set and then transferred to SCE containing 10 mM DTT, in which they were incubated at 37 °C for 1-2 hr. Blocks were subsequently transferred to yeast lysis buffer (1% (w/v) lithium dodecylsulphate, 100 mM EDTA, 10 mM Tris.HCl pH 8.0) and incubated over-night at 37 °C. On the following day, blocks were washed 2 x 30 min in 50 mM EDTA/ 10 mM Tris.HCl pH 7.5. Blocks were stored at 4 °C in this solution.

2.2.2.3 Bacterial clone DNA preparation.

Cosmid and PAC clones were supplied as agar stab cultures. These were first streaked out on an L-broth plate (1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 170 mM NaCl, 1.5% (w/v) agar) supplemented with kanamycin (50 ug/ml). A single colony was then used to inoculate a flask containing L-broth and kanamycin. For the preservation of bacterial stocks, a 0.5 ml aliquot of over-night culture in liquid medium 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 5ml of L-broth medium, containing the appropriate antibiotic, with 20 µl of the glycerol stock.

Plasmid, cosmid, and PAC DNA were isolated from over-night 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, 100 mM EDTA) (table below). Bacteria were then lysed by addition of solution II (0.2 M NaOH, 1% (w/v) SDS) 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 (3M KOAc pH 4.8) 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, cosmid, or PAC DNA was decanted into a fresh polypropylene tube. (For small to medium scale preparations of bacterial clone 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, the two phases mixed by shaking, and separated by centrifugation. The upper aqueous phase was decanted and DNA was precipitated by addition of 0.6 volumes of propan-2-ol, followed by washing in 70% (v/v) ethanol. DNA was resuspended in TE (pH 8.0).

	Culture volume		
	1 ml	50 ml	500 ml
Solution I	100 µl	1 ml	20 ml
Solution II	200 µl	2 ml	40 ml
Solution III	150 µl	1.5 ml	30 ml

For large scale preparations, plasmid, cosmid, or PAC DNA was further purified by equilibrium centrifugation through a continuous CsCl density gradient prepared by dissolving CsCl 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 equivalent amounts 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 (ethanol precipitation) followed by centrifugation at 10,000 g, washing in 70% (v/v) ethanol and re-suspension in TE.

2.2.2.4 Total RNA extraction from mammalian cell lines

Extraction of total RNA from mammalian cell lines was carried out following the manufacturer's protocol for Trizol. Sub-confluent cells grown in 10 cm dishes were washed twice in ice-cold PBS which was completely removed by aspiration. Cells were subsequently lysed directly in the dish by the addition of 1 ml Trizol. A disposable cell scraper was implemented to homogenise the cells, and the RNA was solubilised by passing the lysate a few times through a pipette tip before being transferred to a fresh microcentrifuge tube. Chloroform was added (0.2 ml per 1 ml lysate) and the microcentrifuge tube vortexed for 15 s, then incubated on ice for 5 min. The samples were centrifuged at 12,000 g at 4 °C for 15 min after which the upper colourless aqueous phase was transferred to a fresh microcentrifuge tube and an equivalent volume of propan-2-ol added. The samples were stored overnight at -20 °C. The RNA precipitate was pelleted by centrifugation at 12,000g at 4 °C for 25 min. The RNA pellet

was washed once with 1.5 m1 ice-cold 75% (v/v) ethanol by vortexing and centrifugation at 7,500 g at 4 °C for 8 min. The RNA was air-dried and re-dissolved in 50 µl (DEPC-treated) RNA'ase-free water.

2.2.2.5 First strand cDNA synthesis.

First strand cDNA, prepared for RT-PCR, was synthesised using a kit, according to the manufacturer's instructions. Approximately 1 µg of total RNA in DEPC-treated H_20 was combined with 1 x first strand buffer (50 mM Tris.HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 10 mM DTT, and 0.5 mM of each of the four dNTPs (dATP, dCTP, dGTP, dTTP), and incubated for 5 min at 65 °C. The reaction mixture was chilled on ice and 1 µl (200 units) of M-MLV reverse transcriptase added, followed by incubation at 37 °C for 1 hr.

2.2.2.6 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 30min. 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 de-ionised water. DNA concentrations were calculated as described below (2.2.2.7) and the oligonucleotides were then stored at -20 °C until required.

2.2.2.7 Quantitation of nucleic acid concentrations

Nucleic acids were quantified by spectrophotometric determination of their UV light absorbency. 5 µl of sample was added to 495 u1 of de-ionised water and the

absorbency of the solution measured at 260 nm and 280 nm in a quartz cuvette, using de-ionised water 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, 40 μg/ml for RNA, 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.

2.2.3 Polymerase chain reaction (PCR) protocol and analysis of amplification products

2.2.3.1 PCR

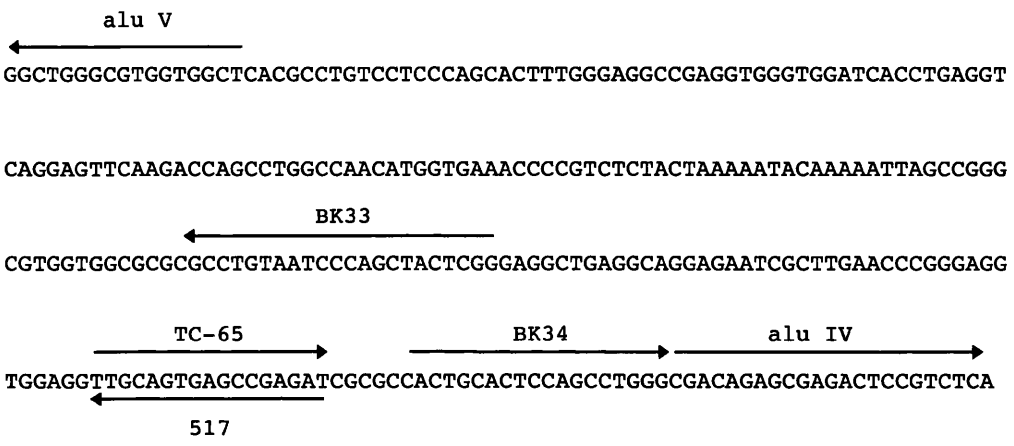
The following conditions were used to perform the majority of PCR amplifications both for allele loss studies, STS content mapping, and SSCP analysis, occasional slight alterations (to the annealing temperature or concentration of magnesium ions) were required for optimal results. 100 ng of human or yeast genomic DNA or 10 pg bacterial clone DNA was subjected to PCR amplification using 1μM oligonucleotide primers, 1.5 mM $MgCl_2$, 50μM dNTPs, 1 x reaction buffer (50 mM KCl, 10 mM Tris.HCl pH 8.0), and 1 unit *Taq* polymerase in a total reaction volume of 25 μl. 1 μCi [α^{32}P]-dCTP per reaction was included for *in situ* radiolabelling of amplification products when desired. 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). Following PCR, amplification products were digested where appropriate by direct addition of 10 units of restriction enzyme without change or modification of the buffer and incubated for several hours at the appropriate temperature.

2.2.3.2 Inter*Alu* PCR

PCR amplification of DNA regions between opposed *Alu* repeats (inter*Alu* PCR) (Figure 2.1) was performed according to Nelson *et al.* (1989). PCR was carried out in a total volume of 50 μl with 100 ng of YAC DNA or 10 pg of cosmid DNA, 1 μM of the oligonucleotide primers shown in Table A3 (with the exception of 517 used at a

concentration of 0.1 μM), 1 x reaction buffer, 300 μM dNTPs, and 2.0 units of *Taq* polymerase for 35 cycles of 94 °C denaturation (1 min), 55 °C annealing (45 s), and 72 °C extension (3 min). Initial denaturation was 4 min at 94 °C.

A)



B)

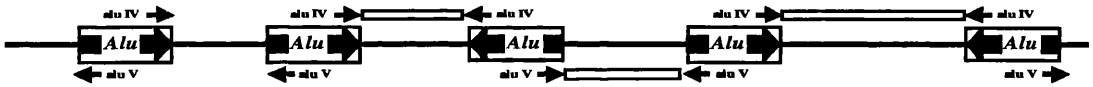


Figure 2.1. Consensus *Alu* sequence and location of primers for amplification (A). The consensus *Alu* sequence is derived from Kariya *et al.* (1987). PCR primers are shown as arrows to indicate 5′ to 3′ orientation relative to the *Alu* sequence. Primers TC-65 and alu IV possess 5′ extensions, which include a *Not* I cloning site and an *Eco*R I cloning site respectively. Scheme for amplification (B). After Nelson *et al.* (1989).

2.2.3.3 Denaturing polyaclylamide gel electrophoresis.

Radiolabelled PCR products from amplified polymorphic markers were resolved on 6% (w/v) polyacrylamide gels under denaturing conditions. A gel solution was prepared from Sequagel stock solutions which contained 6% (w/v) acrylamide, 0.2% (w/v) bisacrylamide, 1 x TBE, and 8 M urea. 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 was allowed to set at room temperature. Gels were pre-run at 100 W for 45 min prior to the loading of samples to warm the gels to approximately 50 °C. 5μl PCR

product was mixed with 5 µl of STOP buffer (95% formamide, 200mM EDTA pH 8.0, 0.01% (w/v) xylene cyanol and bromophenol blue) and denatured by heating at 94 °C for 10 min, followed by quenching on ice. 3 µl was then subjected to electrophoresis at 100 W for 2-3 hr depending upon the size of the PCR product. The plates were then separated and the gel transferred to a sheet of Whatman 3MM paper, followed by drying under vacuum at 80 °C for 45 min, prior to detection of PCR products by autoradiography using x-ray film in a cassette with intensifying screens at -70 °C.

2.2.3.4 Tumour allele loss studies and segregant deletion analysis

100 ng template DNA from paired normal (peripheral blood) and surgically excised breast carcinomas, or SUSM-1 and segregant cell line DNA were subjected to PCR using the standard reaction conditions above (2.2.3.1); amplification products were radiolabelled *in situ*. 30 cycles of amplification was determined to be in the linear part of the amplification process (*i.e.* before product saturation; data not shown), permitting the assumption that the ratio of the optical densities arising from two alleles would be the same for both normal and tumour DNA samples if no LOH occurred. PCR products were resolved on 6% (w/v) polyacrylamide denaturing gels and visualised by autoradiography (section 2.2.3.3). Allele loss was determined by visual examination of autoradiographs.

2.2.3.5 Non-denaturing polyacrylamide gel electrophoresis

For non-denaturing polyacrylamide gels, a 30 % (w/v) stock solution of acrylamide, comprising 29 parts acrylamide to 1 part N,N'methyl-bisacrylamide was diluted to give a 6% (w/v) gel forming solution containing 1 x TBE and 3% (v/v) glycerol. This 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. After mixing, the solution was poured into vertical plates with 0.4 mm spacing and allowed to set at room temperature. 4 µl PCR product was mixed with 8 µl of STOP buffer and denatured by heating at 94 °C for 10 min, followed by quenching on ice. 3 µl was then subjected to electrophoresis at a constant 400 V for run lengths (of several hours) determined by the migration of the xylene cyanol dye front and the size of the PCR product. Gels were

fixed by heating at 80 °C under vacuum, and exposed directly to X-ray film for detection by autoradiography.

2.2.3.6. SSCP-heteroduplex analysis

For SSCP-heteroduplex analysis, 100 ng DNA prepared from tumours, tumour-derived cell lines, or immortal SUSM1/*Hytk7* segregant cell lines were subjected to PCR using the standard reaction conditions above (2.2.3.1). Radiolabelled amplification products were then resolved on 6% (w/v) non-denaturing polyacrylamide gels (section 2.2.3.5) and visualised by autoradiography. Electrophoretic mobility shifts in either single or double stranded DNA products were visually determined from the autoradiographs.

2.2.3.7 Automated chain terminator sequencing.

Both cloned DNA and PCR products were sequenced using a Biosystems ABI 373A automated DNA sequencer operated as a core service by staff at the BICR. 0.3–0.5 μg of plasmid or 50–100 ng of a PCR product was mixed with 20 ng of sequencing primer, 8 μl of 'Dyedeoxy' reaction mix in a total reaction volume made up to 20 μl with water. DNA was subjected to 'cycle sequencing' in a DNA thermal cycler (Perkin Elmer Cetus) for 25 cycles (each cycle comprises 15 s at 96 °C to denature DNA, 1 s at 50 °C for annealing, and 4 min at 60 °C to extend), and the products were then ethanol precipitated, washed with 70% (v/v) ethanol, and air dried prior to being re-suspended in loading buffer (95% (v/v) formamide, 25 mM EDTA pH 8.0, 1.5 mg/ml dextran blue). Samples were then denatured by heating at 94 °C, chilled on ice, and subjected to electrophoresis as advised by the manufacturer. Sequence was analysed using the Sequencing Analysis program v3.0.

2.2.4 Recombinant DNA techniques

2.2.4.1 Restriction digestion of bacterial clone or genomic DNA

Approximately 1 μg of plasmid DNA, 5 μg of cosmid or PAC DNA, or 10–20 μg of mammalian genomic DNA was digested in a final volume sufficient to dilute the

volume of restriction enzyme added 10-fold. Bacterial clone digests were routinely performed using 10 units of enzyme per μg of DNA in each reaction and were incubated at the recommended temperature for at least 1 hr. Genomic DNA was digested overnight with at least 10 units of enzyme being added for each μg of DNA to be digested. A fresh aliquot of enzyme (10 units/μg DNA) was added to genomic digests the following morning and the digest continued for a further 3 hrs. Digest reactions were stopped by the addition of EDTA to a final concentration of 20 mM. Genomic DNA was ethanol precipitated as previously described and then air dried prior to being resuspended in an appropriate volume of TE for subsequent loading on an agarose gel. For digests of DNA requiring the addition of two restriction enzymes, either a buffer compatible for both enzymes was selected or the DNA was digested in sequential reactions separated by ethanol precipitation.

2.2.4.2 Ligation of DNA/PCR fragments into plasmid DNA

Plasmid DNA was digested as described above. The DNA fragment to be inserted was also digested as above to produce blunt ends or complementary sticky ends with the vector, and then isolated by gel electrophoresis and purified as described in Section 2.2.5.3. To prevent the restricted plasmid DNA from re-ligating without an insert, it was first dephosphorylated. Plasmid was digested with the required restriction enzyme in the presence of 5 units calf intestinal alkaline phosphatase. Enzyme was then removed by extraction with an equivalent volume of equilibrated phenol/chloroform, followed by a chloroform extraction, and plasmid DNA was then ethanol precipitated. For ligation, 100 ng of vector and two times the molar amount of insert DNA were mixed on ice in a reaction containing ligase buffer (66 mM Tris.HCl pH 7.5, 5mM $MgCl_2$, 1 mM ATP, 1 mM DTT) and T4 DNA ligase (5-10 units), and incubated at room temperature for a minimum of 3 hours. PCR products were cloned using a TA-cloning kit according to the manufacturer's instructions.

2.2.4.3 Transformation of bacterial cells with recombinant plasmid DNA

Competent *E. coli* (DH5α [*supE*44 Δ*lac*U169(φ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1], INVαF′ [F′ *supE*44 Δ*lac*U169(φ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1]) were thawed on ice, and aliquots transferred to pre-

chilled 1.5 ml screw-cap microcentrifuge tubes. Approximately 10 ng of recombinant plasmid DNA was added to the cells and gently mixed 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, 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 briefly pulsing in a microcentrifuge, 450 µl of supernatant was discarded and the cells resuspended in the remaining 50 µl. Cells were then spread on L-broth plates containing 1.5% (w/v) agar supplemented with the appropriate antibiotic. Plates were incubated in an inverted position overnight at 37 °C.

2.2.4.4 Colony lifts

Hybond N membranes were layered onto plates containing recombinant *E. coli* and left for 1 min. Five orientation marks were made with a needle through the membrane into the agar. After transfer, the filter was carefully lifted ensuring not to drag the membrane across the plate, and inverted over 3MM filter paper wetted with denaturation solution (1.5 M NaCl, 0.5M NaOH) for 5 min, and then transferred to 3MM filter paper wetted with neutralisation solution (1.5 M NaCl, 0.5M Tris.HCl pH 8.0) for 5 min. Finally, the filter was rinsed in 2 x SSC, briefly blotted with 3MM filter paper, and UV-crosslinked using a UV Stratalinker 1800 (Stratagene). Filters were probed as described below (Section 2.2.5.6) and exposed overnight to Kodak X-OMAT AR film with intensifying screens at -70 °C. The films were orientated against the filters using the needle marks, and positive clones determined using the strongest signals on the film. Plasmid DNA was prepared from positively hybridising colonies as described in Section 2.2.2.3.

2.2.5 Blotting and hybridisation protocols

2.2.5.1 Agarose gel electrophoresis of restriction digested bacterial clone or genomic DNA and unlabelled PCR products

DNA restriction fragments from plasmids or genomic DNA or unlabelled (cold) PCR products were separated on non-denaturing agarose gels and visualised by staining

with ethidium bromide and UV transillumination. Typically, gels were prepared by dissolving between 1 and 2% electrophoresis grade agarose in 1 x TAE (40 mM Tris.HCl pH 8.0, 20 mM sodium acetate, 2 mM EDTA). 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) prior to 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, either a *Hind* III digest of bacteriophage λ DNA or an *Hae* III digest of bacteriophage ϕX174 DNA. DNA was visualised by UV transillumination and the gel photographed.

2.2.5.2 PFGE

YAC chromosomal DNA was resolved and isolated by PFGE. Blocks of LMP agarose containing YAC DNA (Section 2.2.2.2) were attached to the teeth of a comb by a bead of molten 1% (w/v) agarose in 0.5 x TBE cooled to 55 °C. The remainder of the molten agarose was then cast around the comb in a gel former, and allowed to set. Once the gel had set, the comb was removed leaving the LMP agarose blocks in place. Samples along with *S. cerevisiae* size standards were then subjected to PFGE in a contour clamped homogeneous electrical field (CHEF) apparatus (Bio-Rad Laboratories, Hercules, CA, USA) with a field strength of 6 V/cm over a 24 hr run time, an initial switchtime of 60 s and a final switch time of 120 s. 0.5 x TBE, used as running buffer, was chilled to 14 °C and constantly recirculated. Following electrophoresis, the pulse-field gel was stained with 5 μg/ml ethidium bromide in dH_2O, and DNA visualised by UV transillumination.

2.2.5.3 Punfication of DNA fragments from agarose gels.

DNA bands of interest were excised from agarose gels using a scalpel with the aid of UV transillumination. The excised DNA was extracted from the agarose using a Geneclean kit according to the manufacturer's instructions. Excised agarose blocks were

weighed and their volumes estimated (1 g = 1 ml), they were then chopped into small pieces to aid extraction. 2.5 volumes of 6M NaI was added and the agarose incubated at 55 °C for 5 min to melt the gel. Following this, 5 µl of glassmilk was added for every 5 µg, or less, of DNA. This was mixed and left at room temperature for 5 min to allow binding. The glassmilk was pelleted by brief centrifugation and washed 3 times by mixing with 300 µl NEW wash (a Tris base and EDTA-buffered solution of NaCl, ethanol, and H_20), pelleting the glassmilk and removing the supernatant. Finally, half the desired volume of TE was added to the cleaned pellet and heated to 55 °C for 5 min. The glassmilk was pelleted and the TE containing DNA removed to a fresh microcentrifuge tube. This was repeated to elute all the DNA. Typically for most applications DNA was eluted in a final volume of 20 µl.

2.2.5.4 Preparation of random-primed radiolabelled probes.

The probes used in this study for the analysis of Northern and Southern blots were obtained as follows:

For *CAVEOLIN-1*, a 1 µl aliquot of phage supernatant (10^7 pfu/µl) from the senescent human fibroblast cDNA library prepared by Dr George Reid was subjected to PCR using the above reaction conditions and 1µM each of the following oligonucleotide primers: L: CATGTCTGGGGGCAAATACG, R: CTTTCTGCAAGTTGATGCGG which amplify the entire open reading frame of *CAVEOLIN-1*.

For *hF11A10.5*, again an aliquot of phage supernatant (10^7 pfu/µl) from the senescent human fibroblast cDNA library was subjected to PCR with 1µM each of the following oligonucleotide primers: L: CGGATGATCCCTTCTCCC, R: GTCATGCTTTCTTGCGGTCC derived from coding sequence.

All probes (with the exception of riboprobes) were radiolabelled with [α^{32}P]-dCTP via the method of random priming using a HighPrime kit. Following each labelling reaction, unincorporated nucleotides were removed by spin-column chromatography using MicroSpin S-200 HR columns according to the manufacturer's

instructions. Before being used in reactions, all double-stranded probes were denatured by addition of an equivalent volume of 0.4 M NaOH.

2.2.5.5 Preparation of riboprobes from cosmid inserts

The cloning site of the cosmid vector, Lawrist 4, is flanked by promoters recognised by T7 and Sp6 bacteriophage RNA polymerases, which can be utilised in the production of radiolabelled *in vitro* run-offs (riboprobes) corresponding to the ends of cosmid clone inserts. Riboprobes were prepared using the Sp6/T7 combination system according to the manufacturer's instructions. 1 μg of cosmid DNA digested with *Rsa* I as described in Section 2.2.4.1 was used as template in a 20 μl reaction volume comprising 1 x transcription optimized buffer, 10 mM DTT, 20 units recombinant RNasin ribonuclease inhibitor, 0.5 mM ATP, GTP, and CTP, 12 uM UTP, 50 uCi [α^{32}P]-UTP, and 20 units of either T7 or Sp6 RNA polymerase. The reaction was incubated at 37 °C for an hour, after which time unincorporated nucleotides were removed by spin-column chromotography.

2.2.5.6 Southern analysis

Following electrophoresis of DNA samples, DNA was depurinated by soaking the gel in freshly prepared 0.25M HCl for 15 min at room temperature with constant shaking. The gel was rinsed in de-ionised H_20 and then soaked for a further 15 min in 0.4 M NaOH to denature the DNA strands. DNA was transferred overnight onto Hybond N+ membrane by upward capillary blotting using 0.4 M NaOH, essentially as described in Maniatis *et al.* (1989).

Membranes were pre-hybridised at 65 °C for four hours in hybridisation solution (5 X SSPE, 5 X Denhardt's, 0.5% (w/v) SDS, 1 mg/ml torula yeast RNA type VI), and then hybridised overnight at 65 °C in a minimal volume of fresh hybridisation solution with added radiolabelled probe (5 x 10^6 cpm/ml). Following hybridisation, probe bound non-specifically to the membrane was removed by washing the membranes twice in 0.2 x SSC/0.1% (w/v) SDS for twenty min at 65 °C, and then once in 0.1 x SSC/0.1% (w/v) SDS for a further twenty min at 65 °C. Excess liquid was removed and membranes wrapped in Saranwrap before being exposed to X-ray film (X-OMAT-AR, Eastman

Kodak Co., Rochester, New York, USA) in a cassette with an intensifying screen at -70 °C for detection of hybridisation by autoradiography.

For successive hybridisations to the same membranes, radiolabelled probe was removed from the membranes by washing in a boiling 0.1% (w/v) SDS solution. Membranes were then agitated at room temperature until the SDS solution had cooled to room temperature. Blots were then wrapped in Saranwrap and stored at 4 °C until further required.

2.2.5.7 Northern analysis

Total RNA samples (20 μg) were mixed with 3 volumes RNA loading buffer (2ml contains 350 μl formaldehyde, 1 ml formamide, 150 μl RNA loading dye {50% (v/v) glycerol, 0.1 mM EDTA, 0.6% (w/v) bromophenol blue, 0.6% (w/v) xylene cyanol} 30 μl ethidium bromide—10 mg/ml stock, 200 μl 5 x Mops buffer containing 0.1 M Mops, 40 mM Sodium acetate, 5 mM EDTA, pH 7.0), and heated for 10 min at 65 °C. The samples were loaded onto a 1% (w/v) agarose gel containing 20% (v/v) 5 x Mops, 17.5% (v/v) formaldehyde. Electrophoresis was performed overnight at 5 V/cm in 1 x Mops buffer which was constantly recirculated. The RNA was visualised by UV transillumination and photographed before the gel was soaked in de-ionised H_20 for 10 min to leach out the formaldehyde.

RNA was transferred to Hybond N membrane by upward capillary blotting, as described in Maniatis *et al.* (1989), using a 20 x SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) solution as transfer buffer. The membrane was UV-crosslinked using a UV Stratalinker 1800 (Stratagene). Membranes were prehybridised in 10 ml of hybridisation solution (200 mM $NaPO_4$, 1mM EDTA, 15% (v/v) formamide, 7% (w/v) SDS, 0.1% bovine serum albumin) at 65 °C for a minimum of 5 hours. The hybridisation solution was then replaced with fresh solution containing the radiolabelled probe (prepared as in section 2.2.5.4) at a concentration of 5 x 10^6 cpm/ml, and hybridised at 65 °C overnight. The membrane was washed at low stringency (2 x SSC, 0.05% (w/v) SDS) several times at room temperature and then at high stringency (0.1 x SSC, 0.1% (w/v) SDS) at 50 °C for 40 min with one change of fresh wash solution. Excess solution was shaken from the membrane, and the blot covered in Saranwrap and exposed to X-ray film at -70 °C with intensifying screens.

Radioactive probe was stripped from the membrane by gentle agitation of the blot for 10 min in dH_2O containing 0.5% (w/v) SDS which had been heated to 100 °C. The solution was allowed to cool before the membrane was removed. The membrane was stored wrapped at -20 °C.

2.2.5.8 Screening the human chromosome 7 cosmid and human PAC library

Nylon membranes supporting a cosmid library prepared from flow-sorted chromosome 7 isolated from a 4X lymphoblastoid cell line, LCL 127 (Nizetic *et al.*, 1994) and a human PAC library constructed from digests of DNA from a male fibroblast cell line (Ioannou *et al.*, 1994) were pre-hybridised at 65 °C for a minimum of four hours in hybridisation buffer (5 x SSPE, 5 x Denhardt's, 0.5% (w/v) SDS, 1 mg/ml torula yeast RNA type VI), and then hybridised overnight at 65 °C with radiolabelled probe in fresh buffer. Prior to hybridisation of the cosmid library with YAC DNA, both probe and filters were competitively hybridised with excess sonicated, denatured human placental DNA in order to suppress non-specific binding through repetitive DNA. Denatured probe was mixed with 10 µl of placental DNA in 0.5 ml of hybridisation buffer, and incubated at 65 °C for 4 hours, while filters were pre-hybridised in the presence of 100 µg/ml placental DNA. Following hybridisation, membranes were washed twice in 0.2 x SSC/0.1% (w/v) SDS for twenty min at 65 °C, and then once in 0.1 x SSC/0.1% (w/v) SDS for a further twenty min at 65 °C. The filters were then exposed to X-ray film overnight at -70 °C in a cassette with an intensifying screen.

2.2.5.9 Western analysis of Caveolin-1 expression

Cells were washed twice in ice-cold PBS, and lysed in a solution comprising 20 mM MES, 150 mM NaCl, 1% triton, 0.1% SDS, 1 mM PMSF, and 2 mM benzamidine, using a disposable cell scraper to assist homogenisation. Lysates were centrifuged at 14,000 rpm for 15 min at 4 °C in a microcentrifuge to pellet cell debris. The resultant supernatants were stored in aliquots at -70 °C until needed. Protein concentrations were determined by performing a bicinchoninic acid colourimetric assay. 10 µl aliquots of a 1:9 dilution of whole cell lysates were incubated with 200 µl of reaction mix (1 volume of 4% (w/v) $CuSO_4$ to 50 volumes bicinchoninic acid solution) at 37 °C for 45 min. Absorbency was measured at 590 nm wavelength light using a Dynatech MR7000

Spectrophotometer. Calibration was performed using a range of BSA standards (80–2000 μg/ml).

For detection of Caveolin-1 by immunoblotting, 20 μg of proteins were resolved by electrophoresis through a 12% (w/v) acrylamide gel containing SDS. 20 ml of a 30% (w/v) acrylamide solution (19:1 acrylamide to bisacrylamide) was combined with 6.25 ml 3 M Tris.HCl pH 8.8, 18 ml water, and 500 μl 10% (w/v) SDS. Polymerisation was initiated by the addition of 250 μl of 20% (w/v) ammonium persulphate and 100 μl of TEMED. This solution was cast to within ~3cm of the top of two glass plates separated by 2mm spacers to allow for the pouring of a stacking gel. Prior to setting butan-1-ol was poured on top of the resolving gel solution to eliminate bubbles that would interfere with the formation of a smooth interface between the stacking and resolving gels. Once the gel was set, the butan-1-ol was washed away with de-ionised water. A stacking gel was then prepared which contained 4% (w/v) acrylamide, 0.125M Tris.HCl (pH 6.8), 0.1% SDS, 0.05% (w/v) ammonium persulphate and 0.002% (v/v) TEMED. This was poured on top of the resolving gel, a comb inserted and the solution allowed to set at room temperature for 15–20 min. After polymerisation, the gel former, comb and gasket were removed and the glass plates containing the gel transferred to a gel tank containing 1 x gel running buffer (50 mM Tris.HCl, 1% (w/v) glycine, 0.25% (w/v) SDS).

Samples were prepared for electrophoresis by mixing 20 μg of protein with an equivalent volume of 2 x Laemmli buffer (100 mM Tris.HCl pH 6.8, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, 5% (v/v) β-mercaptoethanol), boiling the samples for 10 min, chilling briefly on ice, and then loading into the wells of the stacking gel alongside an aliquot of prestained 'rainbow' protein markers (2,350–46,000 Da), employed for protein molecular weight determination. Gels were run at 40 V for several hours. Proteins were then transferred from the gel to ImmobilonP membrane via semi-dry electroblotting for 1 hr using layers of Whatman 3MM paper which had been soaked in blotting buffer (60 mM Tris.HCl, 50 mM glycine, 1.6 mM SDS, 20% (v/v) methanol). Before membranes were probed with antibodies, the efficiency of transfer and the relative loading per lane was assessed by staining the membrane with Ponceau S stain. Membranes were agitated in 10 ml of 1 x stain (0.2% (w/v) Ponceau, 3% (w/v) trichloroacetic acid, 3% (w/v) sulphosalcylic acid in dH_2O) for ~3-5 min and then rinsed with dH_2O until the protein bands became visible. Once analysed, the remainder of the stain was washed away with copious amounts of dH_2O before the membrane was probed as outlined below.

Blots were first blocked for 1 hr in PBS containing 5% (w/v) non-fat milk powder, 0.1% Tween 20, and subsequently incubated in solution with a 1:4000 dilution of an anti-caveolin-1 polyclonal antibody (C13630) for 1 hr. Following incubation with a 1:3000 dilution of a horseradish peroxidase conjugated anti-rabbit Ig secondary antibody, proteins were visualised using enhanced chemiluminescence according to the manufacturer's instructions.

2.2.6 cDNA library screening

A custom HeLa cDNA library constructed in the ZAP II vector was purchased from Stratagene, while a senescent fibroblast cDNA library was prepared by Dr George Reid, in our group, also in the ZAP II vector. Plating and screening of these libraries was carried out essentially as described in the Stratagene instruction manual, outlined briefly below.

2.2.6.1 Preparation of plating cultures

E. coli XLl-Blue (*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1 *lac*$^-$ F′ [*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*(*tet*^{r})]) carry the F′ episome, which is required for both colour selection (not utilised in this case), and the *in vivo* excision process (see section 2.2.6.4). The Tn*l0* tetracycline resistance gene is also located on the F′ episome, therefore, in the presence of tetracycline, the episome is selectively maintained. XLl-blue cells were streaked onto an L-broth agar plate containing 12.5 μg/ml tetracycline, and grown at 37 °C until colonies appeared. A colony was used to innoculate 50 ml L-broth supplemented with 0.2% (w/v) maltose and l0 mM $MgSO_4$ and grown overnight with shaking at 30 °C. This temperature ensures that cells will not overgrow since phage can adhere to dead cells. The culture was centrifuged at 3000 rpm for 10 min, and the pelleted cells gently resuspended in 15 ml of l0 mM $MgSO_4$. The cells were diluted to OD_{600} = 0.5 with l0 mM $MgSO_4$ and stored at 4 °C for 7 days maximum. Approximately 2 ml of cells at OD_{600} = 0.5 was needed for each 245 mm square plate and 600 μl of OD_{600} = 0.5 cells for each l40 mm plate.

2.2.6.2 Titering phage

The Lambda ZAP II phage was diluted in 4 serial 10-fold dilutions in SM buffer (10 mM $MgSO_4$, 50 mM Tris.HCl pH 7.5, 10 mM NaCl, 0.01% (w/v) gelatin). In a 15 ml sterile tube, 600 µl of XL1-blue cells diluted to OD_{600} = 0.5 were mixed with 1 µl of each Lambda phage dilution and incubated at 37 °C for 15 min to allow the phage to attach to the cells. 8 ml molten top agar (0.7% (w/v) agarose in L-broth cooled to 50 °C) was added to the culture-phage mix and poured immediately onto warmed (37 °C) 140 mm L-broth plates. The plates were left to set at room temperature and then incubated at 37 °C overnight. The number of plaques per plate were counted and the titre calculated using the formula:

titre = (no. plaques x 1000 x dilution factor) pfu/ml.

2.2.6.3 Screening cDNA library

For both the HeLa and senescent fibroblast cDNA library, approximately 150,000 pfu were plated on each of four large 245mm square L-broth plates with 2 ml of OD_{600} = 0.5 XL 1-Blue cells/plate and 30m1 top agar/plate. These were incubated overnight at 37 °C. The plates were refrigerated for 2 hours at 4 °C prior to taking lifts (Section 2.2.4.4), as this prevents the top agar from sticking to the nitrocellulose filter. Filters were prehybridised, hybridised and washed as described in section 2.2.5.6. They were then wrapped in Saranwrap and exposed overnight to Kodak X-OMAT AR film with intensifying screens at -70 °C. The films were orientated against the filters using the needle marks, and positive clones determined using the strongest signals on the film. The end of an inverted pasteur pipette was used to core the putative clones from the stock agar plates, and the agar plug placed into an microcentrifuge tube with 500 µl SM buffer and 20 µl chloroform. The tube was briefly vortexed and incubated overnight at 4 °C to elute the phage. The phage stock is stable for 1 year at 4 °C.

For each positive clone, 1 µl of a 200-fold dilution of eluted phage in SM buffer was added to 600ul XLl-Blue cells at OD_{600} = 0.5, incubated at 37 °C for 15 min, mixed with 8 ml molten top agar, and poured onto l40 mm L-broth plates. Transfer to nitrocellulose filters and screening was carried out as described above. This procedure was repeated for a total of three platings, such that single positive plaques were isolated.

2.2.6.4 In vivo excision of the pBluescript phagemid

In vivo excision of the cloned insert is dependent upon the simultaneous infection of XLl-blue cells with both the lambda vector (containing cloned insert) and the M13 helper phage (ExAssist). The F′ episome is required for pili formation, necessary for superinfection with the ExAssist helper phage. The M13 phage proteins recognise the sites of initiation and termination for DNA synthesis, which have been subcloned separately into the Lambda ZAP II vector. The newly synthesised phagemid is secreted from the *E. coli,* and rescued by transformation into SOLR cells. Subsequent bacterial colonies contain the pBluescript double-stranded phagemid with the cloned DNA insert. Helper phage will not grow, since they are unable to replicate in Su^- (non-suppressing) SOLR strains and do not contain ampicillin-resistance genes. SOLR cells are also resistant to lambda phage infection, preventing lambda DNA contamination.

Single plaques, isolated as described above, were placed in 500 µl SM buffer with 20 µl chloroform and incubated overnight at 4 °C. In a 15 ml conical tube 200 µl of OD_{600} = 1.0 XLl-Blue cells, prepared as in section 2.2.6.1, were combined with 100 µl eluted phage stock and 1ul ExAssist helper phage, and incubated at 37^0C for 15 min. After which, 3 ml of terrific broth (for 1 litre: 12g bacto tryptone, 24g bacto yeast extract, 4ml glycerol. After autoclaving add 100 ml solution B {0.17 M KH_2PO_4, 0.72 M K_2HPO_4 }) was added and incubated at 37 °C with shaking for 2½ hours. The tube was heated at 70 °C for 15 min, and centrifuged for 5 min at 4000g. The supernatant containing the pBluescript phagemid, packaged as phage particles, was stored at 4^0C.

To rescue the phagemid, 1 µl supernatant from above was added to 200 µl OD_{600} = 1.0 SOLR host cells, and incubated at 37 °C for 15 min. (The SOLR cells were prepared as described for XL1-Blue cells, see section 2.2.6.1, with the following changes: SOLR cells were streaked on L-broth agar plates containing 50 µg/ml kanamycin. Overnight cultures were grown in L-broth with no supplements.) Following incubation, 10 µl and 100 µl of the phage/SOLR mix was spread onto L-broth plates containing 50 µg/ml ampicillin, and incubated overnight at 37 °C. Minipreparations of plasmid DNA were prepared as described in section 2.2.2.3.

CHAPTER 3

RESULTS

Tumour LOH analysis and clone coverage of a minimally deleted region

3. Results

3.1 Loss of heterozygosity in breast carcinomas

The first task in any positional cloning project is establishing a chromosomal interval within which the gene of interest lies. In the case of TSGs mutated during the development of sporadic tumours, which have not as yet been (and may never be) associated with familial cancer predisposition and so where no form of linkage analysis can facilitate their mapping, then delineating a chromosomal region on the basis of frequent LOH in tumour samples presents a viable alternative (Fearon *et al.*, 1990; Hahn *et al.*, 1996[a]). LOH analysis can detect a wider variety of inactivating events than karyotypic analysis, for example microdeletions and mitotic recombination with a defective chromatid. In addition, due to the abundance of polymorphic markers available, LOH analysis can be used to map TSG loci with higher resolution than is possible with cytogenetics alone. RFLP markers, which were used to establish the first generation maps of the human genome, and which were the original choice for LOH analysis have largely been superseded by simple sequence repeats (SSRs): DNA sequences comprising direct tandem repeats of two, three, or four nucleotides (Weissenbach *et al.*, 1992; Sheffield *et al.*, 1995). The number of repeat units at a particular locus, which translates into a length variation, underlies the polymorphism, and an individual SSR can have many more allelomorphic forms than the two possible for any RFLP, accounting for the generally high frequency of heterozygosity among this class of polymorphic markers. Several genetic and physical maps which utilise these markers have now been established for the whole genome (Murray *et al.*, 1994; Chumakov *et al.*, 1995; Hudson *et al.*, 1995; Dibs *et al.*, 1996; Gyapay *et al.*, 1996), and for individual chromosomes, including chromosome 7 (Bouffard *et al.* 1997). These maps, which are available in electronic form at various web-sites (Materials and Methods), are proving to be invaluable research resources.

Following published reports of LOH on chromosome 7q (Bieche *et al.*, 1992; Kuniyasu *et al.*, 1994; Zenklusen *et al.*, 1994[b]; Zenklusen *et al.*, 1994[c]; Takahashi *et al.*, 1995; Zenklusen *et al.*, 1995[a]; Zenklusen *et al.*, 1995[b]), we selected a number of SSR polymorphic markers from this chromosomal arm to perform LOH analysis with paired normal and breast tumour DNA samples. Initially 25 normal-tumour DNA pairs were examined with SSR markers derived by the CHLC spread evenly across the D7S1797–

D7S1807 interval (corresponding to cytogenetic bands 7q21.1-7qter, or the interval 72.8–165 cM or 430–657 cR from 7pter, a physical distance of ~60–100 Mbp, Table A1). The results are summarised in Table 3.1. From this pilot study, the smallest common deleted region appeared to be the interval between D7S1817–D7S1835 (7q22–7q31.3; corresponding to 495–510 cR from 7pter, a physical distance of ~ 4 Mbp). This interval was selected for further analysis using additional SSR markers published by Genethon, and a total of 88 tumour–normal DNA pairs were examined. Representative examples of LOH using these markers are shown in Figure 3.1, and the results are summarised in Table 3.2. Overall 35/88 (39.8%) of tumour samples displayed LOH at one or more informative markers; the most frequently deleted marker was 17TA-5/17B-RE3, which was lost in 18/55 (32.7%) of informative cases. (17TA-5/17B-RE3 maps to intron 17b of the *CFTR* gene [Zielenski *et al.*, 1991].) From the distribution of losses, the D7S522–17TA-5/17B-RE3 interval (7q31.1; 125.1–125.2 cM) emerges as a region that potentially harbours a TSG locus. From available mapping information, this interval is estimated to be less than 1 Mbp in extent.

3.2 Clone coverage of the D7S522–17TA-5/17B-RE3 interval

3.2.1 YAC clones

The second stage in positional cloning is establishing clone coverage of the defined interval. YACs (Burke *et al.*, 1987), which can be used to propagate large inserts of genomic DNA (in the case of CEPH megaYACs inserts can exceed 1 Mbp), have greatly facilitated positional cloning projects by allowing rapid assembly of cloned DNA fragments which are contiguous and reconstitute a large interval. The WICGR-CEPH YAC STS content map was searched for CEPH megaYAC clones encompassing the D7S522–17TA-5/17B-RE3 interval. Four YAC clones were selected on this basis: 746-h5, 905-g2, 921-b4, and 976-b5. An STS content map for these four clones is shown in Figure 3.2. It is apparent from the STS content of clone 921-b4 that this YAC has undergone an interstitial deletion whose extent can be estimated to be 200-300 Kbp. Yeast chromosomes were resolved in a 1% agarose gel using pulse-field electrophoresis in a contour-clamped homogeneous electric field

Tumour	Polymorphic marker												
	D7S1797	D7S821	D7S796	D7S1799	D7S1841	D7S1817	D7S2847	D7S2203	D7S1835	D7S1801	D7S2197	D7S1837	D7S1807
1	R	R	R	R	R	R	R	R	R	NI	R	NI	R
2	R	R	R	R	NI	NI	R	R	R	R	R	R	R
3	R	R	L	L	R	L	L	NI	R	R	NI	R	R
4	R	R	R	R	NI	R	L	L	L	L	R	R	R
5	R	R	R	NI	R	R	NI	R	R	NI	NI	R	R
6	R	R	R	R	R	R	R	R	R	R	R	R	R
7	R	R	R	NI	R	NI	R	NI	NI	NI	R	R	NI
8	R	NI	R	R	R	R	R	NI	NI	R	NI	R	R
9	R	R	R	NI	R	R	R	NI	NI	R	R	NI	R
10	R	R	R	R	R	R	R	R	R	R	R	R	R
11	NI	R	R	R	R	NI	NI	L	R	R	NI	R	R
12	R	R	R	NI	R	L	L	R	R	R	R	R	R
13	NI	R	R	R	R	NI	R	NI	NI	R	R	R	R
14	R	R	R	NI	R	R	NI	R	R	R	R	R	NI
15	R	R	R	R	R	R	R	R	R	R	NI	R	R
16	R	NI	R	NI	R	NI	R	R	R	R	R	R	R
17	NI	NI	R	R	R	R	R	R	R	R	NI	R	NI
18	R	R	R	R	R	R	L	L	R	R	L	R	R
19	R	R	R	R	R	R	R	R	R	R	R	R	R
20	R	R	R	R	R	NI	NI	NI		R	R	R	R
21	R	R	R	R	R	R	R	R	R	NI	NI	R	R
22	R	NI	R	R	R	R	R	R		R	R	R	R
23	R	R	R	R	R	R	R	R	L	R	R	R	R
24	R	L	L	NI	R	NI	NI	NI	NI	NI	R	L	NI
25	R	R	R	NI	L	L	L		L	L	L	R	R

Table 3.1. Allele loss in breast carcinoma samples between the markers D7S1797–D7S1807. The maximum extent of allele loss that can be deduced for the tumour samples is shaded. L, loss of heterozygosity; R, retention; NI, non-informative.

Tumour	Polymorphic Marker								
	D7S 1817	D7S 486	D7S 522	D7S 633	17TA-5/17B-RE3	AFMA 073ZB9	GATA 44F09	D7S 643	D7S 1835
3	L	L	NI	NI	L	L	L	NI	R
4	R	NI	L	NI	NI	L	L	L	L
8	R	NI	NI	NI	L	R	R	R	NI
14	R	NI	R	NI	L	R	NI	NI	R
15	R	NI	NI	NI	L	NI	R	NI	R
18	R	R	NI	NI	L	L	L	L	R
23	R	R	R	L	NI	L	L	L	NI
24	NI	L	NI	NI	NI	L	NI	NI	NI
25	L	L	L	NI	NI	L	L	L	L
26	L	L	NI		NI	L	L	L	L
30	NI	L	L	R	NI	R	NI	R	NI
31	L	L	NI		L	L	L	L	L
37	L		L	NI	L	L	L	L	R
38	R	R	L	NI	NI	R	R	NI	NI
39	L		L	L	L	NI	L	L	R
42	R	R	L	NI	NI	NI	L	L	NI
43	R	L	R		L	R	L	R	L
45	NI	L	L	NI	L	R	R	R	R
47	L		NI	NI	NI	L	L	L	NI
49	R	L	NI	NI		R	R	NI	R
59	NI	NI	R	R	L	R	R	NI	R
60	R	NI	L	NI	L	L	L	NI	R
62	R	R	NI	NI	L	R	R	R	R
63	NI	R	L	NI	NI	L	R	NI	R
67	NI	R	NI	NI	L	R	NI	R	NI
68	NI	NI	L	NI	L	L	L	R	R
69	R	R	NI	NI	NI	L	R	R	R
78	R	NI		L	L	NI	L	L	L
85	R		NI	NI	L	R	NI	NI	NI
91	R		L	NI	NI	NI	R	R	R
92	R		R	L	L	NI	R	R	R
94	L	L	L	NI	L	L	L	NI	L
97	R	NI	L	NI	L	R	NI	R	NI
100	R	L	L		NI	NI	L	L	NI

Table 3.2. Allele loss in breast carcinoma samples between the markers D7S1817–D7S1835. The maximum extent of allele loss that can be deduced for the tumour samples is shaded. L, loss of heterozygosity; R, retention; NI, non-informative.

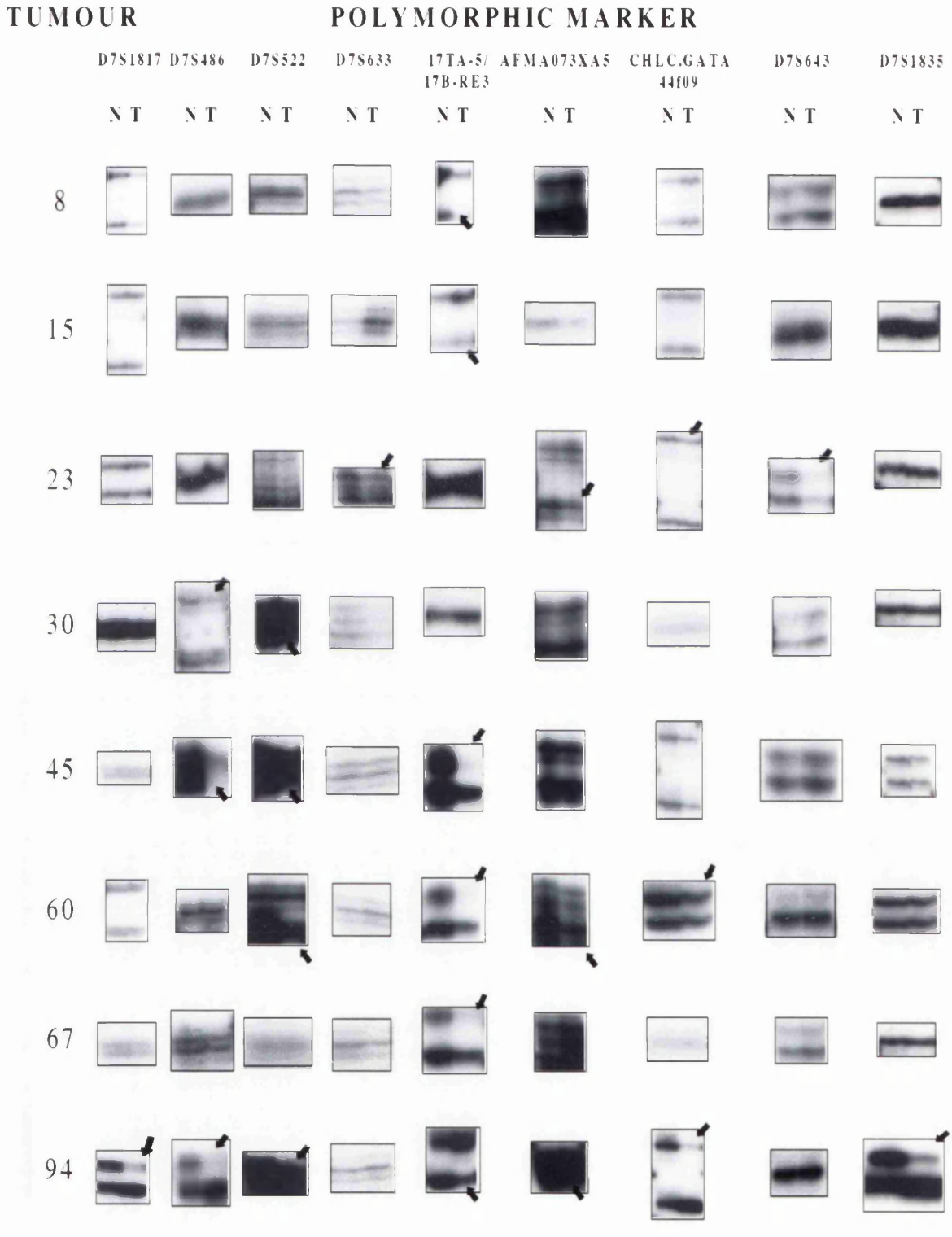


Figure 3.1. Representative examples of breast carcinoma samples showing allele loss. Autoradiographic images for 7q microsatellite markers (shown at the top of respective columns) for matched normal (N) and tumour (T) DNA samples (from cases numbers shown on the left). Arrows point to the loss of an allele (LOH) in tumours compared to corresponding normal DNA.

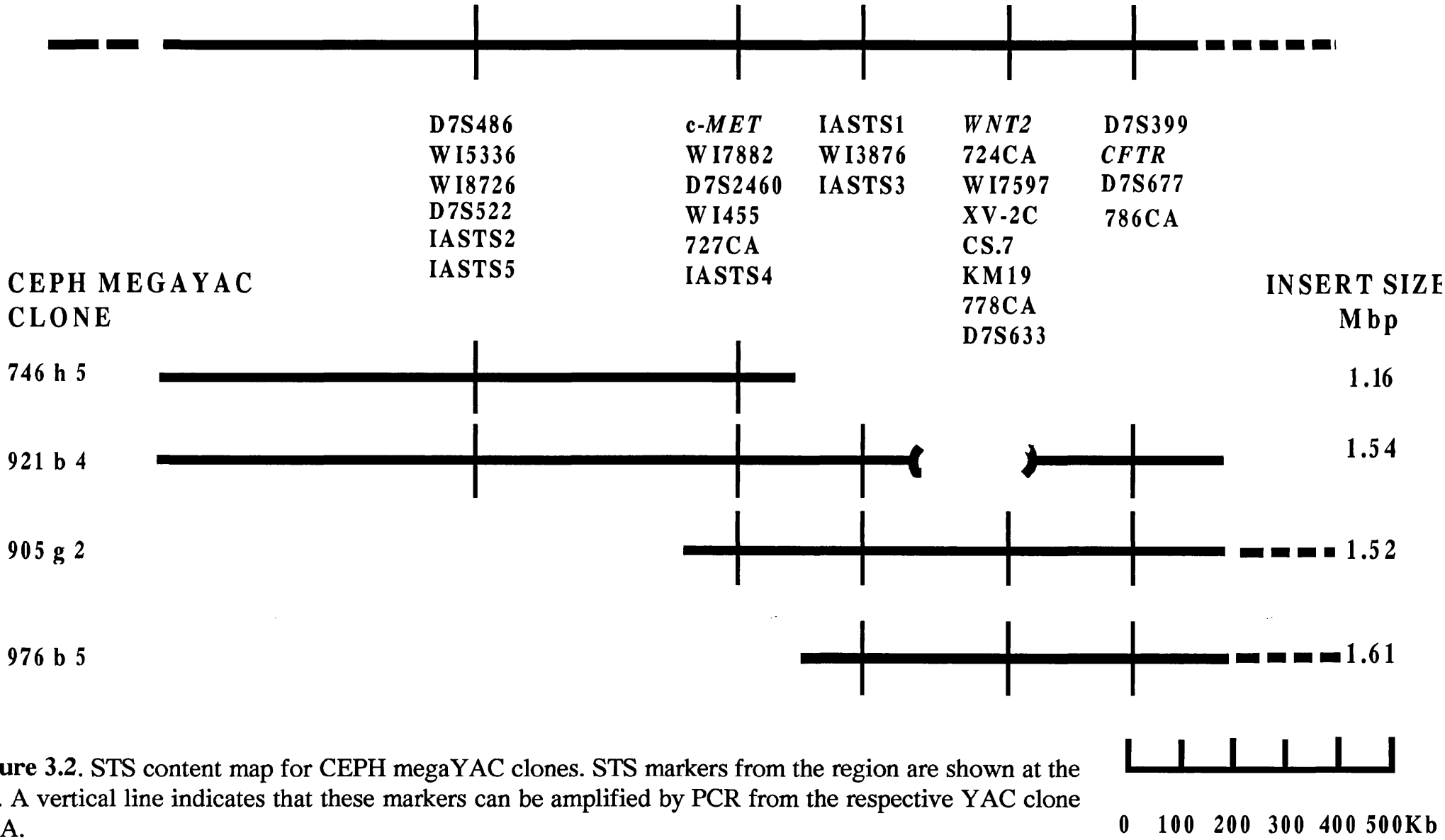


Figure 3.2. STS content map for CEPH megaYAC clones. STS markers from the region are shown at the top. A vertical line indicates that these markers can be amplified by PCR from the respective YAC clone DNA.

(CHEF) apparatus. DNA was transferred onto a nylon membrane and hybridised with radiolabelled probe prepared from human genomic DNA. YAC DNA was revealed by autoradiography in order to establish insert size (Figure 3.3).

3.2.2 Cosmid clones

YACs possess many inherent deficiencies and limitations as cloning vehicles. These include a high rate of chimaerism (co-ligation of non-consecutive DNA fragments—almost 50% in the case of CEPH YACs), insert instability resulting in deletions and rearrangements, and difficulties preparing large amounts of DNA. These failings impede efforts to determine short-range map order, and YAC DNA is difficult to use in the isolation of genes by the methods employed in positional cloning (see Chapter 5). In an attempt to partially overcome these problems, YAC DNA from clones 905-g2 and 921-b4, which between them encompass the entire region defined in the breast carcinoma LOH study, were used to identify cosmid clones corresponding to this interval. Cosmids, plasmid based cloning vectors which possess bacteriophage lambda *cos* sites for *in vitro* packaging of recombinant DNA in phage heads (Collins and Hohn, 1978), may only accommodate 1/20th the DNA of YACs, and certain clones demonstrate instability, nonetheless they surpass YACs in terms of the frequency of chimaerism (which is negligible) and the ease with which they can be prepared in bulk (standard alkaline lysis). The smaller size of cosmid inserts affords the potential to generate finer mapping detail than is possible with YACs.

Radiolabelled YAC DNA was prepared from clones 905-g2 and 921-b4 and hybridised to nylon filters supporting a densely gridded array (144 x 144) of cosmid clone DNA prepared from flow-sorted chromosome 7 isolated from a 4X lymphoblastoid cell line, LCL 127 (Nizetic *et al.*, 1994). The library filters were obtained from the Resource Centre/Primary Database of the German Human Genome project (Lehrach *et al.*, 1990). A total of 114 cosmids were identified in this way (Figure 3.4) and requested from the Resource Centre/Primary Database. The (x,y) co-ordinates of the cosmid clones ordered are given in Table 3.3.

Cosmid DNA was prepared and 5 μg digested with *Pvu* II. Restriction digest fragments were resolved on a 1% agarose gel and then transferred onto nylon membranes and probed with YAC DNA as above. 89% (101/114) of cosmids gave a

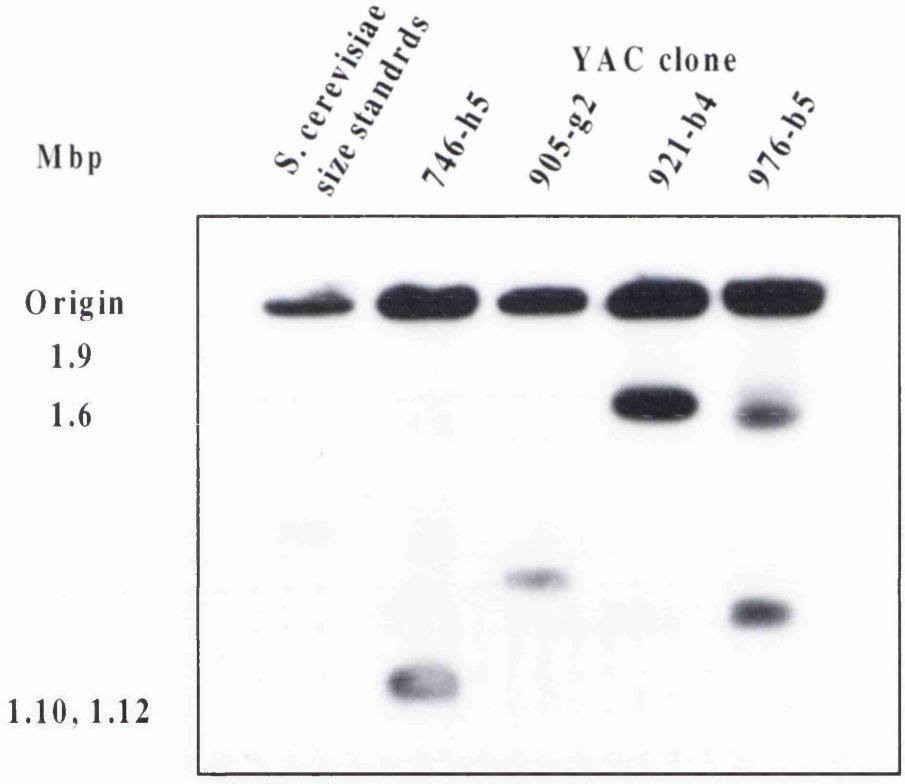


Figure 3.3. Southern blot of CEPH megaYAC clone DNA. Yeast chromosomes were resolved on a 1% agarose gel by pulse-field gel electrophoresis, transferred over-night onto a Hybond N+ membrane, pre-hybridised and probed with total human genomic DNA, radiolabelled by the method of random priming. (The larger [1.61 Mbp] of the two YACs in yeast clone 976-b5 was subsequently determined to be derived from 7q31 by performing PCR analysis of STS content on DNA excised from the pulse-field gel and purified as described in Materials and Methods.)

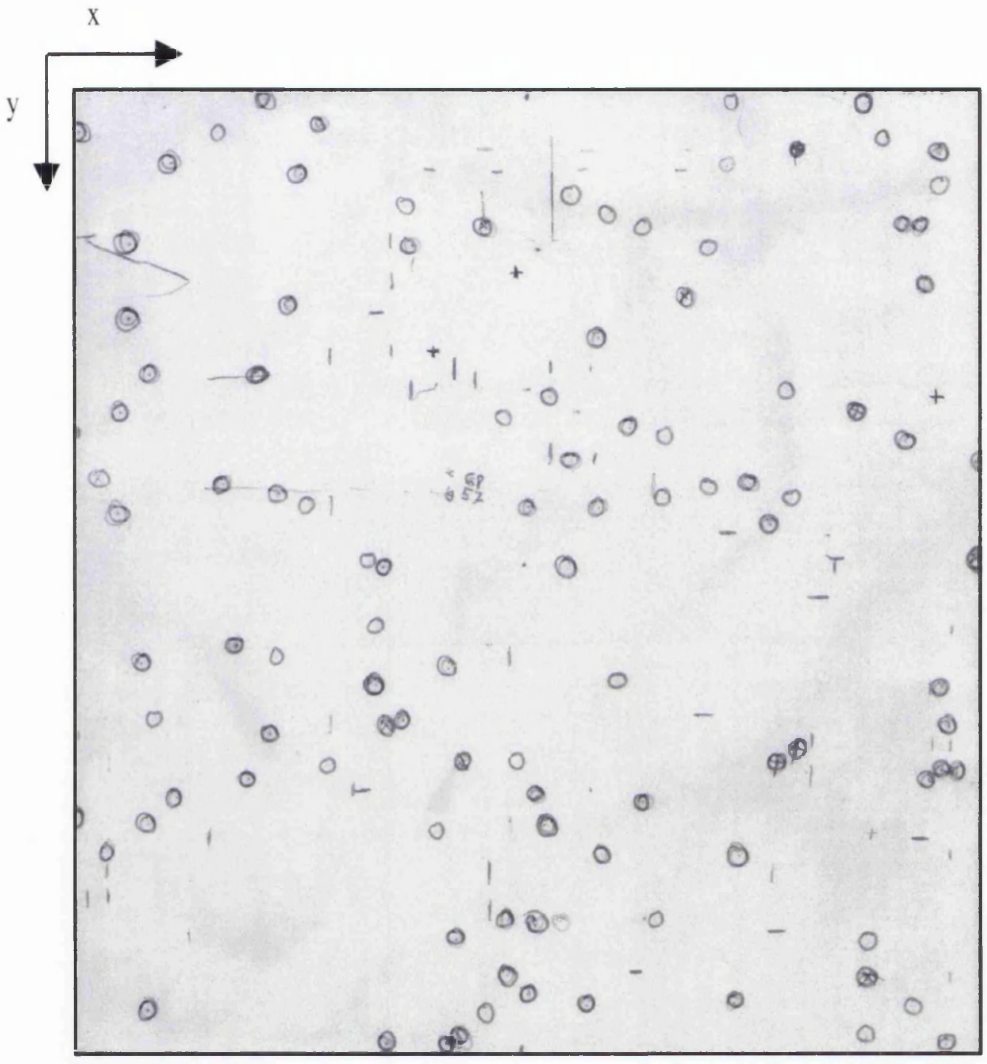


Figure 3.4. Screening of the human chromosome 7 specific cosmid library. Autoradiographs following hybridisation of the library filters with radiolabelled DNA prepared from CEPH megaYAC clones 905-g2 and 921-b4 have been superimposed. Positively hybridising spots have been circled. The origin of the filters is indicated by the the arrows marked x and y.

Cosmid c-	x	y	Clone ICRFc113-	Positive for hybridisation	Positive for STS	Positive for riboprobe	Positive for fingerprint
905-g2 alone							
14	129	99	P0616Q4	-	-		-
71	124	3	P072Q4	-	-		+
83	101	12	M158Q4	-	-		-
90	11	39	D2110Q4	-	-		-
106	67	57	NO21Q4	+	-		-
921-b4 alone							
1	133	14	L0421Q4	+	+	+	-
2	111	45	B1214Q4	+	+	+	+
3	85	89	C2025Q4	+	-		+
4	69	50	P0232Q4	+	-	+	-
5	102	2	P1533Q4	+	+	+	+
6	84	19	J2152Q4	+	-		+
7	66	139	B0355Q4	+	+		+
8	34	85	D1338Q4	-	-		-
9	125	7	N07464Q4	+	-		+
11	70	101	O0124Q4	+	+	+	-
18	89	21	J198Q4	+	-	+	+
19	53	18	K0710Q4	+	-		+
20	130	138	C053Q4	-	-		-
21	49	81	F081Q4	-	-		-
23	92	52	O1850Q4	+	+		+
34	91	125	G1822Q4	+	+	+	+
35	99	24	I1614Q4	+	+		+
67	42	102	O1118Q4	+	+	+	+
68	99	60	M1619Q4	+	+	+	+
69	78	16	K2352Q4	+	+		-
70	92	61	L1850Q4	+	+	+	-
73	58	112	K0542Q4	+	-		+
77	25	7	N1641Q4	+	+	+	-
78	34	61	L1338Q4	+	+		+
107	16	95	A1920Q4	+	+		+
108	111	61	L1256Q4	+	+	+	+
905-g2 and 921-b4							
10	140	55	N0250Q4	+	-	+	-
12	39	66	K1213Q4	+	-		-
13	60	144	A0518Q4	+	+	+	+
15	75	111	L2416Q4	+	+		+
16	9	115	J2255Q4	+	+	+	+
17	15	139	B2055Q4	+	-		+
22	62	142	A0449Q4	+	-	+	-
24	134	102	O049Q4	+	+	+	+
25	51	96	A0813Q4	+	+	+	+
26	26	60	M167Q4	+	+	+	+
27	82	63	L216Q4	+	+		+
28	75	46	A2452Q4	+	+		+

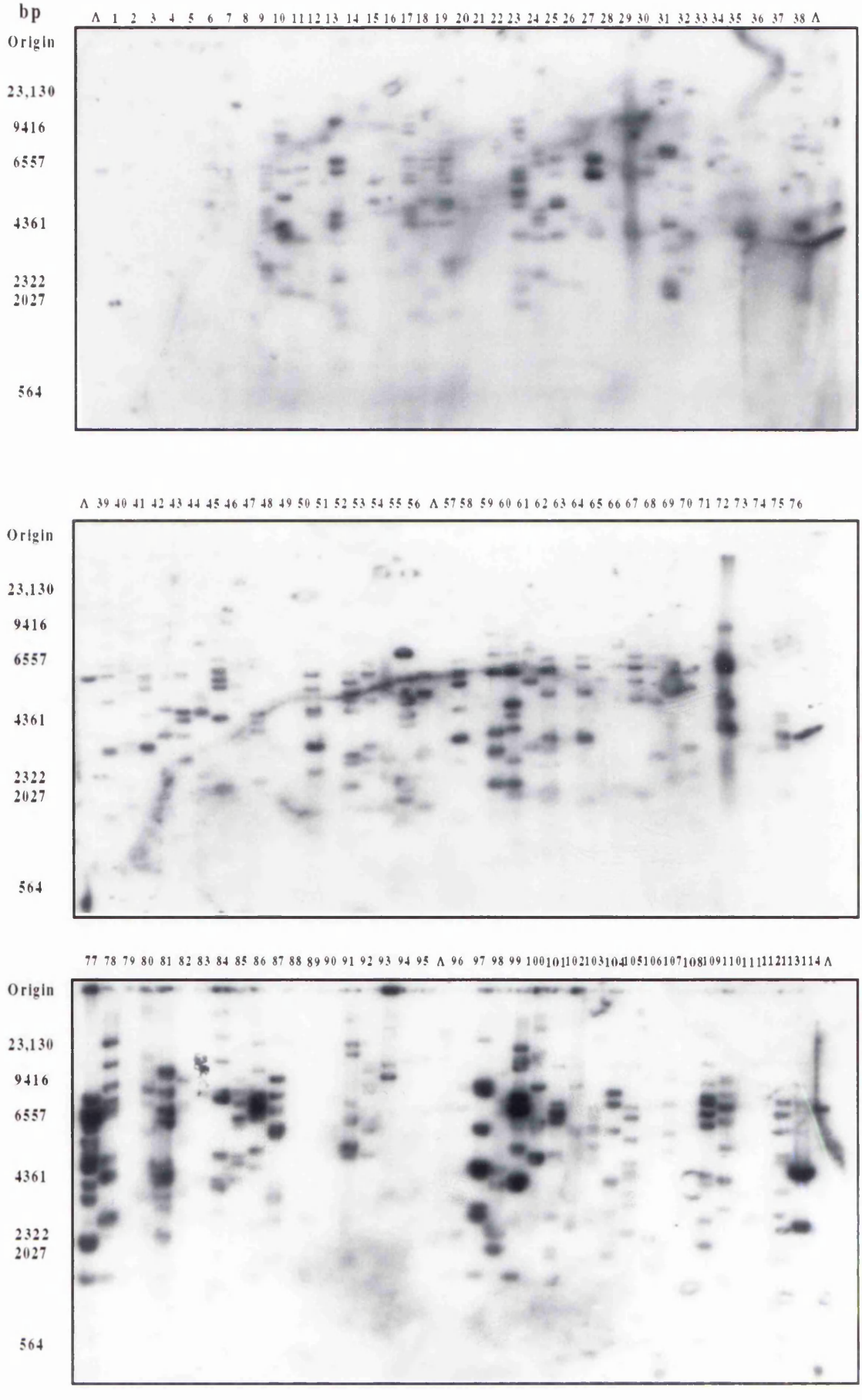
29	132	103	N0553Q4	+	-		-
30	4	7	N2341Q4	+	-		+
31	139	70	I0243Q4	+	+	+	+
32	73	106	M2440Q4	+	+		+
33	103	115	J1440Q4	+	-		-
36	68	57	N027Q4	+	-		+
37	14	87	D207Q4	-	-		-
38	135	95	A0437Q4	+	+	+	+
39	141	53	O0237Q4	+	+	+	+
40	3	98	P2436Q4	+	+	+	+
41	30	104	N1536Q4	+	-		+
42	10	64	K2138Q4	+	+	+	+
43	10	49	P2138Q4	+	+		-
44	15	43	B2054Q4	-	-		-
45	69	133	D0255Q4	+	+		+
46	131	29	G0527Q4	+	-	+	-
47	123	133	D0853Q4	+	+		-
48	77	125	G2328Q4	+	-		-
49	89	107	M1928Q4	-	-	+	-
50	53	95	A0726Q4	+	+		+
51	128	20	J0627Q4	-	-		-
52	61	128	F0424Q4	+	+		+
53	82	37	D2139Q4	+	+	+	+
54	12	24	I2117Q4	+	-	+	+
55	15	111	L2018Q4	+	-		+
56	18	12	M1917Q4	+	-	+	+
57	72	136	C0155Q4	+	+		+
58	33	97	P1455Q4	-	-		+
59	103	137	C1422Q4	+	+	+	+
60	32	2	P1429Q4	+	+	+	-
61	121	48	A082Q4	+	+	+	+
62	108	65	K1337Q4	+	+		+
63	18	11	M1935Q4	-	-		-
64	12	35	E2135Q4	+	+	+	+
65	60	87	D0513Q4	+	+	+	+
66	72	63	L0113Q4	+	+		+
72	49	90	C081Q4	+	+	+	+
74	83	115	J2147Q4	+	-		+
75	73	126	G243Q4	+	-	+	+
76	105	59	M1437Q4	+	+		-
79	2	28	G2448Q4	+	+		+
80	128	52	O0650Q4	+	+	+	+
81	3	52	O2451Q4	+	-	+	+
82	123	142	A0853Q4	+	-		-
84	40	6	O114Q4	+	+	+	+
85	65	21	J0310Q4	+	-		+
86	134	90	C0412Q4	+	+		+
87	81	138	C2216Q4	+	+	+	+

88	77	72	I2312Q4	-	-		-
89	144	140	B0134Q4	-	-		-
91	133	9	N042Q4	+	+		+
92	112	99	P113Q4	+	+		+
93	112	9	N112Q4	+	-	+	+
94	136	102	O033Q4	+	-		+
95	32	44	B1429Q4	+	-		-
96	109	101	O1222Q4	+	-		-
97	87	51	P2019Q4	+	+	+	+
99	54	24	I0717Q4	+	+		+
100	36	33	F1317Q4	+	+	+	+
101	37	13	L1241Q4	+	+	+	+
102	95	31	F1746Q4	+	-		-
103	135	143	A0434Q4	+	+		+
104	62	101	O0430Q4	+	+	+	+
105	28	84	E151Q4	+	+	+	+
109	69	125	G0236Q4	+	+	+	+
110	50	72	I087Q4	+	+		+
111	4	110	L2324Q4	+	+	+	+
112	19	107	M1824Q4	+	+		-
113	51	144	A0818Q4	+	+	+	+
114	130	20	J0521Q4	+	-		+

Table 3.3. Cosmids identified by hybridisation of YAC DNA to the chromosome 7 cosmid library filters. + indicates the clones was confirmed positive by either hybridisation, STS content mapping, through hybridisation with a ripobrobe generated from another cosmid insert, or by restriction digest fingerprinting.

positive hybridisation signal as revealed by autoradiography (Figure 3.5, Table 3.3). Of five cosmids which were selected owing to their apparent hybridisation with DNA from YAC clone 905-g2 alone, only one (20%) was positive on subsequent screening, this compares to 22/25 (88%) cosmids positive for 921-b4 alone, and 76/84 (90%) with DNA from both clones. It is likely that cosmid clones which were negative on subsequent hybridisation screening resulted from human error in determining the (x,y) co-ordinates of the original hybridisation spots on the library filters, since these cosmids also proved to be negative for any STS or riboprobe (below). This appears to be especially true of the few cosmid clones positive for 905-g2 alone.

Figure 3.5. Hybridisation of YAC clones 905-g2 and 921-b4 to isolated cosmid clones. DNA samples from isolated cosmid clones were digested with *Pvu* II and resolved on 1% (w/v) agarose gels before being transferred onto Hybond N+ membranes. Following pre-hybridisation, cosmid DNA was probed with radiolabelled DNA prepared from YAC clones 905-g2 and 921-b4, and visualised by autoradiography. Λ, lamba phage/*Hind* III molecular weight marker.


bp
Λ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 Λ
Origin
23,130
9416
6557
4361
2322
2027
564
Λ 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 Λ 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76
Origin
23,130
9416
6557
4361
2322
2027
564
77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 Λ 96 97 98 99 100 101 102 103 104 105 106 107 108 109 110 111 112 113 114 Λ
Origin
23,130
9416
6557
4361
2322
2027
564

3.2.3 'Contiguation' of cosmid clones

3.2.3.1 STS content mapping of cosmid clones

Having identified numerous cosmid clones corresponding to the interval of LOH, it was necessary to order the cosmid clones relative to one another and to establish overlaps, *i.e.* to determine which clones contain genomic inserts that are contiguous. This was determined in the first instance by STS content mapping. Various databases were plumbed for possible STSs; two types can be distinguished: those which are polymorphic and therefore of potential use in LOH studies as well, including the Genethon markers already mentioned in addition to several RFLPs which have been adapted to allow PCR amplification (Table A1), and non-polymorphic STSs (Table A2). PCR-amplification of these markers was performed using a standard reaction protocol. To minimise the number of PCR reactions needing to be performed, cosmid DNA samples were first pooled in such a way that each clone appeared in two distinct pools (one each from 1–11 and A–K). Pooled DNA was then subjected to PCR amplification. In the example given for the marker D7S2742 (Figure 3.6), after the first round of screening there is a product in lanes 2, 6, 8, and 11, and D, E, F, I and J, which could have arisen from cosmid clones c35, c39, c41, c44, c46, c50, c52, c55, c57, c61, c63, c66, c90, c94, c96, c99, c101, c105, c107, or c110. The PCR assay was then repeated for all the possible individual DNA samples to establish the identity of the positive clones, which in this example were c41 (8D), c50 (6E), c57 (2F), c99 (11I) and c110 (11J). The results for STS content mapping of the cosmid clones are given in Table 3.4. 57% (66/114) of cosmid clones proved to be positive for one or more STSs from the cloned interval, including novel STSs (below) (Table 3.3). The discrepancy between this value of positivity and that determined by hybridisation of YAC DNA to filters of cosmid DNA most probably reflects the density and non-uniform distribution of the markers available, which does not allow for complete representation of the cloned interval.

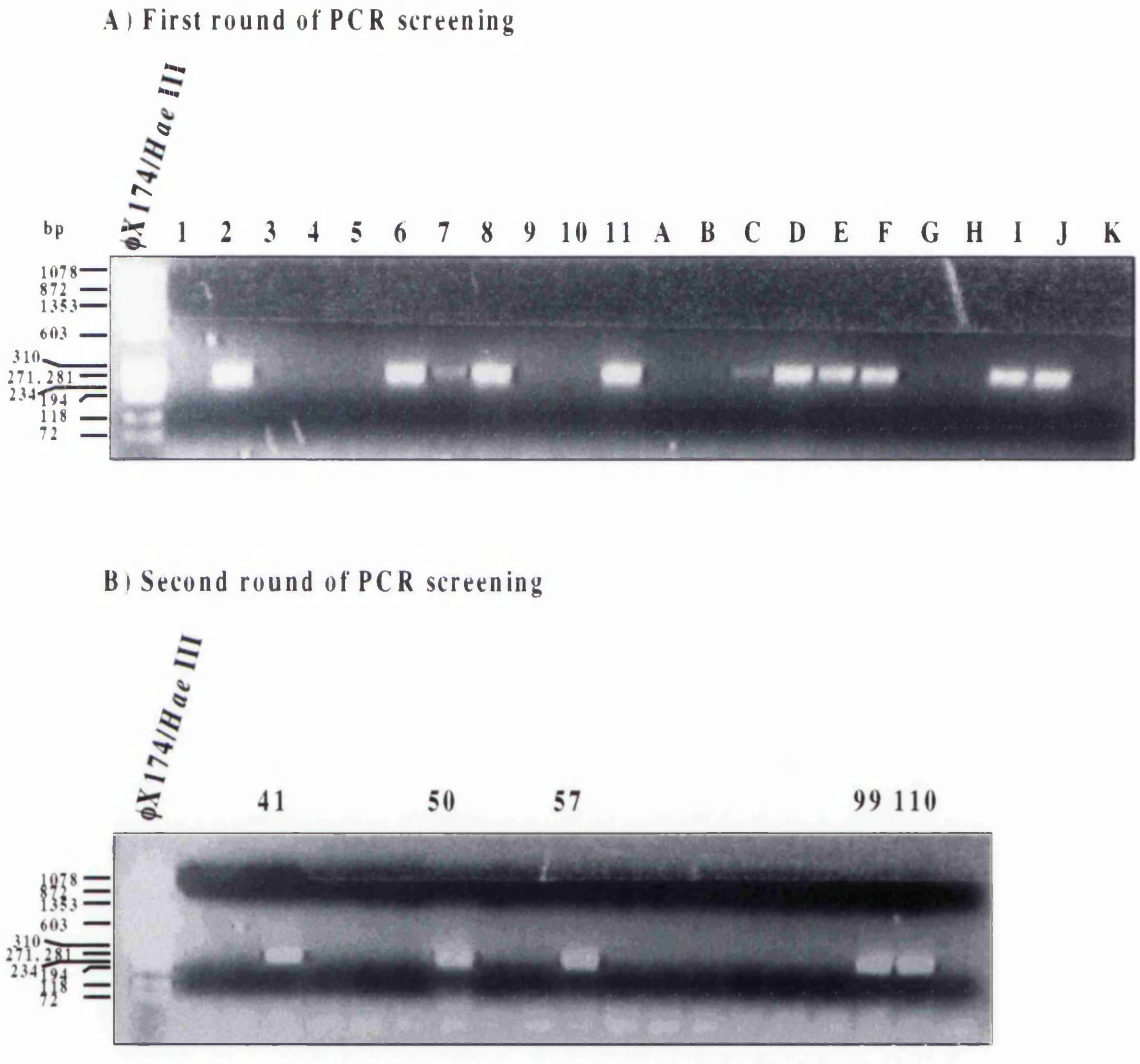


Figure 3.6. STS content mapping of isolated cosmid clones. The D7S2742 STS PCR assay was performed first on pools of cosmid clone DNA, and then on individual putative positive cosmid clones. PCR products were resolved on 2% (w/v) agarose gels and stained with ethidium bromide before visualisation on a UV transilluminator.

Table 3.4. STS content mapping of YAC-selected cosmid clones. Cosmid clone numbers are shown in the left hand column and the STS assay (marker) at the top of the table: 1) D7S486 2) WI-8726 3) WI-5336 4) D7S522 5) WI-10385 6) WI-455 7) D7S2460 8) METD 9) METH 10) 727CA 11) sWSS305 12) 715CA 13) WI-7882 14) IASTS4 15) sWSS2899 16) WI-18209 17) IASTS1 18) sWSS1398 19) sWSS843 20) sWSS3428 21) AFMB316XB9 22) sWSS2710 23) WI-3876 24) sWSS2099 25) IASTS3 26) sWSS377 27) sL12 28) sWSS844/845 29) 778CA 30) Cdaozb11 31) sWSS1948 32) sWSS1948 33) WI-7597 34) XV-2C 35) CS.7 36) D7S23/740CA 37) KM-19 38) D7S633 39) D7S399/sWSS849 40) sWSS850 41) sWSS915 42) CFTRx1 43) D7S677 44) CFTRx3 45) CFTRx4 46) CFTRx6 47) CFTRx10 48) sWSS376 49) CFTRx12 50) 17TA-5/17B-RE3 51) CFTRx20 52) D7S2742 53) 786CA. + indicates clone is positive for this assay.

Cosmid		Marker																																																		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53
135	E2454Q4		+																																																			
77	N1641Q				+					+																																												
70	L1850Q					+																																																
87	C2216Q						+																																															
39	O0237Q						+	+	+																																													
53	D2139Q						+	+	+																																													
28	G2328Q						+	+	+																																													
64	E2135Q						+	+	+																																													
38	A0437Q							+	+																																													
104	O0430Q							+	+																																													
112	M1824Q								+																																													
62	K1337Q						+	+	+	+																																												
31	I0243Q								+	+																																												
35	I1614Q								+	+																																												
69	K2352Q								+	+																																												
80	O0650Q									+																																												
113	A0818Q									+	+																																											
11	O0124Q										+																																											
25	A0813Q										+																																											
27	L216Q4										+																																											
34	G1822Q										+																																											
91	NO42Q4											+																																										
103	A0434Q											+																																										
15	L2416Q												+	+																																								
107	A1920Q													+	+																																							
72	C081Q4									+					+																																							
79	G2448Q																+	+																																				
43	P2138Q																+																																					
7	B0355Q																						+																															
23	O18550Q																						+																															
67	O1118Q																									+																												
111	L2324Q4																																																					
84	O114Q4																										+	+	+																									
78	L1338Q																												+	+	+																							
97	P2019Q									+																				+	+	+																						
108	L1256Q																													+	+	+																						
105	E151Q4																															+																						
13	A0518Q																																																					
24	O049Q4																																																					
59	C1422Q																																																					
105	E151Q4																																																					
60	P1429Q																																	+																				
26	M167Q4																																			+	+																	
40	P2436Q																																			+	+																	
109	G0236Q																																			+	+																	
2	B1214Q																																			+	+	+																
1	L0421Q																																							+														
61	A082Q4																																							+														
65	D0513Q																																							+														
16	J2255Q																																									+	+											
32	M2440Q																																									+	+	+	+									
52	F0424Q																																									+	+	+	+									
76	M1437Q																																													+	+	+						
47	D0853Q																																															+						
92	P113Q4																																																	+	+			
66	L0113Q																																																		+			
100	F1317Q4																																																					
5	P1533Q4																																																			+		
42	K2138Q																																																			+		
68	M1619Q																																																			+		
50	A0726Q																																																			+	+	
99	I0717Q																																																				+	+
110	I087Q4																																																				+	+
41	N1536Q																																																				+	+
45	D0255Q																																																				+	+
57	C0155Q																																																				+	+
86	C0412Q																																																					+

3.2.3.2 Chromosome walking with cosmid insert-end riboprobes

Overlaps between cosmid clones were also established by chromosome walking. The Lawrist 4 vector used in the construction of these cosmids possesses T7 and Sp6 phage promoters flanking the insert DNA, which allow the generation of RNA molecules (riboprobes) corresponding to the ends of the insert when *Rsa* I digested cosmid DNA is used as the template for *in vitro* transcription in the presence of radioactive ribonucleotides (see Materials and Methods). Several cosmids were selected as templates for generating riboprobes. Initially, these were hybridised to Southern blots containing *Pvu* II-digested DNA prepared from the 114 cosmids identified above. Subsequently, the original cosmid library filters were also hybridised with riboprobes when it became clear from STS content mapping that various cosmid clones spanning the region were absent (indeed, 14/56 [25%] of the STS assays attempted failed to identify any positive clones). Positively hybridising cosmids were visualised by autoradiography, and in the case of the library the (x,y) co-ordinates ascertained (Figure 3.7); the results of riboprobing are given in Table 3.5. Cosmids corresponding to novel co-ordinates were requested from the Resource Centre/Primary Database. 21 additional cosmids were requested (Table 3.6). 43% (49/114) of the previously identified cosmids hybridised with one or more of the riboprobes assayed from this sample set. In eight of these incidences the cosmid had not previously been identified as being positive for an STS.

Restriction digestion followed by fluorescent tagging (Brenner and Livak, 1989) was also used to construct cosmid contigs on the basis of a shared restriction digest profile when the fragments were resolved by electrophoresis (Dr Lisa O'Neill, The Sanger Centre, Cambridge, UK). 76 of the 114 original cosmids were placed in 21 contigs of two or more cosmids (the cut-off point is for an approximately 50% or greater overlap; fingerprinting is therefore a conservative measure of the degree of contiguity). 18 of the cosmid contigs could be ordered relative to each other, while 3 contigs were orphans, *i.e.* could not be mapped or orientated because there was no information on STS content (contig 7: c98 and c114; contig 13: c9, c17, c19, c55, and c85; contig 20: c54 and c56). Moreover, there were 20 incidences where a contiguated cosmid had not previously been identified positive by either STS content mapping or riboprobing. Thus overall 94/114 (82%) of the original set of cosmids identified through hybridsation with DNA from the selected YAC clones were confirmed, independently of subsequent

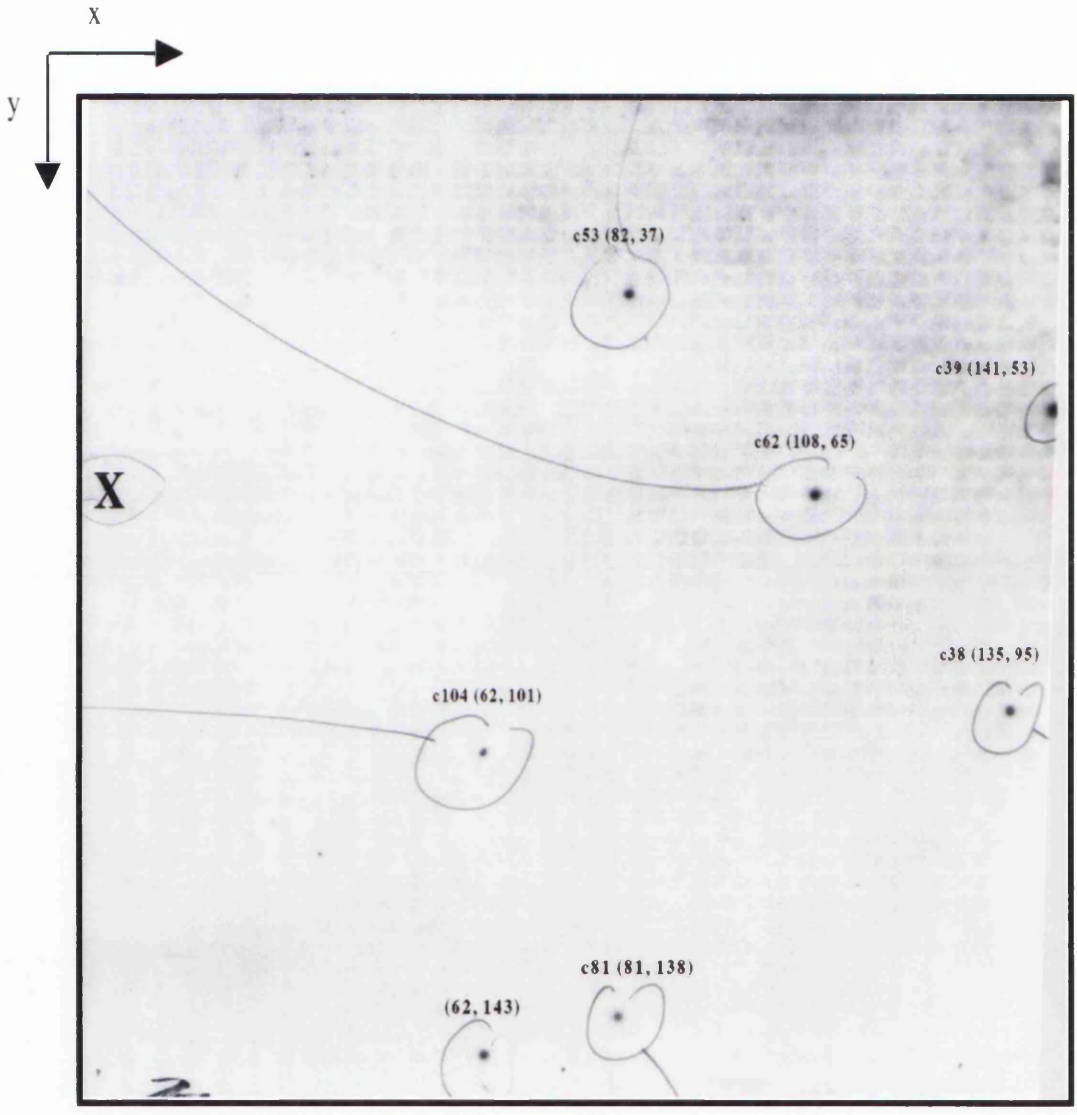


Figure 3.7. Riboprobing the chromosome 7 cosmid library. The cosmid library filter was pre-hybridised and then probed with the c62T7 riboprobe. Positively hybridising spots were visualised by autoradiography; co-ordinates and corresponding clone number are shown. The X conceals a positively hybridising spot from a previous probing.

Riboprobe	Positively hybridising cosmids
c1Sp6	c4, c16
c1T7	c61, c65
c2Sp6	c26, c40, c109, (48,72)
c2T7	c6, (33,97)
c13Sp6	c24, 59, c60, c105
c13T7	c97
c17Sp6	(37,100), (115,32)
c17T7	
c24Sp6	c13, c97
c24T7	c59, c60
c27Sp6	c11, c25, c113
c27T7	
c34Sp6	
c34T7	c75, c34, c49, c10
c35Sp6	(25,105), (52,60), (77,84), (118,66)
c35T7	(25,50), (38,67)
c40Sp6	c2, c26, c109
c40T7	
c50Sp6	c31, c80
c50T7	c5, c42, c68, c80
c54Sp6	c54, c56, (54,18), (59,118), (62,16), (68,107), (87,123), (90,42)
c54T7	c53, c54, c56, c87, (5,11), (112,68)
c60Sp6	
c60T7	c13, c24, c59, c81, c105
c62Sp6	
c62T7	c38, c39, c53, c87, c104, (62, 143)
c65Sp6	c61, c93
c65T7	c1, (45,109)
c68Sp6	c5, c42, c72, c77
c68T7	c70, c100
c77Sp6	(16,14), (25,7), (34,24), (107,38), (118,13), (134,30)
c78Sp6	c84
c78T7	c97, c108
c84Sp6	c18, c101, (1,67), (32,43), (82,25)
c84T7	c78, c97,c108
c87Sp6	c22, c38, c39, c46, c53, c64, c104
c87T7	c54, c56, (48,90)
c97Sp6	c24, c81, c105
c97T7	c78, c84
c101Sp6	c18, c84
c101T7	c67, c111

Table 3.5. Contiguation with riboprobes. The probe name is derived from the cosmid used as template and the bacteriophage polymerase. In the righthand column are shown cosmids which demonstrate positive hybridisation with these probes. In parenthesis are the co-ordinates of clones which hybridise from the filters.

Cosmid c-	X	Y	Clone ICRFc113-
115	87	123	H2016Q4
116	90	42	C1914Q4
117	77	84	E2312Q4
118	54	18	K0717Q4
119	32	43	B1448Q4
120	62	16	K0448Q4
121	68	107	M0230Q4
122	82	25	H2139Q4
123	118	66	K096Q4
124	48	72	I0913Q4
125	33	97	P1455Q4
126	46	25	H0941Q4
127	66	108	M0318Q4
128	12	19	J2154Q4
129	1	67	J2438Q4
130	143	140	B0128Q4
131	52	60	M071Q4
132	25	105	N165Q4
133	38	67	J1244Q4
134	25	50	P1620Q4

Table 3.6. Additional cosmids identified from the chromosome 7 cosmid library through hybridisation with insert-ends generated riboprobes.

hybrisation screening, to correspond to the region cloned within the YACs. Further, all these cosmid clones are assumed to map to 7q31 for the obvious reason that the cosmid library at least is chromosome 7 specific and because the likelihood of two non-contiguous regions of chromosome 7 being co-ligated in the same CEPH YAC clone (derived from total genomic DNA) is remote.

3.3 Deriving novel STS.

3.3.1 Inter*Alu*-directed PCR

In order to facilitate further contiguation of cosmid clones, novel STSs, both polymorphic and non-polymorphic, were sought. A plentiful and accessible source of unique (single copy) DNA sequences, ideal for the generation of random genomic STSs, is contained within regions between closely spaced *Alu* repeats. DNA aliquots from YACs 905-g2 and 921-b4 were subjected to amplification by PCR using oligonucleotide

primers (BK34, TC65, alu IV, and alu V; Table A3) directed against the *Alu* repeat consensus sequence (according to the scheme shown in Figure 2.1 of Materials and Methods). PCR products were then TA-cloned in the pCR2.1 vector. Plasmid inserts from transformed colonies of INVαF′ *E. coli* were sequenced using vector-directed M13 reverse and T7 primers (Table A4). Sequences were edited to remove vector-derived and *Alu*-derived sequences (by performing BLAST homology searches against an *Alu*-sequence database). Finally oligonucleotide primers were designed and synthesised to allow PCR amplification of unique inter*Alu* DNA sequences. Five novel STSs were generated in this way (Table 3.7); PCR amplification from human total genomic DNA using these primers revealed only a single product of the expected size (data not shown).

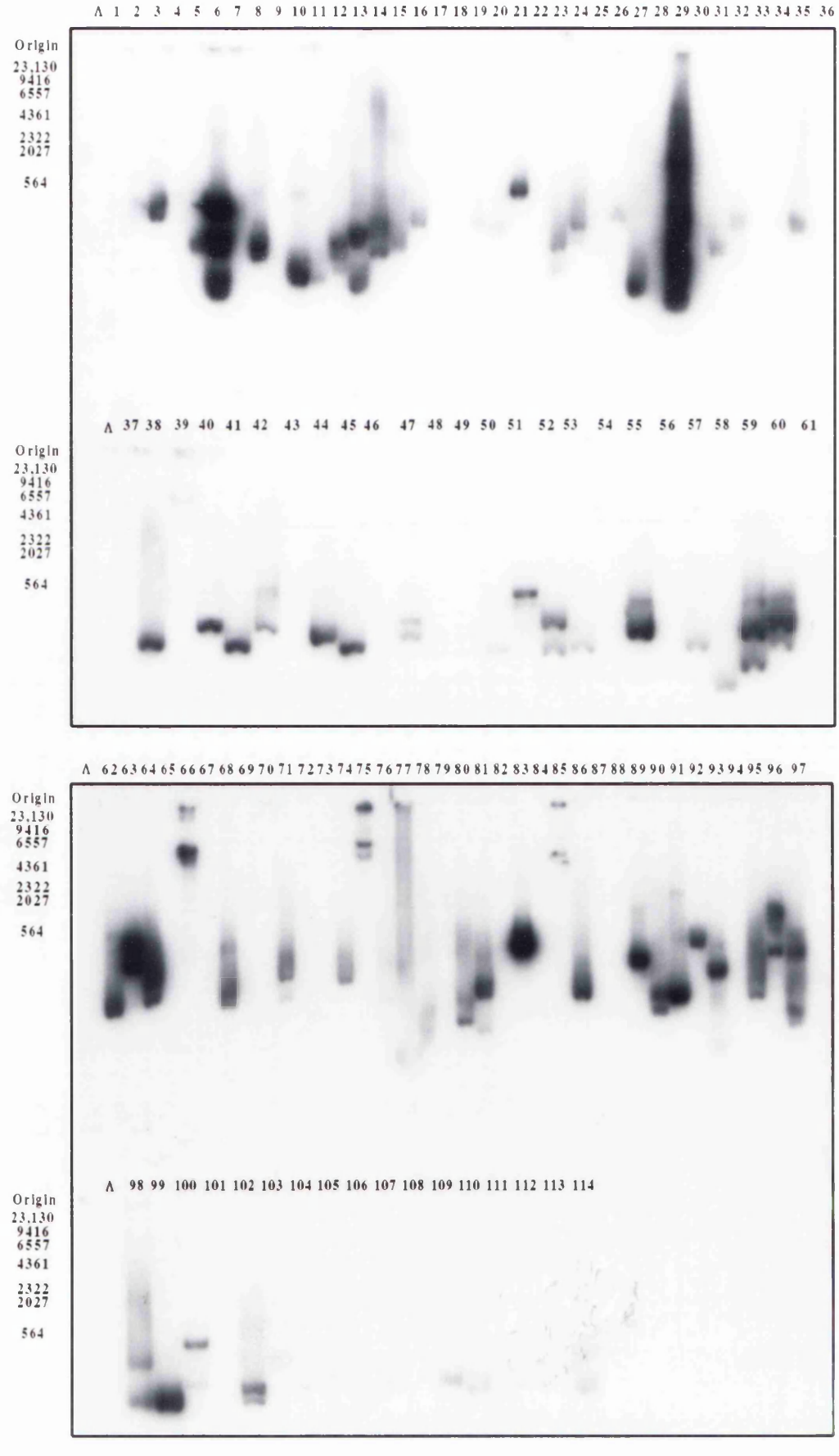
STS	Primers	Size (bp)[a]
IASTS1	L: GAGGAAAGCAGTCATACAGG R: ATGCCTATGTTTCTCACCCAG	869
IASTS2	L: GTTTCATCACCCTCTCAGGG R: GTGGCAGTATCTAAAGGAGC	150
IASTS3	L: TTTTTGAACAGAAGGATGGC R: TTCTATCCAGTGCAGGCTGC	240
IASTS4	L: CAGTATGCCTATTCCAGTTCC R: TGTTTCCTAGGCCGACTGTG	338
IASTS5	L: TTCACCTCCTGCCATGATTGG R: TGAAGGAAATGACAGGGTGG	225

Table 3.7. Primer sequences which amplify novel STS markers from YAC clones covering the 7q31 region, generated by sequencing TA-cloned inter*Alu* PCR amplification products. [a] Sizes for IASTSs are determined precisely from the respective sequences from which the STS was derived. The sequences for IASTS1 and 4 are identical to base-pairs 106,901–107,770 and 49,255–49,593 respectively of a 150,147 bp sequence deposited in GenEMBL (accession number AC002543), while IASTS3 is identical to base-pairs 29407–29647 of a 188,741 bp sequence in GenEMBL (accession number AC002542). The sequences to which these accession numbers refer were generated from 7q31-specific BAC clones as part of the chromosome 7 mapping and sequencing project that is a joint venture between the National Human Genome Research Institute, NHGRI, at the National Institute of Health, NIH, Washington University, and the University of Washington. A continuous update of progress made on this project is accessible on-line through a site on the World Wide Web at http://www.nhgri.nih.gov/DIR/GTB/CHR7/.

3.3.2 Novel CA.GT dinucleotide repeats

SSRs that are CA.GT dinucleotide repeats occur frequently within the human genome—approximately once every 50 Kbp (Weissenbach *et al.*, 1992)—and are generally highly polymorphic, making them invaluable as mapping reagents in LOH studies and equally of service in STS content mapping. We decided that a useful aim would be to clone several novel CA.GT repeats to assist in the cloning of the chromosome 7q31.1 TSG. 5 μg of cosmid DNA was digested to completion with *Sau*3A I restriction enzyme before being electrophoresed on a 1% agarose gel and transferred to nylon membranes. Membranes were then hybridised with a radiolabelled degenerate CA oligonucleotide probe. Positive hybridisation was revealed by autoradiography (Figure 3.8). Digested DNA from cosmids giving positive hybridisation was subcloned in *Bam*H I-restricted pBluescript KS(+/-) phagemid. Colony lifts of transformed DH5α *E. coli* were then hybridised with the degenerate CA probe to identify clones with inserts containing a CA.GT repeat. Plasmid DNA from positive clones was sequenced using KS and SK primers (Table A4) derived from vector sequences. The sequences of 8 novel CA.GT repeats are given in Figure 3.9 and primers designed to allow amplification by PCR under standard reaction conditions are given in Table 3.8. Products for one such novel CA.GT repeat, 724CA, are shown in Figure 3.10 for a number of cell line DNA samples. The results of content-mapping cosmid clones with both novel inter*Alu* STSs and novel CA.GT repeats are also given in Table 3.4. No cosmids were identified as being positive for IASTS2 and IASTS5, this could imply that the inter*Alu* PCR products corresponding to these STSs were generated from chimaeric regions of YAC DNA and do not map, therefore, to the 7q31 region. Alternatively, it could simply imply that cosmid clones corresponding to these genomic segments do not exist in the cosmid library or that if they exist, then they were not successfully identified.

Figure 3.8. Cloning of novel CA.GT dinucleotide repeats. Cosmid DNA was digested to completion with *Sau*3A I restriction enzyme before being electrophoresed on a 1% agarose gel and transferred to nylon membranes. Membranes were then hybridised with a radiolabelled degenerate CA oligonucleotide probe. Positive hybridisation was revealed by autoradiography. Λ, lamba phage/*Hind* III molecular weight marker.


λ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36
Origin
23,130
9416
6557
4361
2322
2027
564
λ 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61
Origin
23,130
9416
6557
4361
2322
2027
564
λ 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97
Origin
23,130
9416
6557
4361
2322
2027
564
λ 98 99 100 101 102 103 104 105 106 107 108 109 110 111 112 113 114
Origin
23,130
9416
6557
4361
2322
2027
564

Marker	Primers	Size (bp)[a]	Heterozygosity % (n)
CAVCA1	L: GATCGTGCCATTGCACTCC R: TCCTAAACTACACCGTGTG	112	ND
CAVCA2	L: GTATGTTCACCACATGGACC R: CCAAAGTCTAGGTTTACAGC	125	0 (8)
715CA	L: GTGTTAAGACAGATGCTACC R: TAAAAGATAGCTTCAGGGGC	399	40 (17)
727CA	L: GATTTTGGGTTCAGTAACAGC R: CCAGGAAATAGAAACAGCAC	144	75 (65)[b]
724CA	L: GCTTTGTTAGGGTTCTCCAG R: CATGTTTTCAGTCCTTCAGC	140	71 (65)[b]
740CA	L: TCCTGACTGGCTGAATTG R: GAGCGACAGCAAAATCAG	205	83 (65)[b]
778CA	L: CTGTAGGATAGATAGGGAGC R: TACAGGAGATTGCATGGG	194	52 (65)[b]
786CA	L: CATAACCGGCTGGCATCATG R: ACACATTCCTTTGGGGCCTC	308	45 (65)[b]

Table 3.8. Primer sequences which amplify novel CA.GT repeats from the 7q31 region. [a] Size given is for PCR product of cloned allele. [b] Heterozygosity calculated by determining ratio of heterozygotes to homozygotes for normal peripheral blood DNA for n samples (H. Russell, unpublished data).

Figure 3.9. Novel chromosome 7q31 CA.GT repeats. CA repeat units are in red. Locations of primers are underlined. The position of a *Pst* I restriction site is shown for 715CA. CAVCA1 and CAVCA2 were derived from PAC162-021.

CAVCA1:

1 GATCGTGCCA TTGCACTCCA GCCTGGGCAA CAAGAGCGAA ACTCTGCCAC

51 ACACACACAA ACACACACAC ACACACACAC ACACACACAC ACACACACGG

101 TGTAGTTTAG GAAGTAAAAA AAAAAAAAAA AAAAAAATCA GATC

Number of repeat units = 18

CAVCA2:

1 GGATCACTTA TGTGTATGTT CACCACATGG ACCTACATCC CCACACACAC

51 ACACACACAC ACACACACAC ATAGAAACAC ACACACGCAC AGAGTCTTTA

101 AAGACGGACT TTATTTTATG CTGTAAACCT AGACTTTGGG AGGCTGAAGT

151 GGAGGGATTG

Number of repeat units = 15

715CA:

1 AGAAACATGT ATGAATGTGT TAAGACAGAT GCTACCTAAG AAGATAAGCT

51 ATATTTACAT ATACATACAT ACACACACAC ACACACACAC ACACACACAC

101 ACACACACAC GTATTTTTTT GAGACAGGGT GTCACTCTGT TGCCCAGGTT

Pst I

151 ACAGTGCAGT GTGTGAATAC AGCTCACTGC AGCCTCGACA TCCTGGGCTC

201 AAGCCATCCT CCTGCCTCAG CCTCTCTAGT AGCTGGGACT ACAGGCGTGT

251 GCCACCATGC CCAGCTAATT TATTTTTTGT AGAGATGGGG TCTCACCATA

301 TTGTCCAGGC TGGTCTCAAA CCCTGGCCAC AACCAACCTT TCTGCCTTGG

351 GCTCCCANAA TGCTGGGATT ACANGCATNG GGCCACCACA CCCAGCCCCT

401 GAAGCTATCT TTTAAAAAAA ATTTTTACT

Number of repeat units = 19

724CA:

1 GTACTCAATC TGCTTTGTTA GGGTTCTCCA GAAAAAACAG CCACCATAGC

51 AGATATTAAT ACACACACAC ACACACACAC ACACACACAC ACACACACAC

101 CCTTTGGCTC CCNAGCTAGA GACCCCAGAA AGCTGAAGGA CTGAAAACAT

151 GGGGAGCCAT GATTTCCCCC

Number of repeat units = 19

727CA:

1 TGGATCAATT ACAGATTTTG GGTTCAGTNA CAGCTTTCAA TAAAAGATGA

51 ATAAATTAAT GAATTTAAAA AGTTTTACAC ACACACACAC ACACACACAC

101 ACACACACAG AGCCATAGTT GTCTAAAGGA ATGATTAGAG TGCTGTTTCT

151 ATTTCCTGGT TTCCATAGC

Number of repeat units = 16

740CA:

1 ACACGCTTTA GGTTCTACAA CTCCTGACTG GCTGAATTGG CCCGTCGATT

51 TACCCTGAGG CAGTTTTGGC GGGGTGGGGG CTGGGATGGG GGAGGCGGTT

101 GTAGTTTTCA AGGTGAATTT ACACACACAC ACACACACAC ACACACACAC

151 ACACACACAC ACGTCTGTGC TAGAGCTGGA GACCAGGCTA GCACGTCTCT

201 CAACAGGATA AGAAATTGGC TGATTTTGCT GTCGCTCAGC TGGATCCCCC

251 GNCCTGCGGC

Number of repeat units = 20

778CA:

1 CTTCTNTTTG TTTTACTGTA GGATAGATAG GGAGCAATTT TTGAAGGTTA

51 TTTTGTNGAT TGCANATGTT AACCTTTCTT TTCTGGGTGG CATTCTCCCC

101 TCTTGGATCC ATCCACACAC ACACACACAC ACACACACAC ACACACATAC

151 ACCCACCCCA CCACCACCAC CAAAGACAAA CATTATTACA GCCCATGCAA

201 TCTCCTGTAA AATGGAGTT

Number of repeat units = 17

786CA:

1 GAGACCAGGT CAACATAACC GGCTGGCATC ATGTTTATCT TGCTCAGCAT

51 TTAACACACA CACACACACA CACACACACA CACACACACA CACACACACA

101 CACACACAAA CTTTTTGGCT CTACTTCTGA CCTTGGCTTT TATATTGTGT

151 TCATTTGTTT TTCAGAGGGG CTTGGTTCTT TTATTTGAAG ATACATCCTA

201 TTTGTTGGAA GAACTTCCAT TAAATTATCT TGTCAGTTCT CACTAAATTT

251 TCTTTTCACA GCTCTTGCTG TCTGGGTTAT AAAAACCCAT GGCAAACATG

301 GGAGGCCCCA AAGGAATGTG TGCTGGGATC TTGCACGGCA

Number of repeat units = 27

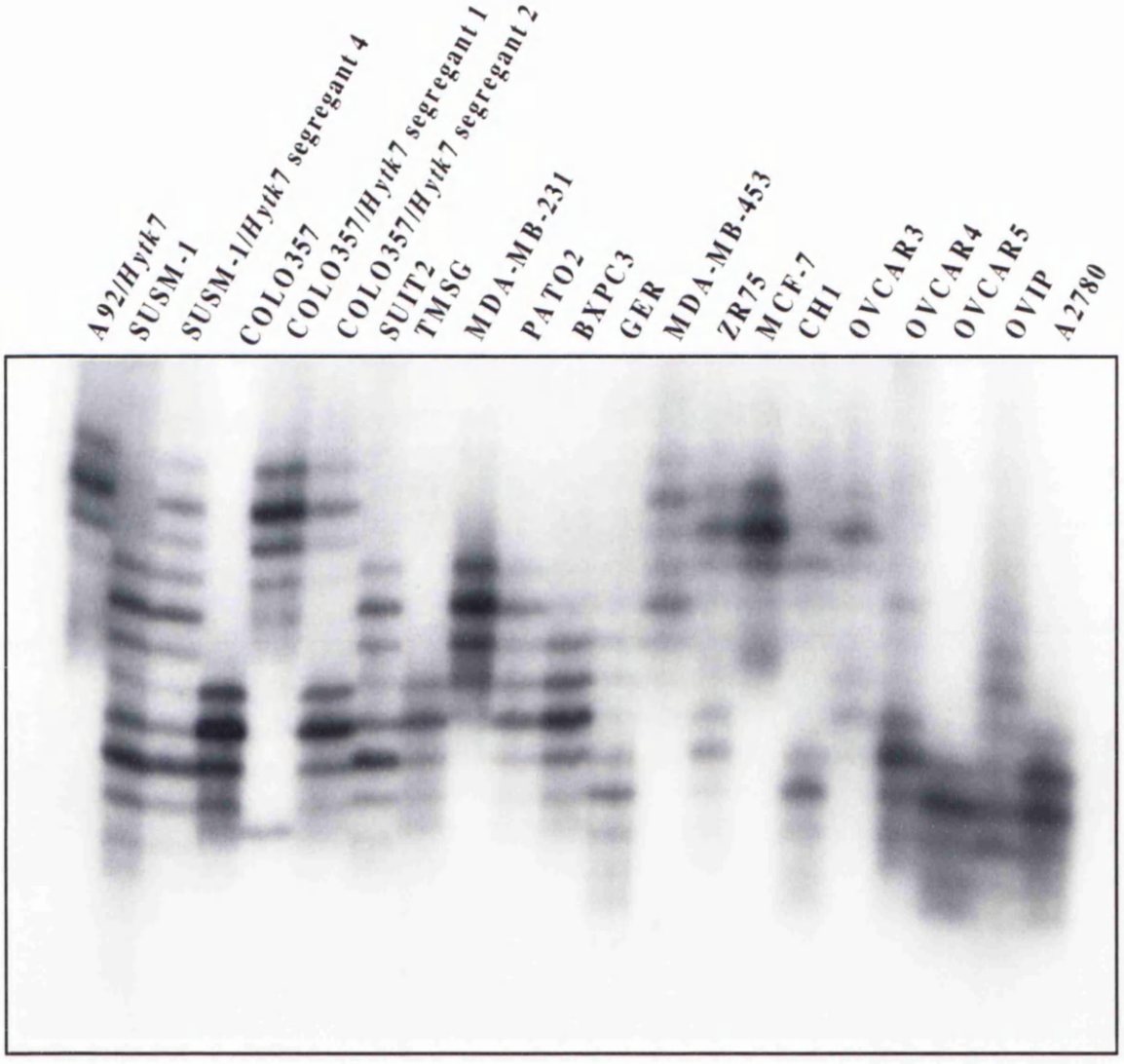


Figure 3.10. PCR amplification products for the novel CA.GT repeat 724CA. DNA from a number of cell lines was subjected to PCR using oligonucleotide primers for the CA.GT repeat 724CA, and radiolabelled in situ. The PCR amplification products were then resolved on a 6% (w/v) polyacrylamide gel, and visualised by autoradiography.

Identification of PAC clones

The PAC vector, pCYPAC-2, incorporates features of both P1 and F-factor cloning systems and allows propagation of inserts in the 100–300 Kbp range (Ioannou *et al.*, 1994). There are no apparent problems with chimaerism or clone instability with this vector. Individual PAC clones were sought to give additional clonal coverage of the region of LOH by hybridisation screening of a human PAC library constructed from digests of DNA from a male fibroblast cell line (Ioannou *et al.*, 1994), obtained from the Resource Centre/Primary Database of the German Human Genome project (Lehrach *et al.*, 1990), and from the UK Human Genome Mapping Project (UKHGMP) resource centre. The library consists of seven independent nylon filters each supporting a 192 x 192 gridded array of PAC DNA. In particular, probes were generated by PCR amplification using primers for WI-3876, Xv-2c, and D7S399 (oligonucleotide primers listed in Table A2). In addition, a probe was prepared from *CAVEOLIN-1* cDNA (see Chapter 5). Positive hybridisation was revealed by autoradiography and the clones corresponding to the deduced co-ordinates requested from the resource centres. 10 clones were requested, PAC clone 162-O21 which was positive for *CAVEOLIN-1* and the clones represented in Table 3.9, and re-screened with the probe used to initially identify them and additonal markers in order to ascertain their positive status and to determine the extent of the region they covered.

A summary of the total clone coverage, inferred from STS content mapping, ribo-probing, and fingerprinting, of the genomic region contained within YACS 905-g2 and 921-b4 is shown in Figure 3.11. The largest contiguous region of clone coverage is approximately 600-700 Kbp and encompasses the markers WI-3876 and exon 4 of *CFTR*.

Marker	PAC clone LLNLP704-								
	H11146	B1719Q	A19152	L2088Q	B0964Q	K11206	I14270	F2012Q	K1448Q
WI-7882	-	-	-	-	-	-	-	-	-
AFMB316XB9	-	-	-	-	-	-	-	-	-
WI-3876	+	+	+	+	+	-	-	-	-
sL12	-	-	+	+	+	-	-	-	-
778CA	-	-	-	-	+	-	-	-	-
WI-7597	-	-	-	-	-	+	+	-	-
Xv-2c	-	-	-	-	-	+	+	-	-
CS.7	-	-	-	-	-	+	+	+	-
KM19	-	-	-	-	-	+	+	+	-
D7S633	-	-	-	-	-	-	-	+	-
D7S399	-	-	-	-	-	-	-	+	+
CFTRex1	-	-	-	-	-	-	-	+	+
CFTRex4	-	-	-	-	-	-	-	+	-

Table 3.9. STS content mapping of PAC clones.

Figure 3.11. Clone coverage of the D7S486–786CA interval. Clone coverage and STS content map of the D7S486–786CA interval are shown. Symbols beneath the tie line represent different varieties of STS marker (polymorphic/non-polymorphic, expressed/non-expressed). The shaded region beneath the tie line depicts the smallest common region of allele loss in breast carcinomas.

Novel STS
Novel CA.GT repeat
Published STS
Genethon CA.GT repeat
RFLP
EST
7cen
7qter
sWSS2099
WI-3876
sWSS3428
AFMB316XB9
sWSS2710
sWSS843
sWSS1398
IASTS1
WI-18209
sWSS2899
IASTS4
WI-7882
715CA
sWSS305
727CA
METH
METD
D7S2460
WI-455
SHGC-10385
CAV1CA2
CAV1CA1
D7S522
WI-5336
WI-8726
D7S2543
D7S486
SHGC-8708
786CA
D7S2742
Exon20
17B-R1/17B-RE3
Exon12
sWSS376
Exon10
Exon6
Exon4
Exon3
D7S677
Exon1
sWSS915
D7S399
sWSS850
D7S633
KM19
CS.7
740CA
Xv-2c
WI-7597
sWSS1948
724CA
778CA
Cda0zb11
sWSS845
sWSS844
sL12
sWSS377
IASTS3
K1448Q
F2012Q
114270
H11146
B1719Q
8-1.24
162-024
195-P4
198-09
162-021
A19152
L2088Q
B0964Q

CHAPTER 4

RESULTS

Allele loss analysis of SUSM-1/*Hytk*7 immortal segregants

4. Results

4.1 Segregant deletion analysis

As stated in the introduction, a replicative senescence gene that is inactivated during the immortalization of cell lines within complentation group D is believed, from monochromosomal transfer studies, to map to human chromosome 7. Further, two complementation group D cell lines, SUSM-1 and KMST-6, display loss of genetic material from the 7q31 region (Ogata *et al.*, 1993). Based upon the proximity of these losses with those found in tumour samples, and also upon the probable role of replicative senescence in tumour suppression, we formulated the hypothesis that the complementation group D replicative senescence gene, *SEND*, was the target for inactivation (as demonstrated by LOH) in tumours *in vivo*, and presented a new avenue by which to clone the chromosome 7q31.1-TSG. The approach we adopted combines two aspects: phenotypic selection and somatic cell genetics.

The strategy chosen (schematically represented in Figure 4.1) entails introducing intact copies of human chromosome 7 into SUSM-1 cells by microcell mediated monochromosome transfer. The donor chromosome is tagged with a selectable fusion gene, *Hytk*, which is derived from a hygromycin phosphotransferase gene, *Hph*, conferring hygromycin resistance and allowing positive selection of transfected cells, and the herpes simplex virus thymidine kinase gene, *tk*, that allows negative selection (Cuthbert *et al.*, 1995). As previously reported (Ogata *et al.*, 1993; Nakabayashi *et al.*, 1997), Dr George Reid, then in our group, responsible for performing the microcell fusions, found that introduction of human chromosome 7 into SUSM-1 induced replicative senescence in the majority of recipient cells (as determined by delayed growth arrest, and induction of s.a-β-galactosidase activity; data not shown). However, also as previously noted, he observed that a proportion of microcell-fused hybrids escaped replicative senescence and appeared to retain (or revert to) their immortal phenotype (capable of being serially passaged indefinitely). We reasoned that this would most likely be due to inactivation of *SEND* on the *Hytk*-tagged chromosome 7 segregating in immortal variant clones. If such inactivation resulted through deletion or mitotic recombination then it should be possible to map these events by determining the pattern of retention of informative polymorphic markers (markers in which the alleles arising from the *Hytk*-tagged chromosome differ from those on the endogenous

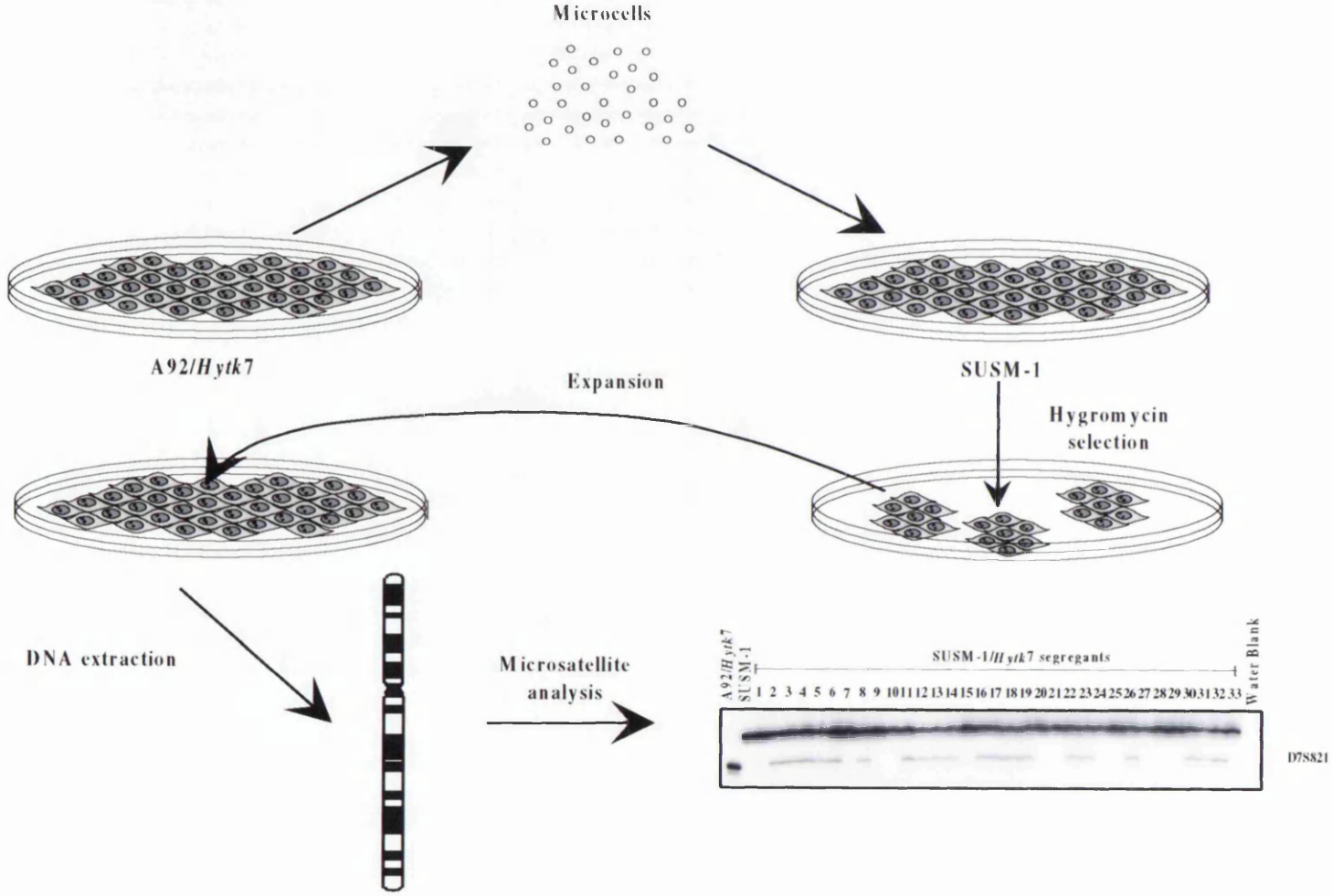


Figure 4.1. Segregant deletion analysis. Microcells are prepared by colcemid treatment followed by centrifugation of donor rodent cells which contain a single *Hytk*-tagged copy of human chromosome 7. Microcells are fused to SUSM-1 cells using polyethylene glycol and cells subsequently placed on drug selection. Hygromycin resistant cells are then examined microscopically for growth potential and, when colonies are macroscopic, are harvested by ring-cloning and expanded for segregant deletion analysis.

chromosomes) in immortal SUSM-1/*Hytk7* segregants. Potential advantages of this gene mapping strategy when compared to examining LOH in tumour DNA samples are one, the unequivocal nature of the data (tumour LOH studies are complicated by the presence of contaminating normal stromal tissue within the tumour sample, making detection of reductions in band intensity more subjective); two, the association of allele loss with an observable phenotypic effect; and three, since the end product is an immortal cell line, there is an indefinite source of DNA, RNA, and proteins for further molecular analysis.

Dr George Reid generated 30 independent immortal SUSM-1/*Hytk7* segregants and Dr Robert F Newbold (Department of Biology and Biochemistry, Brunel University, Uxbridge, UK) kindly provided us with three more. Over one hundred polymorphic markers (Table A1 and in addition the novel CA.GT repeats I generated [Chapter 3.4.2]) from the D7S2490–D7S1807 interval were assayed to determine whether alleles were informative between SUSM-1 and A92/*Hytk7*; only thirty four markers were clearly informative. Immortal segregants were then analysed with these polymorphic markers. Representative examples of segregant deletion analysis are shown in Figure 4.2 and the results are summarised in Table 4.1. From Table 4.1, it is apparent that this mode of analysis is hampered by noise: several segregants present with multiple losses (the nature of these losses—deletion *vs.* mitotic recombination— has not been established, although for some markers *e.g.* D7S1804 loss of an endogenous allele in a number of segregants argues that mitotic recombination might be operating [data not shown]. Zenklusen *et al.* (1996)[b] and Gupta *et al.* (1997) suggest that mitotic recombination is the most likely mechanism accounting for the majority of LOH in tumours). One possible explanation for at least some of this noise is that the order of markers has not yet been established correctly. Ascertaining the correct order of markers will ultimately depend upon knowledge of the nucleotide sequence for this chromosome.

Overall 17/33 (51.5%) of immortal segregants demonstrated loss of one or more markers on chromosome 7q. The most frequently lost marker was D7S2555 (98.1 cM from 7pter; 7q21), which was lost in 14/33 (42.2%) of immortal segregants, followed by D7S821 (109.1 cM from 7pter; 7q21), D7S633 and 17TA-5/17B-RE3 (both 125.2 cM from 7pter; 7q31), which were each lost in 13/33 (39%) of immortal segregants. A graph of frequency of allele loss against distance along the long arm of chromosome 7 is shown in Figure 4.3. Again, this graph demonstrates the existence of three 'hot spots' of allele loss in immortal SUSM-1/*Hytk7* segregants. The smallest solitary interstitial losses detected were in SUSM-1/*Hytk7* segregant 4 and segregant 5. The extent of allele

Table 4.1. Summary of segregant deletion analysis of SUSM-1/*Hytk7* immortal segregants. -, donor allele lost; +, donor allele retained; *, endogenous allele lost.

Marker	SUSM-1 Segregant																
	1	4	5	7	9	10	15	20	21	24	25	27	28	29	31	32	33
D7S1797	-	+	+	-	+	-	-	+	+	-	-	-	-	+	+	+	-
D7S820	-	+	+	-	-	-	-	+	+	+	-	-	-	+	+	+	-
D7S2540	-	+	-	-	-	-	-	+	+	+	-	-	-	+	+	+	-
D7S2555	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
D7S1820	-	+	+	-	+	-	-	+	+	+	-	-	-	-	+	+	-
D7S2431	-	+	+	-	+	-	-	-	-	-	-	-	-	-	+	+	-
D7S821	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	-
D7S2539	-	+	+	-	+	-	-	-	+	-	-	-	-	+	+	+	-
D7S1796	-	+	+	-	+	-	-	+	+	-	-	-	-	+	+	+	-
D7S554	-	+	+	-	-	-	-	-	+	-	-	-	-	+	+	+	-
D7S477	-	+	+	-	+	-	-	+	+	-	-	-	-	+	+	-	-
D7S515	-	+	+	-	+	-	-	+	-	-	-	-	-	+	-	-	-
D7S666	-	+	+	-	+	-	-	+	+	-	-	-	-	+	-	-	-
D7S518	-	+	+	-	+	-	-	+	-	-	-	-	-	+	-	-	-
D7S796	-	+	+	-	+	-	-	+	+	+	-	-	-	+	-	-	-
D7S501	-	+	+	-	+	-	-	+	+	+	-	-	-	+	-	-	-
D7S692	-	+	+	-	+	-	+	+	+	+	-	-	-	+	-	-	-
D7S523	-	+	+	-	+	-	+	+	-	-	-	-	-	-	-	-	-
D7S687	-	+	+	-	+	-	+	-	+	+	-	-	-	-	-	-	+
D7S486		+	+		+		-	-	+	+	-	-	-	-	-	-	
MET	-	+	+	-	+	-	-	-	-	+	-	-	-	-	-	-	+
778CA	-	+	+	-	+	-	-	-	-	+	-	-	-	-	-	-	+
724CA	-	+	+	-	+	-	-	-	-	+	-	-	-	-	-	-	+
D7S633	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	+
17B-TA5/17B-RE3	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	-	+
786CA	-	+	+	-	+	-	-	-	-	+	-	-	-	-	-	-	+
CAREP3	-	+	+	-	+	-	-	-	-	+	-	-	-	-	-	-	+
AFMA073ZB9	-	+	+	-	+	-	-	-	-	+	-	-	-	-	-	-	+
GATA44F09	-	+	+	-	+	-	-	-	-	+	-	-	-	-	-	-	+
D7S643	-	+	+	-	+	-	+	-	-	-	+	-	-	-	-	-	+
D7S650	-	+	+	-	+	-	+	-	-	-	+	-	-	-	-	-	+
D7S1809	-	+	+	-	+	-	+	-	-	+	+	-	-	-	-	-	+
D7S2197	-	+	+	-	+	-	+	+	+	+	+	-	-	+	-	+	+
D7S1804	-	+	+	-	+	-	+	+	+	+	+*	-	-	+	-	+	+
GATA63F08		+	+	-	+	-	+	+	-	-	-	-	-	-	+	+	+
D7S794	-	+	+	-	+	-	+	+	+	+	-*	-	-	+	+	+	+
D7S1805	-	+	+	-	-	-	+	-	-	+	-*	-	-	-	+	+	+

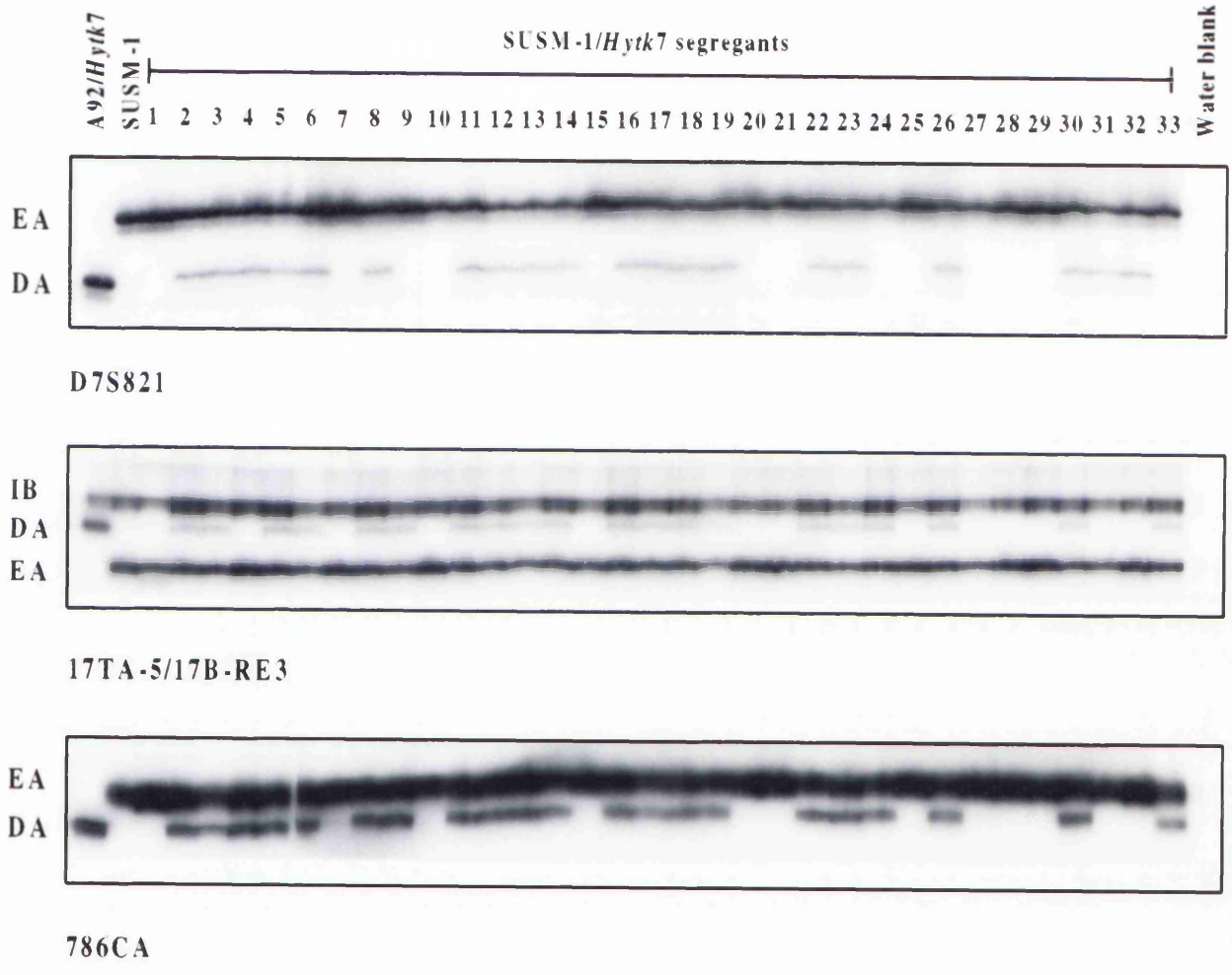


Figure 4.2. Representative allele loss analysis in immortal SUSM-1/*Hytk7* segregants. Autoradiographs from radiolabelled PCR products of three amplified microsatellite markers, D7S821, 17TA-5/17B-RE3, and 786CA, are shown. The SUSM-1 cell line is homozygous at each of these loci. DA, donor allele; EA, endogenous allele; IB, invariant band (PCR artefact).

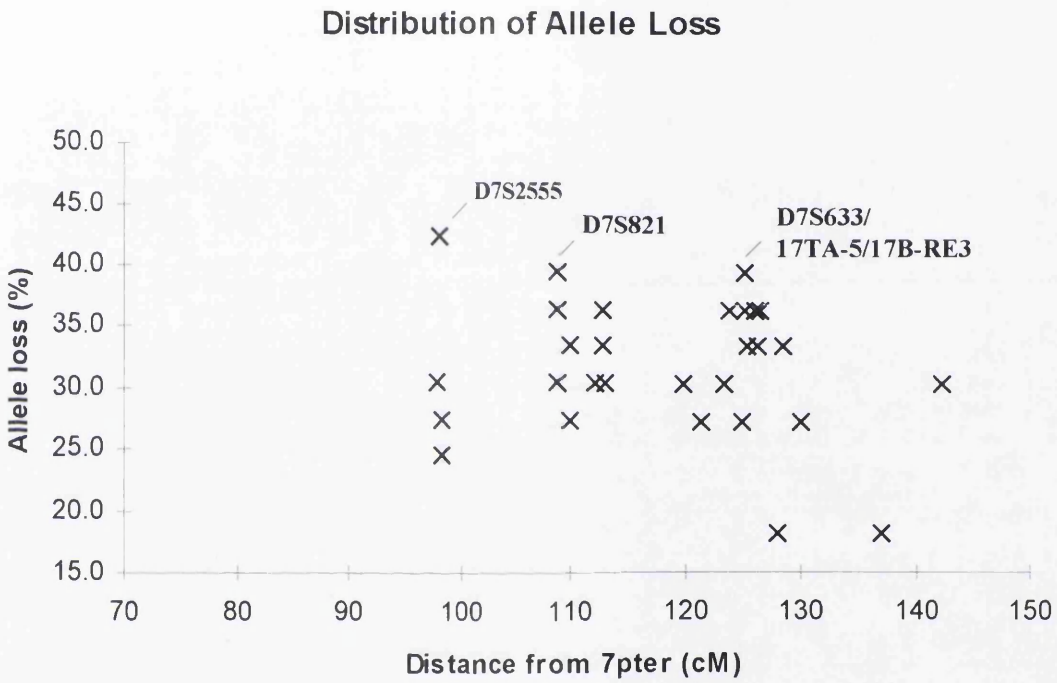


Figure 4.3. Allele loss *vs.* genetic distance on chromosome 7q in SUSM-1/*Hytk7* immortal segregants. Percentage allele loss in SUSM-1/*Hytk7* immortal segregants is shown plotted against the genetic distance in Kosambi cM from 7pter of the corresponding polymorphic marker (X). Three 'hot spots' of allele loss correspond to D7S2555, D7S821, and D7S633–17TA-5/17B-RE3.

loss in segregant 5 has not been finely delineated, but is within the order of a megabase. The allele loss in segregant 4, between the two novel markers 724CA and 786CA, is approximately 500 KBp in extent and is nested within the region of LOH defined by the tumour LOH studies. This region of loss also lies within the 7q31-qter region of LOH identified in KMST-6 (Ogata *et al.*, 1993). Cosmid and PAC clones covering this interval in segregant 4 have been identified (Figure 4.4).

Figure 4.4. Overlap of regions of allele loss in breast carcinomas and immortal SUSM-1/*Hytk7* segregants. Symbols beneath the tie line represent different varieties of STS marker (polymorphic/non-polymorphic, expressed/non-expressed). The shaded regions, beneath the tie line, depict the smallest common region of allele loss in breast carcinomas (lighter) and the region of allele loss in immortal SUSM-1/*Hytk7* segregant 4.

Novel STS
Novel CA.GT repeat
Published STS
Genethon CA.GT repeat
RFLP
EST
7cen
7qter
sWSS2099
WI-3876
sWSS3428
AFMB316XB9
sWSS2710
sWSS843
sWSS1398
IASTS1
WI-18209
sWSS2899
IASTS4
WI-7882
715CA
sWSS305
727CA
METH
METD
D7S2460
WI-455
SHGC-10385
CAV1CA2
CAV1CA1
D7S522
WI-5336
WI-8726
D7S2543
D7S486
SHGC-8708
786CA
D7S2742
Exon20
17B-R1/17B-RE3
Exon12
sWSS376
Exon10
Exon6
Exon4
Exon3
D7S677
Exon1
sWSS915
D7S399
sWSS850
D7S633
KM19
CS.7
740CA
Xv-2c
WI-7597
sWSS1948
724CA
778CA
Cda0zb11
sWSS845
sWSS844
sL12
sWSS377
IASTS3
H11146
B1719Q
8-L24
162-024
195-P4
198-09
162-021
A19152
L2088Q
B0964Q
K1448Q
F2012Q
I14270

CHAPTER 5

RESULTS

Identification, characterisation, and mutation analysis of genes from the minimally deleted region in tumours and immortal segregants

5. Results

5.1 The identification of genes within the D7S522-17TA-5/17B-RE3 interval

Having identified that the genomic region just proximal to the *CFTR* locus is deleted in a high proportion of breast tumours and immortal SUSM-1/*Hytk7* segregants, and having established YAC and bacterial clone coverage of this genomic region, we moved to the next stage in positional cloning: identifying candidate genes that map to this interval. A number of different approaches were adopted.

5.1.1 The cloning of *CFTR*

With many of the mapping targets of the human genome project now achieved, it will prove increasingly difficult to find a 1.5 Mbp region of the genome that is entirely uncharted and devoid of known genes. The D7S522-17TA-5/17B-RE3 interval is no exception, having come under close scrutiny once already during the successful positional cloning of the gene responsible for cystic fibrosis—*CFTR* (Rommens *et al.*,1989). The cloning of this gene, which was hotly contested for several years, was a *tour de force*: *CFTR* was the first gene to be positionally cloned without the aid of a constitutional cytogenetic alteration, the cloning strategy being solely reliant on chromosome walking and jumping—methods largely superseded by STS content mapping. Moreover, the travail left a rich legacy of information for those who have come afterwards, including long and short range restriction maps of the region, probes and markers, and the identification of additional genes, including *WNT2*, which maps approximately 200 Kbp centromeric of *CFTR*. In addition to *CFTR* and *WNT2*, two other characterised genes are known to map to the D7S522-17TA-5/17B-RE3 interval: c-*MET* and *CAPZA2*.

5.1.2 *hF11A10.5*

During the cloning of *CFTR*, Rommens *et al.* identified, in addition to *CFTR* and *WNT2*, three other genomic regions of conserved homology, as determined by cross-

species hybridization with DNA on a zoo blot. Cross-species hybridization can reveal the presence of an underlying gene, since expressed sequences frequently demonstrate evolutionary conservation. A probe, G-2, from one of these regions, which maps 60 Kbp centromeric of the 3´-end of *WNT2*, detected a 3.7 Kbp transcript when hybridized to RNA from SV40-Transformed human fibroblasts. Based on the mapping distance (and the average insert size of a cosmid), we reasoned that this transcript should correspond to sequences contained within cosmids 78, 97, and 108. Mr Robert McFarlane shotgun subcloned c97, following digestion with *Eco*R I and *Hind* III, into the prokaryote phagemid vector, pBluescript. Sequencing of one of these fragments, 97HR13, revealed significant homology (80% similarity at the amino-acid level) to a gene present in the genome of *C. elegans* (*F11A10.5*; GenEMBL accession number Z68297), in addition to a number of human and rodent EST sequences (accession numbers: N58116, W02490, W13940). Subsequently Robert was able to use this subcloned genomic fragment to isolate 5 independent overlapping cDNA clones from a senescent fibroblast full-length cDNA library prepared by Dr. George Reid. The cDNA clones were sequenced and confirmed to be derived from the human homologue of *F11A10.5* (*hF11A10.5*). Based upon the human cDNA sequence, a probe was generated by PCR corresponding to the central region of *hF11A10.5* using cDNA as a template (see Materials and Methods). The PCR product was then used to probe a northern blot containing total RNA from a number of tumour and non-tumourigenic immortal cell lines, as well as RNA from senescent fibroblasts (70 mean population doublings). This analysis revealed an abundant and ubiquitously expressed transcript, which is present in a single form of approximately 3.7 Kbp (Figure 5.1), strongly suggesting that we had identified the same gene as Rommens *et al.*

5.1.3 R14.4E1

The other region of cross-species homology identified by Rommens *et al.*, corresponding to an approximately 1 Kbp *Eco*R I-digest fragment, R14.4E1, maps 100 Kbp telomeric of the 5´-end of *WNT2*, between *WNT2* and *CFTR* and within the deleted region identified in SUSM-1/*Hytk7* segregant 4. The authors noted that this region contains recognition sites for the restriction enzyme *Bss*H II as well as a high G/C content as determined by sequencing, suggesting that it might be a CpG island. CpG islands are discrete units up to 2 Kbp long with a disproportionately high G/C content

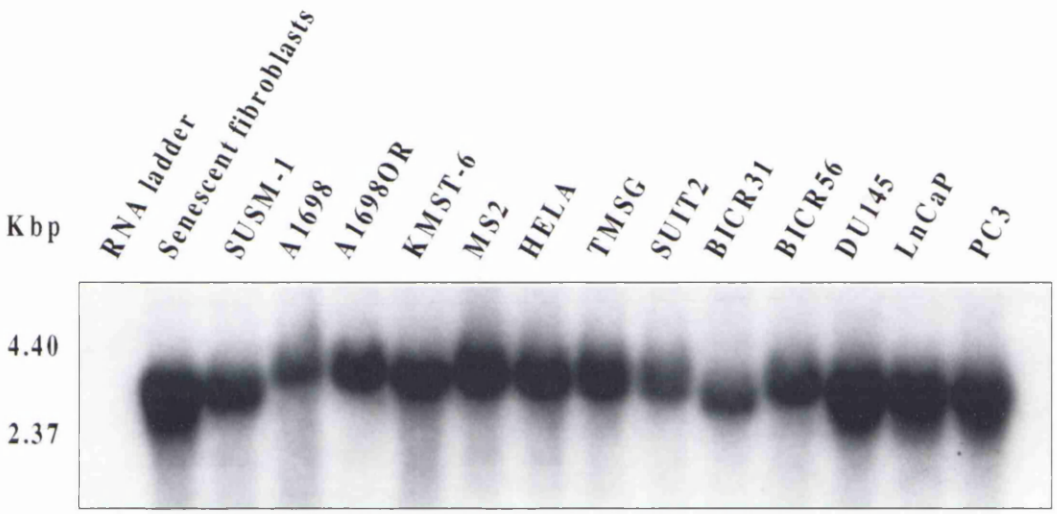


Figure 5.1. Northern blot analysis of *hF11A10.5* expression in cell lines. RNA from a number of tumourigenic and non-tumourigenic immortal cell lines, as well as RNA from senescent fibroblasts (70 mean population doublings) were transferred to Hybond N membrane and hybridised with a radiolabelled probe corresponding to *hF11A10.5*. Hybridisation was detected by autoradiography. RNA from the (cervical carcinoma) cell line MS2 was supplied by Dr A. Malliri, BICR, and from the (head and neck squamous cell carcinoma) cell lines BICR31 and BICR56 by Dr J. Munro, BICR. (See Materials and Methods for the origin of the other cell lines.)

and are virtually free of methylation at CpG dinucleotides. Such regions are associated with over half the genes in the human genome, both tissue-specific and house-keeping, and in particular with the site of transcription initiation and the first exon(s) (Bird, 1986). Over 80% of the sites cleaved by *Bss*H II, whose recognition sequence is GCGCGC, would be expected to occur within CpG islands. Again, we conjectured that this sequence could be contained within cosmids 1, 61, and 65. These cosmids, therefore, were digested with *Eco*R I and *Bss*H II, and c61 was found to contain an approximately 1 Kbp *Eco*R I-digest fragment, which could be cleaved by *Bss*H II, which agrees with the description of the CpG island above (Figure 5.2). This fragment and adjacent *Eco*R I-digest fragments (based upon the published restriction map of Rommens *et al.*, 1989) of c61 were subcloned into pBluescript. Sequencing of the 1 Kbp *Eco*R I fragment revealed a high CpG content and in addition it confirmed the presence of three closely clustered *Bss*H II sites. A BLAST homology search against known genes and ESTs revealed no significant homologies. This fragment in addition to the flanking two *Eco*R I fragments (4.5 Kbp and 7.5 Kbp) failed to detect a transcript on the northern blot used above and failed to detect a corresponding cDNA when used to probe lifts of the senescent fibroblast cDNA library. Rommens *et al.* also failed to detect an RNA transcript or cDNA with R14.4E1, and suggested that this might indicate that the transcript is restricted in tissue or developmental specificity, or that the cross-species hybridization signal generated with this probe resulted from non-specific hybridization of the CpG-rich sequence.

5.1.4 The human gene map

Another source of information we have been able to exploit in the search for genes which map to the D7S522-17TA-5/17B-RE3 interval is the human gene map (Schuler *et al.*, 1996). The starting point for the generation of this map—a collaborative venture between genome mapping centres or groups at the Whitehead Institute for Biomedical research, the Sanger Centre, Genethon, Stanford University, Oxford University, the University of Colorado Health Sciences Centre, and informatics centres at the National Centre of Biotechnology Information and the European Bioinformatics Institute—was the UniGene dataset. This dataset strives to represent each gene in the human genome as a single entry (*i.e.* to be non-redundant) by concentrating solely on the 3′ ends of genes: either characterised genes or 3′-reads of

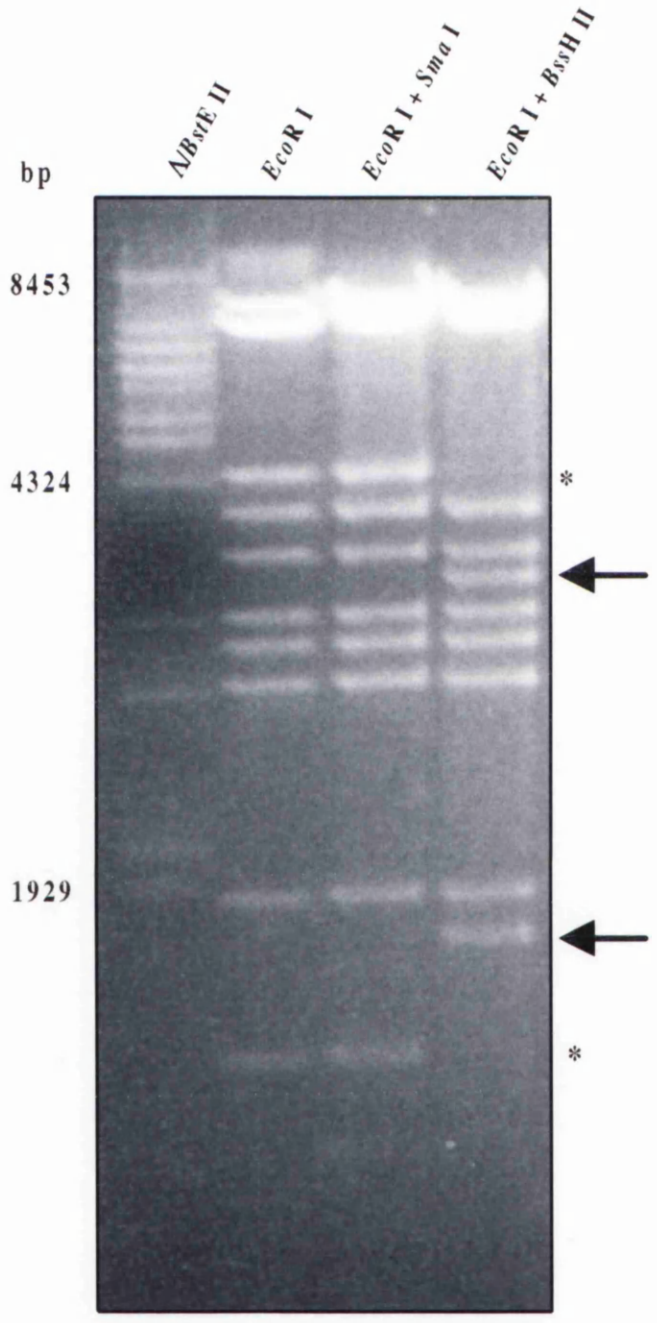


Figure 5.2. Identification of a CpG island in c61 corresponding to R14.4E1. DNA samples prepared from cosmid clone 61 were digested in parallel with the restriction enzymes shown at the top of the panel and then resolved by electrophoresis on an agarose gel. Bands were visualised by staining with Ethidium bromide. The asterisks indicate *Eco*R I fragments which are also cleaved by *Bss*H II. The upper band (approximately 4.5 Kbp) is derived from the cosmid vector, Lawrist 4; the arrows indicate the smaller fragment resulting from *Bss*H II cleavage of this fragment. The smaller band (approximately 1 Kbp) marked with an asterisk corresponds to R14.4E1.

novel genes in the form of ESTs. The great majority of EST sequences (over 500,000) have been generated by Washington University Genome Sequencing Centre with the sponsorship of Merck pharmaceuticals, using cDNAs prepared by the IMAGE consortium. Entries in the UniGene database were converted into STSs and then placed on radiation hybrid mapping panels (either Stanford G3 or Genebridge 4) to create the human gene map.

The human gene map is accessible in electronic form through a site on the World Wide Web (http://www.ncbi.nlm.nih.gov/SCIENCE96/), and allows one to search within bins corresponding to physical intervals on a radiation hybrid map defined by Genethon microsatellite markers. We conducted a search between the markers D7S522 and D7S655; this generated 32 returns (Table 5.1). Oligonucleotide primers were ordered for certain of these markers and then used to screen YACs 905-g2 and 921-b4. The results of this screening are given in Table 5.1. EST-derived STSs for which YACs were positive were then assayed using cosmid DNA as template; cosmids identified as being positive for these markers are also shown in Table 5.1. Overall 10 EST-derived STSs were identified as mapping to the YACs, and at least one cosmid corresponding to each of these was identified. Finally, these markers were used to screen senescent human fibroblast cDNA by taking duplicate 1 μl aliquots of phage supernatant (10^7 pfu/μl) from the senescent human fibroblast cDNA library prepared by George and subjecting them to PCR amplification, to assess their expression status in a population of senescent cells; these results are also given in Table 5.1.

Map entity	Within YAC(s)	Within cosmid(s)	Expressed in senescent fibroblasts
SHGC-8678	-		-
SHGC-8668	-		-
A001X05/ Cda1bd01/ SHGC-8711	-		-
A004D18	-		-
A005X13	-		-
SHGC-13594	-		-
SHGC-12021/ SHGC-8664	-		-
SHGC-13610	-		-
stSG401/ A001W15	-		+
SGC33824	-		-
WI-18254	ND	ND	ND
WI-17786	-		-
WI-18408	-		-

stSG10289	-		-
stSG3600	ND	ND	ND
stSG464	-		-
stSG4807	-		-
WI-8693	-		-
WI-8726	921-b4	c135*	-
SHGC-10385/ D29206	921-b4 905-g2	c70	+
WI-12662	ND	ND	ND
SHGC-31795	ND	ND	ND
WI-7882	921-b4 905-g2	c15, c107	ND
WI-18209	921-b4 905-g2	c43, c79	-
Cda0zb11/ SHGC-5660	921-b4 905-g2	c78, c97, c108	+†
WI-7597	921-b4 905-g2	c60	ND
D7S2742	921-b4 905-g2	c41, c45, c50, c57, c99, c110	ND
SHGC-8708	921-b4 905-g2	c45, c86	-
A005W09/ Cda19h02	-		+
A004D07/ SHGC-5654	-		-
A004D12/ SHGC-10294	-		-

Table 5.1. EST-derived STS from the human gene map corresponding to the 7q31 region. ND, either not determinable (failure to find the correct conditions for amplification) or not done; +, PCR product; -, no PCR product; * Identified by Dr. Edward Tobias in our group; †trace product .

Novel EST sequences, from which markers mapping to the selected YAC clones had been generated, were used as starting points in the generation of sequence contigs. Essentially an EST would be used to search for homology with other EST or cDNA sequences deposited in the GenEMBL database using the BLAST algorithm. Matches showing significant homology (near identity) which were not due to repetitive DNA and which extended the sequence 5′ were used to search the database afresh, and so the process was re-iterated. In this way, it was established that the marker SHGC-10385 corresponds to the 3′ of a gene, *CAVEOLIN-1* (Figure 5.3). Walking in the EST database with H63719 (WI-18209), Z39076 (Cda0zb11), or Z39427 (SHGC-8708) did not reveal any shared homology with known genes, and further suggested that they are expressed in only a narrow range of tissues (brain or fetal liver and spleen).

```
        10                  30                  50
GAATTCCGGAGTTTTCATCCAGCCACGGGCCAGCATGTCTGGGGGCAAATACGTAGACTC
                                  M  S  G  G  K  Y  V  D  S   9
        70                  90                 110
    ▼
GGAGGGACATCTCTACACCGTTCCCATCCGGGAACAGGGCAACATCTACAAGCCCAACAA
 E  G  H  L  Y  T  V  P  I  R  E  Q  G  N  I  Y  K  P  N  N  30
       130                 150                 170
CAAGGCCATGGCAGACGAGCTGAGCGAGAAGCAAGTGTACGACGCGCACACCAAGGAGAT
 K  A  M  A  D  E  L  S  E  K  Q  V  Y  D  A  H  T  K  E  I  50
       190                 210                 230
                                                 ▼
CGACCTGGTCAACCGCGACCCTAAACACCTCAACGATGACGTGGTCAAGATTGACTTTGA
 D  L  V  N  R  D  P  K  H  L  N  D  D  V  V  K  I  D  F  E  70
       250                 270                 290
                                   C
AGATGTGATTGCAGAACCAGAAGGGACACACAGTTTTGACGGCATTTGGAAGGCCAGCTT
 D  V  I  A  E  P  E  G  T  H  S  F  D  G  I  W  K  A  S  F  90
                                   H
       310                 330                 350
CACCACCTTCACTGTGACGAAATACTGGTTTTACCGCTTGCTGTCTGCCCTCTTTGGCAT
 T  T  F  T  V  T  K  Y  W  F  Y  R  L  L  S  A  L  F  G  I  110
       370                 390                 410
CCCGATGGCACTCATCTGGGGCATTTACTTCGCCATTCTCTCTTTCCTGCACATCTGGGC
 P  M  A  L  I  W  G  I  Y  F  A  I  L  S  F  L  H  I  W  A  130
       430                 450                 470
                                             C
AGTTGTACCATGCATTAAGAGCTTCCTGATTGAGATTCAGTGCATCAGCCGTGTCTATTC
 V  V  P  C  I  K  S  F  L  I  E  I  Q  C  I  S  R  V  Y  S  150
                                             T
       490                 510                 530
CATCTACGTCCACACCGTCTGTGACCCACTCTTTGAAGCTGTTGGGAAAATATTCAGCAA
 I  Y  V  H  T  V  C  D  P  L  F  E  A  V  G  K  I  F  S  N  170
       550                 570                 590
TGTCCGCATCAACTTGCAGAAAGAAATATAAATGACATTTCAAGGATAGAAGTATACCTG
 V  R  I  N  L  Q  K  E  I  *                                 179
       610                 630                 650
ATTTTTTTTCCTTTTAATTTTCCTGGTGCCAATTTCAAGTTCCAAGTTGCTAATACAGCA
       670                 690                 710
ACGAATTTATGAATTGAATTATCTTGGTTGAAAATAAAAAGATCACTTTCTCAGTTTTCA
       730                 750                 770
TAAGTATTATGTCTCTTCTGAGCTATTTCATCTATTTTTGGCAGTCTGAATTTTTAAAAC
       790                 810                 830
CCATTTATATTTCTTTCCTTACCTTTTTATTTGCATGTGGATCAACCATCGCTTTATTGG
       850                 870                 890
CTGAGATATGAACATATTGTTGAAAGGTAATTTGAGAGAAATATGAAGAACTGAGGAGGA
       910                 930                 950
AAAAAAAAAAAAAAAGAAAAGAACCAAAAACCTCAACTGCCTACTCCAAAATGTTGGTCAT
       970                 990                1010
TTTATGTTAAGGGAAGAATTCCAGGGTATGGCCATGGAGTGTACAAGTATGTGGGCAGAT
      1030                1050                1070
TTTCAGCAAACTCTTTTCCCACTGTTTAAGGAGTTAGTGGATTACTGCCATTCACTTCAT
      1090                1110                1130
AATCCAGTAGGATCCAGTGATCCTTACAAGTTAGAAAACATAATCTTCTGCCTTCTCATG
      1150                1170                1190
ATCCAACTAATGCCTTACTCTTCTTGAAATTTTAACCTATGATATTTTCTGTGCCTGAAT
      1210                1230                1250
ATTTGTTATGTAGATAACAAGACCTCAGTGCCTTCCTGTTTTTCACATTTTCCTTTTCAA
      1270                1290                1310
ATAGGGTCTAACTCAGCAACTCGCTTTAGGTCAGCAGCCTCCCTGAAGACCAAAATTAGA
      1330                1350                1370
ATATCCATGACCTAGTTTTCCATGCGTGTTTCTGACTCTGAGCTACAGAGTCTGGTGAAG
      1390                1410                1430
CTCACTTCTGGGCTTCATCTGGCAACATCTTTATCCGTAGTGGGTATGGTTGACACTAGC
      1450                1470                1490
CCAATGAAATGAATTAAAGTGGGACCAATAGGGGCTGAGCTCTTCTGTGGGGCTGGGCAG
      1510                1530                1550
TCCTGGGGAAGCCAGCTTTTCCCTGGCCTCTTCATCCAACTTGAATTGAGGGTCAGCCAT
      1570                1590                1610
GTTCTTATTTCAGCTTTCGTTTTATTTTTTCAAGGAATAATTCACGCTTTTCCTGGAATC
      1630                1650                1670
CAAACTAATCCATCACCGGGGGTGGTTTAGTGGCTCAACATTGTGTTCCCATTTCAGCTGA
      1690                1710                1730
TCAGTGGGCCTTCCAAGGAGGGGGCTGTAAAATGGAGGCCATTGTGTGAGGCCTTTCAGA
      1750                1770                1790
GTTGCTTGGCAAACCTGACCCCTTGCTCAGTAAAGCACNTTGCAACCATCTTGTTATGCC
```

```
      1810                 1830                 1850
TGTGACACATGGCCCCTCCCCCTGCCAGGAGCTTTTGGACCTAATCCAAGCATCCCTTTG
      1870                 1890                 1910
CCCAGAAAGAAGATGGGGGAGGAGGCAGTAATAAAAAGATTGAAGTATTTTGCTGGAATA
      1930                 1950                 1970
AGTTCAAATTCTTCTGAACTCAAACTGAGGAATTTCACCTGTAAACCTGAGTCGTACAGA
      1990                 2010                 2030
AAGCTGCCTGGTATATCCAAAAAGCCTTTTTATTCCTCCTGGCTCATATTGTGATTCTGG
      2050                 2070                 2090
CCCTTTGGGGACTTTTCTTAAACCTTCAGTTATGATTTTTTTTTCATACACCTTATTGGA
      2110                 2130                 2150
ACTCTGCCTGGATTTTTGCCTCTTCCAAGTCTTCCTGACACTTTAATTACCAACCTGTTA
      2170                 2190                 2210
CCTACTTTGACTTTTTGCATTTAAAACAGACACTGGCATGGATATAGTTTTACTTTTAAA
      2230                 2250                 2270
CTGTGTACATAACTGAAAATGTGCTATACTGCATACTTTTTAAAATGGTAAAGATATTTT
      2290                 2310                 2330
TATCTTTATATGAAGAAAATCACTTAGGAAATGGCTTTGTGATTCAATCTGTAAACTGTG
      2350                 2370                 2390
TATTCCAAGACATGTCTGTTCTACATAGATGCTTAGTCCCTCATGCAAATCAATTACTGG
      2410                 2430                 2450
TCCAAAAGATTGCTGAAATTTTATATGCTTACTGATATATTTTACAATTTTTTTATCATGC
      2470                 2490                 2510
ATGTCCTGTAAAGGTTACAAGCCTGCACAATAAAAATGTTTAACGGTTAAAAAAAAA
```

Figure 5.3. The amended cDNA sequence of human *CAVEOLIN-1*. The cDNA sequence and protein translation of human *CAVEOLIN-1* are shown. The cDNA sequence is a composite. The first 839 base pairs are derived from the published cDNA sequence (GenEMBL accession number Z18951), with two alterations: sequencing RT-PCR products and also genomic DNA fragments (see below), we consistently found G instead of C at position 278 and T instead of C at position 465. These changes are anticipated to alter the translated product non-conservatively: the histidine residue, amino acid 82, would be substituted for an aspartate residue, while the threonine residue, amino acid 144, would be substituted for an isoleucine residue. An alternative start codon, position 128, giving rise to the beta-form of Caveolin-1, is also shown (blue), as are the position of splice sites, indicated by ▼ (see below). The membrane associated domain of Caveolin is highlighted in magenta. The remaining cDNA sequence, which is 3′ UTR, is amalgamated from several EST sequences deposited in the EST division (dbEST) of GenEMBL. The position of primers which allow amplification of the EST (GenEMBL accession number T57690)-derived STS, SHGC-10385, are underlined, while the position of the polyadenylation signal is shown in green.

5.1.5 Exon trapping.

The technique of exon-trapping (Buckler *et al.*, 1991), or exon-amplification, is one among several in the armamentarium of a positional cloner for cloning genes. Unlike other techniques such as cDNA selection or hybridisation to a cDNA library or zoo blot where choices have to be made as to the samples to be screened, the success of exon-trapping is completely unaffected by the choice of starting material. DNA is selected simply by virtue of its possessing potential splice donor and acceptor sites which lie in the same restriction digest fragment. Restricted fragments of a genomic clone(s) of interest are subcloned into an exon-trapping vector (pSPL3) (Church *et al.*, 1994), which allows *in vivo* splicing of any exon contained within the insert with exons of a surrogate gene contained in the vector. The spliced donor exon can then be recovered by performing RT-PCR using primers directed against vector sequences (Table A4).

Exon-trapping was undertaken using DNA pooled from cosmids c53, c62, and c87, digested with *Pst* I or *Bam* HI/*Bgl* II. c53 and c62 contain exons fom c-*MET*, and it was hoped that these would serve as a positive control for the technique. Of 20 RT-PCR amplification products TA-cloned into pCR2.1 and sequenced, only one was not derived from vector sequences (Figure 5.4). The sequences flanking this putative trapped-exon were determined and revealed the presence of consensus splice sites. A BLAST search did not detect any homologies to known nucleotide or protein sequences, including c-*MET*. Finally, a probe prepared containing this 143 bp putative exon was used to screen a northern blot, but no transcript was detected, nor was it possible to amplify a product by PCR from senescent fibroblast cDNA corresponding to this putative exon.

5.1.6 Chromosome 7 sequencing consortium

There have been other major inroads into the characterisation of the genomic interval between D7S522 and 17TA-5/17B-RE3, not least among them being recent releases from the chromosome 7 sequencing project which is a collaborative venture between investigators at the National Human Genome Research Institute, NHGRI (a subdivision of the National Institutes of Health, NIH, USA), the University of Washington Genome Research Centre and Washington University Genome Sequencing Centre (St. Louis, MO, USA). A summary of their sequencing data can be accessed

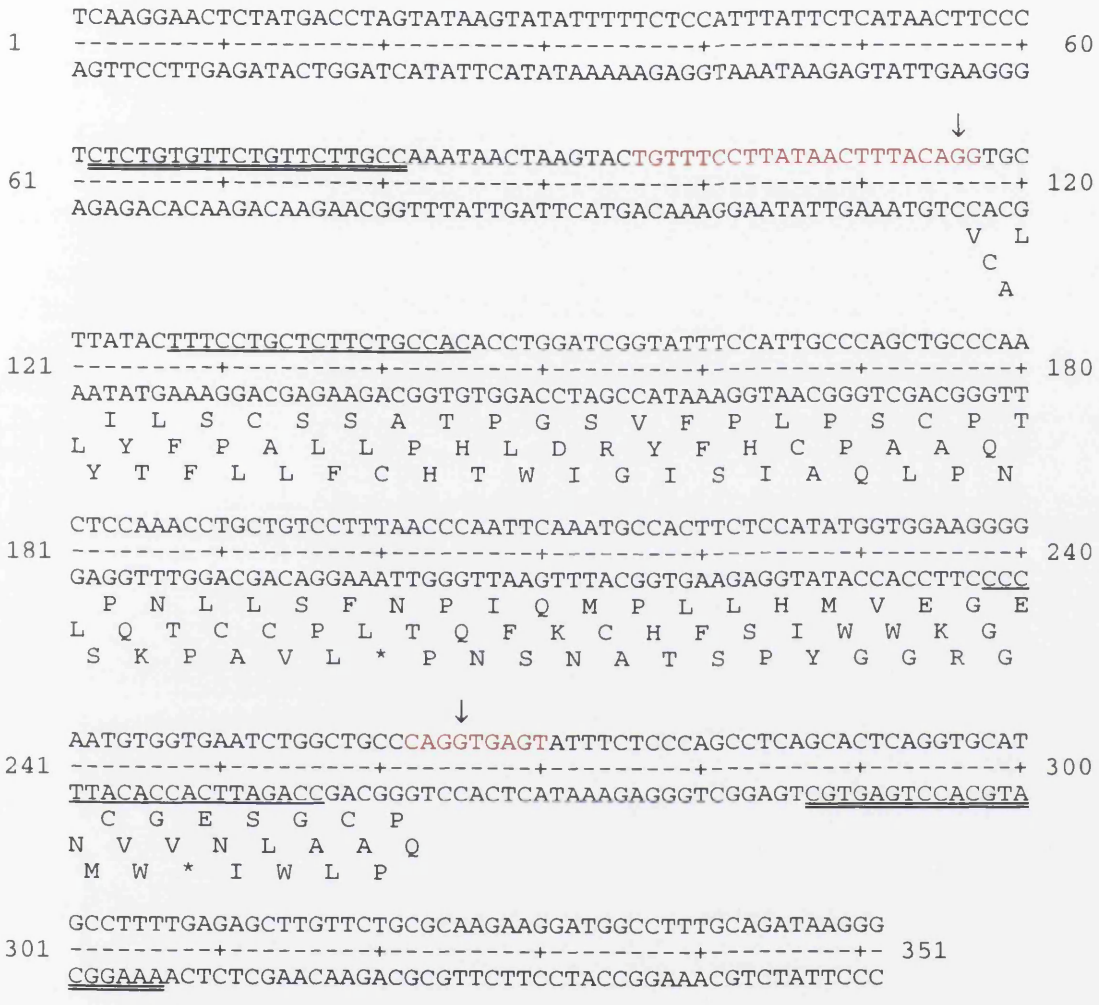


Figure 5.4. Sequence of a novel exon. The sequence shown includes a novel putative exon derived by exon-trapping. The position of the splice acceptor and donor sites are marked (↓). Translations for the sense strand of this exon are given below in single letter annotation for all three frames; * designates a stop codon. Both the first and second reading frame are open. The positions of primers used to extend the genomic sequence, and hence verify the existence of splice site sequences (shown in red) in close agreement with the consensus, are underlined with a single line. The positions of primers used to perform SSCP-heteroduplex analysis are underlined with a double line.

through a site on the World Wide Web at (http://www.nhgri.nih.gov/DIR/GTB/CHR7/). Sequencing of the D7S522–17TA-5/17B-RE3 interval is approaching completion; so far 1 Mbp of sequence has been deposited by the consortium in the GenEMBL databank for public access, including virtually the complete sequence covering the deleted interval in SUSM-1/*Hytk7* segregant 4. In addition to generating primary data, sequences have also been analysed by performing homology searches using the BLAST algorithm (Altschul *et al.*, 1990), and this has revealed various instances of highly significant homology with several human EST and cDNA sequences also deposited in the GenEMBL database, as well as to sequences present in the genomes of other species, such as the nematode *Caenorhabditis elegans* and the budding yeast *Saccharomyces cerevisiae.* Sequence data has also been subjected to analysis using exon prediction programs such as GRAIL (Uberbacher and Mural, 1991) to identify putative exons. This information provides strong evidence for the existence of expressed sequences at positions identified in these ways (although the possibility that these sequences are derived from pseudogenes can not be completely excluded).

The complete sequence for *hF11A10.5* has been deposited by the consortium (GenEMBL accession numbers AC002542 [nucleotides 61,071–185,981]). The *hF11A10.5* gene comprises fourteen exons distributed over 125 Kbp, oriented with its 5´-end towards the centromere, and encoding a protein product with 432 amino acid residues. Sequence corresponding to R14.4E1 has also been submitted (GenEMBL accession numbers AC002465 [nucleotides 147354–148318]). Further residues with homology to mammalian ESTs as well as GRAIL-predicted exons have been identified in the interval defined in the SUSM-1/*Hytk7* segregant 4, which due to their mapping position emerge as very interesting candidates. There are, however, also numerous apparent gaps in the sequence for the D7S522–17TA-5/17B-RE3 interval: as yet there is no deposited genomic sequence for *CAVEOLIN-1*, nor the exon trapped from cosmid 53.

5.2 Mutation analysis of genes within the D7S522-17TA5/B-RE3 interval

Our analysis of the D7S522-17TA-5/17B-RE3 interval indicates the existence of several genes which by virtue of their map positions alone promote themselves as candidates for the 7q31 multi-tissue tumour suppressor/replicative senescence gene.

However, a candidate gene has to satisfy a number of criteria before its role as a TSG can be asserted: the gene should be expressed in tissues from which tumours displaying 7q31 LOH are derived; it should be possible to find loss of expression of the gene in tumours and mutations which would compromise the function of the gene product, including possibly germ-line mutations which predispose carriers to an increased cancer susceptibility; ultimately, it should be possible to demonstrate that restoration of the gene or of its product to tumour cells is able to abrogate some aspect of their transformed phenotype. Based upon the known roles of c-*MET*, *CAPZA2*, *WNT2*, and *CFTR*, these genes were not considered as candidates for the 7q31 tumour suppressor/replicative senescence gene. Further, on the assumption that the 7q31 tumour suppressor and replicative senescence gene were one and the same, we decided to limit our analysis of candidate genes to those that were expressed in senescent cells.

5.2.1 Southern and northern blot analysis

Of the novel genes so far identified, and which possibly map to the D7S522-17TA-5/17B-RE3 interval, only *CAVEOLIN*-1 and *hF11A10.5* were found to be expressed in senescent fibroblasts, both by northern blot analysis (Figures 5.1 and Figure 5.5) and RT-PCR (using the senescent fibroblast cDNA library as a template, Table 5.1). As stated, from the tissue types represented the transcript for *hF11A10.5* appears to be ubiquitously expressed. The level of expression of *CAVEOLIN*-1 appeared to be more variable among the different cell lines studied. Northern blot analysis revealed the presence of a single 2.7 Kbp transcript in the majority of samples examined. However, no transcript was detected in the metastatic prostate carcinoma cell lines, DU145, LnCaP, and PC3. No altered transcripts were detected for either *hF11A10.5* or *CAVEOLIN-1* that might reflect the presence of an underlying mutation. cDNA probes corresponding to these two genes were also used to probe Southern blots of restriction digested DNA prepared from tumourigenic as well as non-tumourigenic immortal cell lines and SUSM-1/*Hytk7* immortal segregants. No evidence was found for re-arrangements or homozygous deletion of either gene (Figure 5.6).

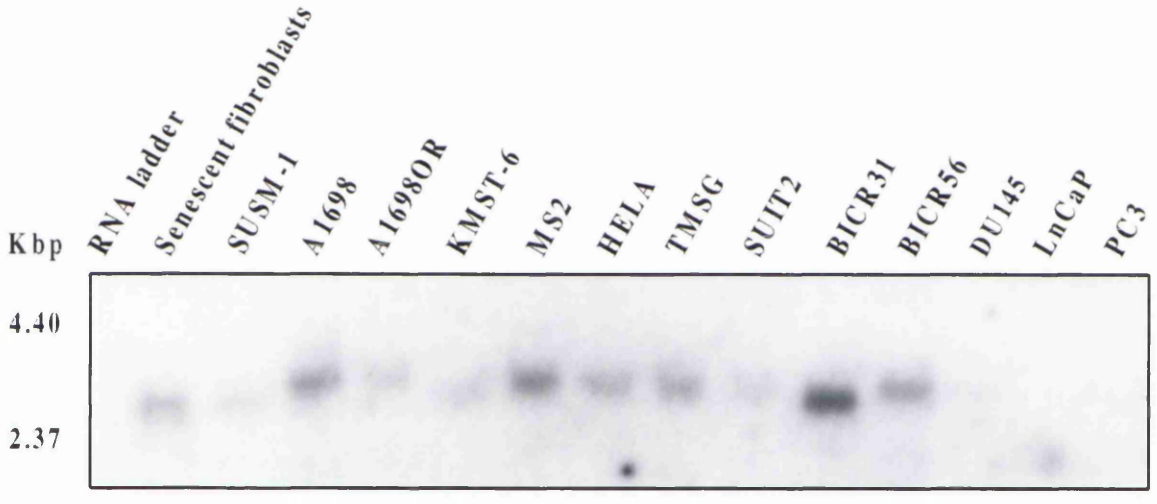
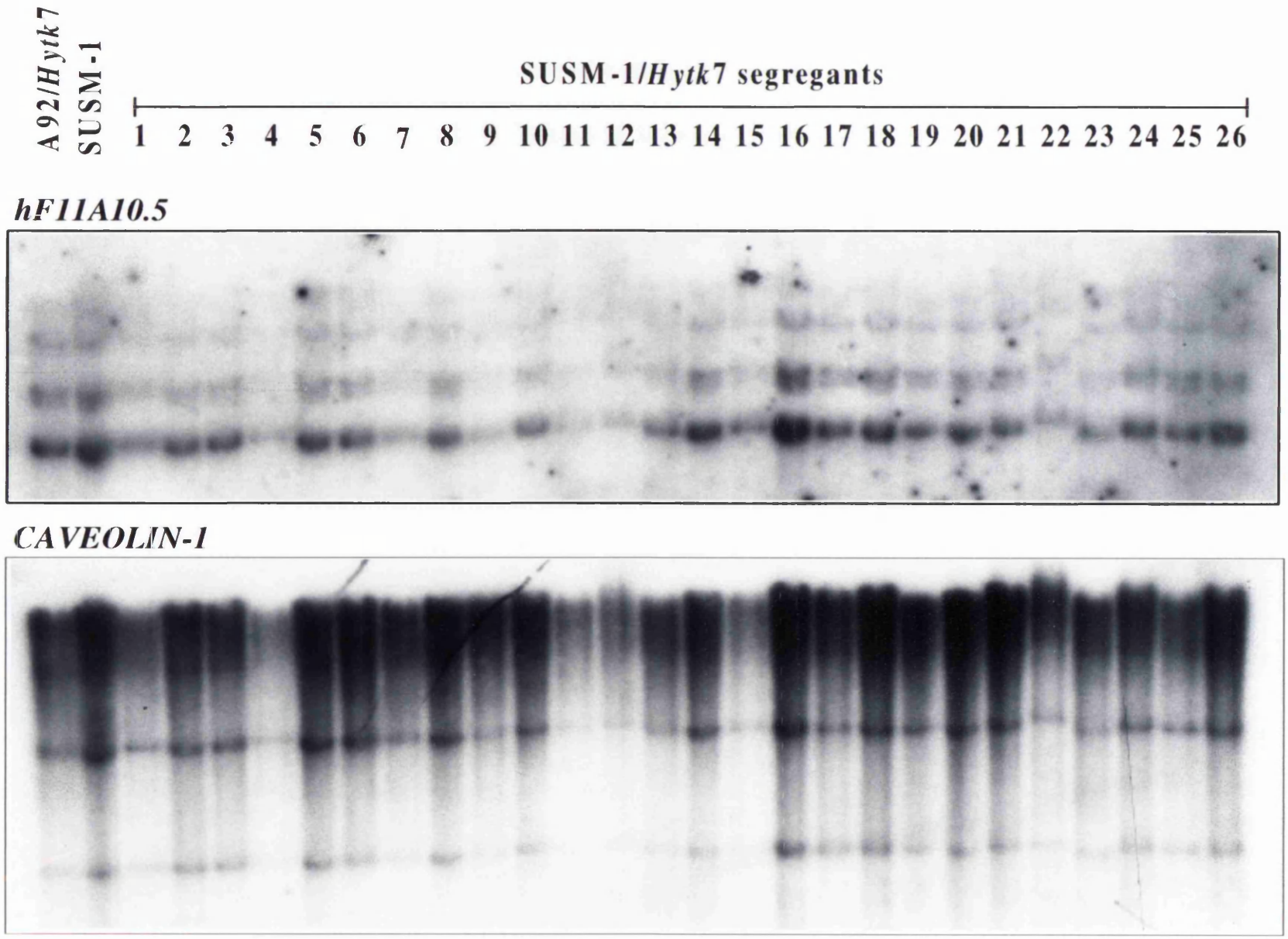


Figure 5.5. Northern blot analysis of *CAVEOLIN-1* expression in cell lines. RNA from a number of tumourigenic and non-tumourigenic immortal cell lines, as well as RNA from senescent fibroblasts (70 mean population doublings) were transferred to Hybond N membrane and hybridised with a radiolabelled probe corresponding to *CAVEOLIN-1*. (See Figure 5.1 for note on samples.)

Figure 5.6. Southern blot analysis of SUSM-1/*Hytk*7 immortal segregants probed with *hF11A10.5* or *CAVEOLIN-1* cDNA. Segregant DNA was digested with *Bam* HI and resolved on a 1% agarose gel, before being transferred onto Hybond N+ membrane. Blots were then prehybridised and probed with either *hF11A10.5* (upper panel) or *CAVEOLIN-1* (lower panel) radiolabelled cDNA. Bands were revealed by autoradiography.


A92/Hytk7
SUSM-1
SUSM-1/Hytk7 segregants
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
hF11A10.5
CAVEOLIN-1

5.2.2 SSCP-heteroduplex analysis

To determine whether there were microscale alterations in the expressed sequences of *CAVEOLIN-1* and *hF11A10.5*, we decided to perform single strand conformation polymorphism (SSCP) and heteroduplex analysis. In order to achieve this, it was first necessary to establish the genomic structures of these two genes; a similar strategy was adopted in both cases. Bacterial clones containing these genes were identified from among the cosmids already selected and by screening the human PAC library filters. Oligonucleotide primers spaced at regular 100 bp intervals along both the sense and antisense strand of the cDNA were synthesised and used to sequence directly from cosmids or PAC DNA. The quality of sequence derived from cosmid clones was consistently good, but was poorer from PAC clones. Nonetheless, even when derived from PAC clones, it was sufficiently good to identify the points at which genomic sequence diverged from cDNA sequence. In every case, it was obvious that these points of divergence corresponded to canonical splice donor and acceptor sites (conforming to the GT..AG rule). In this way, it was possible to determine the genomic structure of all three exons of *CAVEOLIN-1* (Figure 5.7) and the last four exons from *hF11A10.5* (corresponding to nucleotides 173637–173787, 182934–183026, and 185773–185981 of the 7q31 BAC clone RG114A06 [GenEMBL accession number AC002542], and nucleotides 5071–5412 of BAC clone DJ0866N18 [GenEMBL accession number AC003987])

Oligonucleotide primers were synthesised to allow PCR-amplification across exons, including both splice junctions, of *CAVEOLIN-1* and *hF11A10.5* (Table 5.2), using DNA from tumourigenic as well as non-tumourigenic immortal cell lines and SUSM-1/*Hytk*7 immortal segregants as template. (It was possible to amplify DNA corresponding to exons 1 and 3, but not exon 2, of *CAVEOLIN-1*. This failure probably results from the high (62.9%) G/C content of this exon.) Radiolabelled PCR products were resolved on thin polyacrylamide gels under non-denaturing conditions and visualised by autoradiography using X-ray film. None of the PCR products obtained from exons of *CAVEOLIN-1* or *hF11A10.5* demonstrated altered electrophoretic mobility or gave rise to heteroduplexes (Figure 5.8). The absence of mutations in the entire coding sequence (including exon 2) of *CAVEOLIN-1* was confirmed for the cell lines A1698, A1698OR, MDA-MB-231, OVCAR5, and SUSM-1 by sequencing cDNA generated from these cell lines by RT-PCR (data not shown).

Figure 5.7a. The genomic structure of the human *CAVEOLIN-1* gene: Exon 1. Sequence containing the first exon of human *CAVEOLIN-1*, in addition to a portion of the promoter region, is shown. A putative transcription initiation site at position 90 (blue) of the given sequence was calculated using the neural-network promoter prediction algorithm after Reese *et al.* (1996); initiation at this site would give rise to an anticipated messenger RNA molecule approximately 2760 nucleotides long, dependent upon the size of the polyA tail, which is in close agreement with what was found on a Northern blot for *CAVEOLIN-1* message (Figure 5.5). Two CpG islands are also depicted (green); the first, which contains the putative transcription start site, has a 63.4% GC content, while the second, from base-pair 1213 to 1433, has a 52.1% GC content. Multiple *Sma* I sites which fall within these two CpG islands are shown. The position and extent of the CpG islands was calculated using the algorithm after Gardiner-Garden and Frommer (1987). The first exon of *CAVEOLIN-1* encodes only 5% of the alpha-form of the protein. The splice donor site for this exon is indicated by ↓, and the flanking sequence is highlighted in red. The positions of primers used to perform SSCP-heteroduplex analysis are underlined.

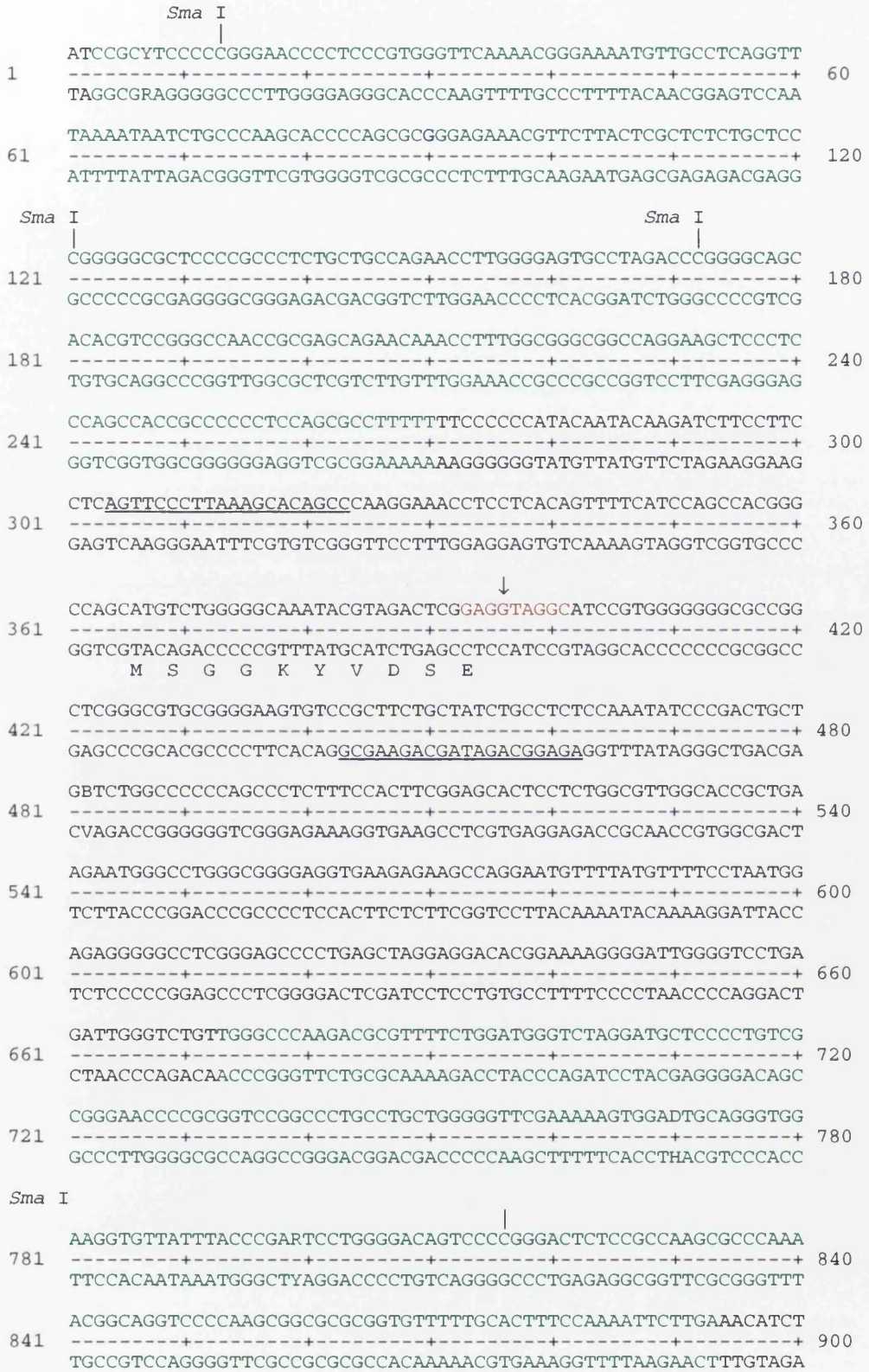
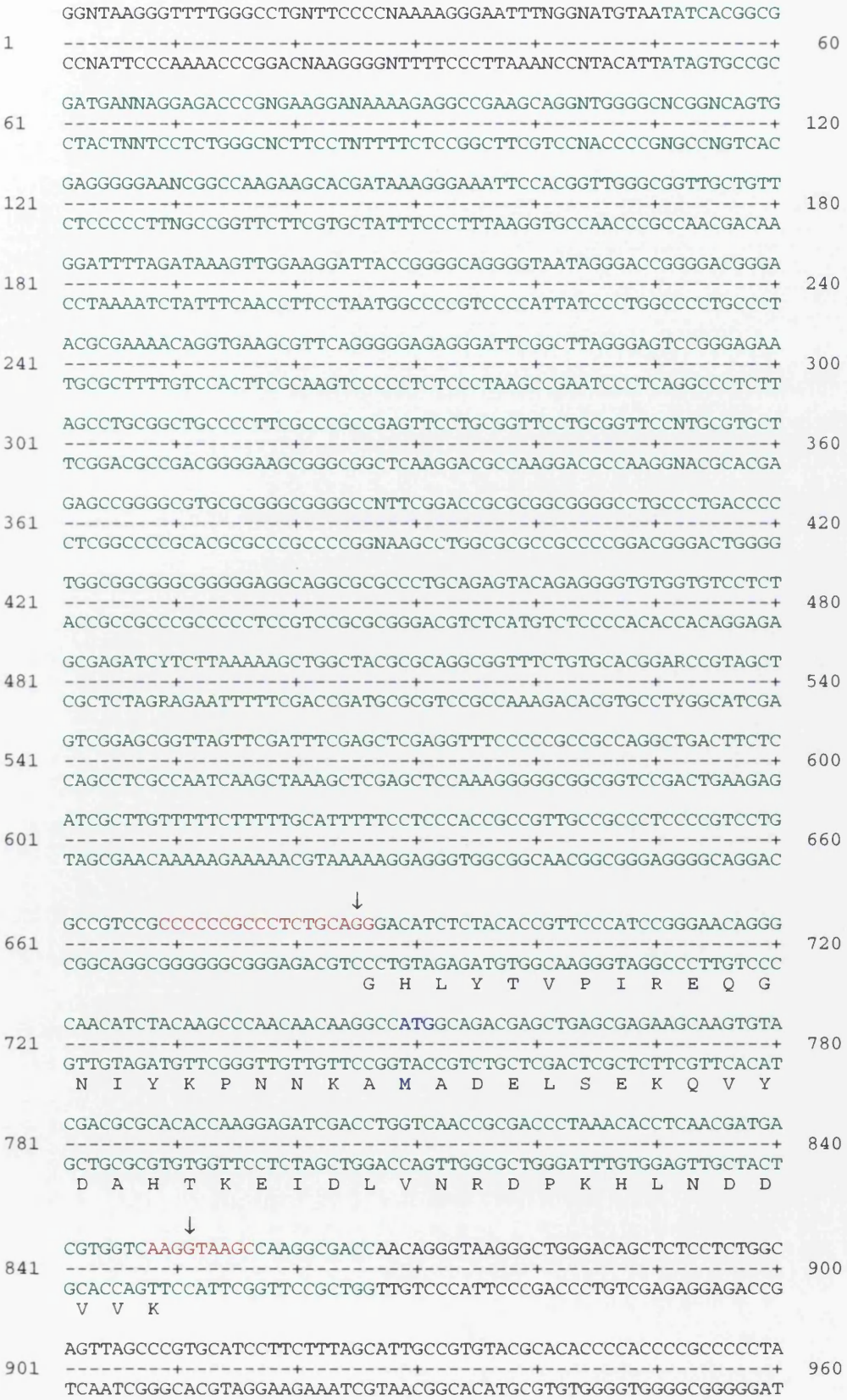

Sma I
ATCCGCYTCCCCCGGGAACCCCTCCCGTGGGTTCAAAACGGGAAAATGTTGCCTCAGGTT
1
60
TAGGCGRAGGGGGCCCTTGGGGAGGGCACCCAAGTTTTGCCCTTTTACAACGGAGTCCAA
TAAAATAATCTGCCCAAGCACCCCAGCGCGGGAGAAACGTTCTTACTCGCTCTCTGCTCC
61
120
ATTTTATTAGACGGGTTCGTGGGGTCGCGCCCTCTTTGCAAGAATGAGCGAGAGACGAGG
Sma I
Sma I
CGGGGGCGCTCCCCGCCCTCTGCTGCCAGAACCTTGGGGAGTGCCTAGACCCGGGGCAGC
121
180
GCCCCCGCGAGGGGCGGGAGACGACGGTCTTGGAACCCCTCACGGATCTGGGCCCCGTCG
ACACGTCCGGGCCAACCGCGAGCAGAACAAACCTTTGGCGGGCGGCCAGGAAGCTCCCTC
181
240
TGTGCAGGCCCGGTTGGCGCTCGTCTTGTTTGGAAACCGCCCGCCGGTCCTTCGAGGGAG
CCAGCCACCGCCCCCCTCCAGCGCCTTTTTTTCCCCCCATACAATACAAGATCTTCCTTC
241
300
GGTCGGTGGCGGGGGGAGGTCGCGGAAAAAAAGGGGGGTATGTTATGTTCTAGAAGGAAG
CTCAGTTCCCTTAAAGCACAGCCCAAGGAAACCTCCTCACAGTTTTCATCCAGCCACGGG
301
360
GAGTCAAGGGAATTTCGTGTCGGGTTCCTTTGGAGGAGTGTCAAAAGTAGGTCGGTGCCC
CCAGCATGTCTGGGGGCAAATACGTAGACTCGGAGGTAGGCATCCGTGGGGGGGCGCCGG
361
420
GGTCGTACAGACCCCCGTTTATGCATCTGAGCCTCCATCCGTAGGCACCCCCCCGCGGCC
M S G G K Y V D S E
CTCGGGCGTGCGGGGAAGTGTCCGCTTCTGCTATCTGCCTCTCCAAATATCCCGACTGCT
421
480
GAGCCCGCACGCCCCTTCACAGGCGAAGACGATAGACGGAGAGGTTTATAGGGCTGACGA
GBTCTGGCCCCCCAGCCCTCTTTCCACTTCGGAGCACTCCTCTGGCGTTGGCACCGCTGA
481
540
CVAGACCGGGGGGTCGGGAGAAAGGTGAAGCCTCGTGAGGAGACCGCAACCGTGGCGACT
AGAATGGGCCTGGGCGGGGAGGTGAAGAGAAGCCAGGAATGTTTTATGTTTTCCTAATGG
541
600
TCTTACCCGGACCCGCCCCTCCACTTCTCTTCGGTCCTTACAAAATACAAAAGGATTACC
AGAGGGGGCCTCGGGAGCCCCTGAGCTAGGAGGACACGGAAAAGGGGATTGGGGTCCTGA
601
660
TCTCCCCCCGGAGCCCTCGGGGACTCGATCCTCCTGTGCCTTTTCCCCTAACCCCAGGACT
GATTGGGTCTGTTGGGCCCAAGACGCGTTTTCTGGATGGGTCTAGGATGCTCCCCTGTCG
661
720
CTAACCCAGACAACCCGGGTTCTGCGCAAAAGACCTACCCAGATCCTACGAGGGGACAGC
CGGGAACCCCGCGGTCCGGCCCTGCCTGCTGGGGGTTCGAAAAAGTGGADTGCAGGGTGG
721
780
GCCCTTGGGGCGCCAGGCCGGGACGGACGACCCCCAAGCTTTTTCACCTHACGTCCCACC
Sma I
AAGGTGTTATTTACCCGARTCCTGGGGACAGTCCCCGGGACTCTCCGCCAAGCGCCCAAA
781
840
TTCCACAATAAATGGGCTYAGGACCCCTGTCAGGGGCCCTGAGAGGCGGTTCGCGGGTTT
ACGGCAGGTCCCCAAGCGGCGCGCGGTGTTTTTGCACTTTCCAAAATTCTTGAAACATCT
841
900
TGCCGTCCAGGGGTTCGCCGCGCGCCACAAAAACGTGAAAGGTTTTAAGAACTTTGTAGA

Figure 5.7b. Exon 2. Exon 2 of *CAVEOLIN-1* encodes 31% of the gene product. Splice donor and acceptor sites are indicated (↓), and flanking sequences highlighted (red). Exon 2 is contained within a CpG island (green) that extends between base-pair 51 and 866 of the given sequence. This CpG island has a 62.9% GC content. The position of an alternative start codon, position 749, giving rise to the beta-form of Caveolin-1, is also shown (blue).


GGNTAAGGGTTTTGGGCCTGNTTCCCCNAAAAGGGAATTTNGGNATGTAATATCACGGCG
CCNATTCCCAAAACCCGGACNAAGGGGNTTTTCCCTTAAANCCNTACATTATAGTGCCGC
GATGANNAGGAGACCCGNGAAGGANAAAAGAGGCCGAAGCAGGNTGGGGCNCGGNCAGTG
CTACTNNTCCTCTGGGCNCTTCCTNTTTTCTCCGGCTTCGTCCNACCCCGNGCCNGTCAC
GAGGGGGAANCGGCCAAGAAGCACGATAAAGGGAAATTCCACGGTTGGGCGGTTGCTGTT
CTCCCCCTTNGCCGGTTCTTCGTGCTATTTCCCTTTAAGGTGCCAACCCGCCAACGACAA
GGATTTTAGATAAAGTTGGAAGGATTACCGGGGCAGGGGTAATAGGGACCGGGGACGGGA
CCTAAAATCTATTTCAACCTTCCTAATGGCCCCGTCCCCATTATCCCTGGCCCCTGCCCT
ACGCGAAAACAGGTGAAGCGTTCAGGGGGAGAGGGATTCGGCTTAGGGAGTCCGGGAGAA
TGCGCTTTTGTCCACTTCGCAAGTCCCCCTCTCCCTAAGCCGAATCCCTCAGGCCCTCTT
AGCCTGCGGCTGCCCCTTCGCCCGCCGAGTTCCTGCGGTTCCTGCGGTTCCNTGCGTGCT
TCGGACGCCGACGGGGAAGCGGGCGGCTCAAGGACGCCAAGGACGCCAAGGNACGCACGA
GAGCCGGGGCGTGCGCGGGCGGGGCCNTTCGGACCGCGCGGCGGGGCCTGCCCTGACCCC
CTCGGCCCCGCACGCGCCCGCCCCGGNAAGCCTGGCGCGCCGCCCCGGACGGGACTGGGG
TGGCGGCGGGCGGGGGAGGCAGGCGCGCCCTGCAGAGTACAGAGGGGTGTGGTGTCCTCT
ACCGCCGCCCGCCCCCTCCGTCCGCGCGGGACGTCTCATGTCTCCCCACACCACAGGAGA
GCGAGATCYTCTTAAAAAGCTGGCTACGCGCAGGCGGTTTCTGTGCACGGARCCGTAGCT
CGCTCTAGRAGAATTTTTCGACCGATGCGCGTCCGCCAAAGACACGTGCCTYGGCATCGA
GTCGGAGCGGTTAGTTCGATTTCGAGCTCGAGGTTTCCCCCGCCGCCAGGCTGACTTCTC
CAGCCTCGCCAATCAAGCTAAAGCTCGAGCTCCAAAGGGGGCGGCGGTCCGACTGAAGAG
ATCGCTTGTTTTTCTTTTTGCATTTTTCCTCCCACCGCCGTTGCCGCCCTCCCCGTCCTG
TAGCGAACAAAAAGAAAAACGTAAAAAGGAGGGTGGCGGCAACGGCGGGAGGGGCAGGAC
GCCGTCCGCCCCCCGCCCTCTGCAGGGACATCTCTACACCGTTCCCATCCGGGAACAGGG
CGGCAGGCGGGGGGCGGGAGACGTCCCTGTAGAGATGTGGCAAGGGTAGGCCCTTGTCCC
G H L Y T V P I R E Q G
CAACATCTACAAGCCCAACAACAAGGCCATGGCAGACGAGCTGAGCGAGAAGCAAGTGTA
GTTGTAGATGTTCGGGTTGTTGTTCCGGTACCGTCTGCTCGACTCGCTCTTCGTTCACAT
N I Y K P N N K A M A D E L S E K Q V Y
CGACGCGCACACCAAGGAGATCGACCTGGTCAACCGCGACCCTAAACACCTCAACGATGA
GCTGCGCGTGTGGTTCCTCTAGCTGGACCAGTTGGCGCTGGGATTTGTGGAGTTGCTACT
D A H T K E I D L V N R D P K H L N D D
CGTGGTCAAGGTAAGCCAAGGCGACCAACAGGGTAAGGGCTGGGACAGCTCTCCTCTGGC
GCACCAGTTCCATTCGGTTCCGCTGGTTGTCCCATTCCCGACCCTGTCGAGAGGAGACCG
V V K
AGTTAGCCCGTGCATCCTTCTTTAGCATTGCCGTGTACGCACACCCCACCCCGCCCCCTA
TCAATCGGGCACGTAGGAAGAAATCGTAACGGCACATGCGTGTGGGGTGGGGCGGGGGAT

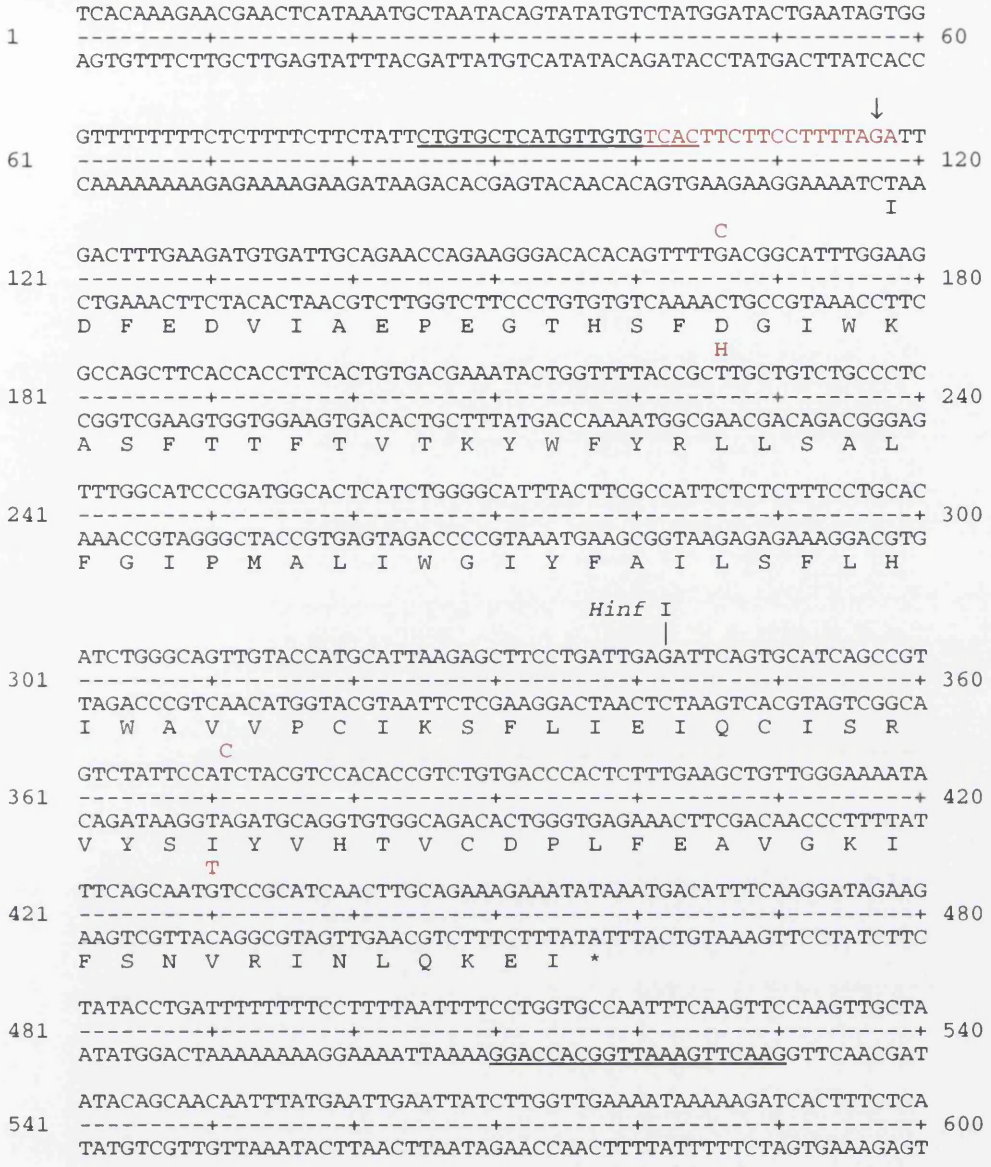


Figure 5.7c. Exon3. The sequence shown contains the third and final exon of *CAVEOLIN-1*, which encodes 63% of the gene product. The splice acceptor site for this exon is indicated by ↓, and the flanking sequence highlighted in red. The positions of primers (underlined), as well as the position of a *Hinf* I site, are shown which were used to perform SSCP-heteroduplex analysis. We consistently found G instead of C at position 278 and T instead of C at position 465 (See Figure 5.3).

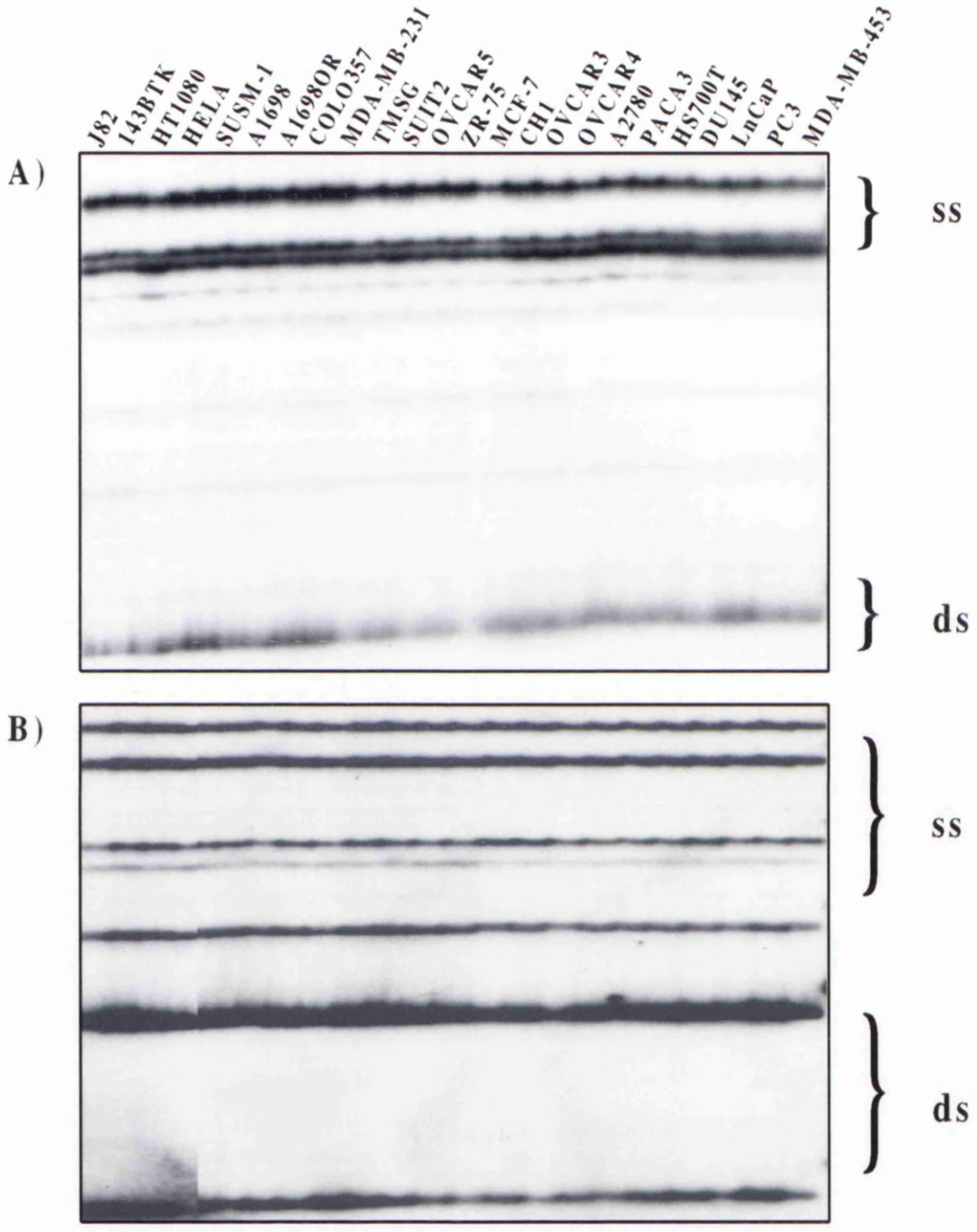


Figure 5.8. Mutation analysis of *hF11A10.5* and *CAVEOLIN-1* by SSCP analysis. PCR amplifications products as revealed by autoradiography are shown for the indicated cell lines for (**A**) exon 11 of *hF11A10.5* and (**B**) exon 3 of *CAVEOLIN-1*. The PCR product for exon 3 of *CAVEOLIN-1* was cleaved with *Hinf* I resulting in two fragments of 257 bp and 187 bp. ss, single-stranded DNA; ds, double-stranded DNA.

CAVEOLIN-1	
Exon 1	L: AGTTCCCTTAAAGCACAGCC R: AGAGGCAGATAGCAGAAGCG
Exon 3	L: CTGTGCTCATGTTGTGTCAC R: GAACTTGAAATTGGCACCAGG
hF11A10.5	
Exon 11	L: TTAGGTATTAACACAAGTGTGTCC R: TTTAGCACCTTTTCATGCTC
Exon 12	L: ACAAACATTGGACATCTCTG R: TTCCAATCCCCACCTTTCAC
Exon 13	L: GAGTGCAGTTTACTCCAGCC R: CTTGGCTTTCCCCATCC
Exon 14	L: GTTTGTTTTATAGTCTTGAACAAGG R: GACTTTCTTCTTTTCTGAAGGC

Table 5.2. Oligonucleotide Primers for SSCP-heteroduplex analysis

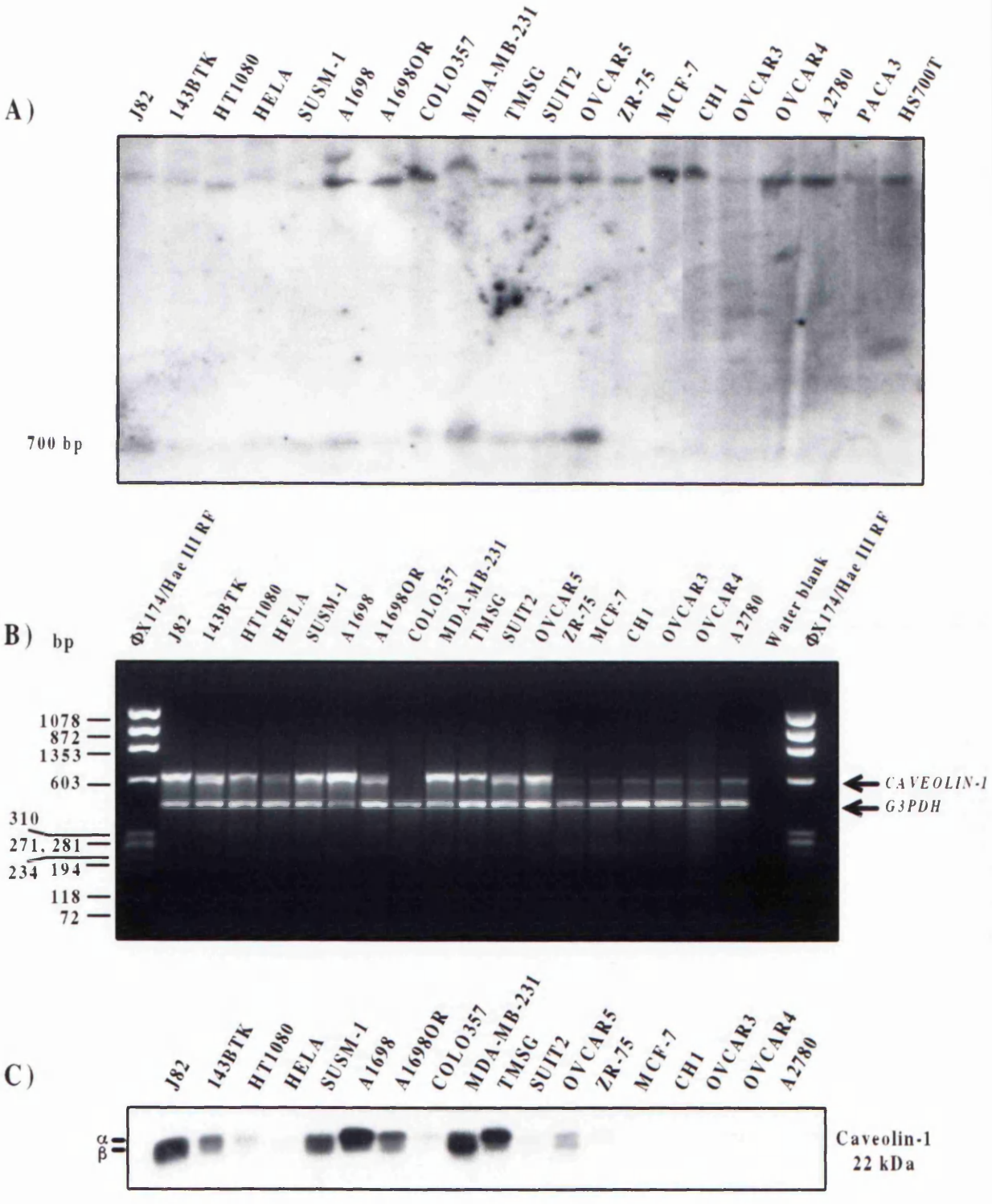
5.3 *CAVEOLIN-1*

5.3.1 Methylation and regulation of gene expression

There have now been several reported instances of genes possessing CpG islands being transcriptionally silenced by methylation during tumour progression, and methylation has been proposed as a possible epigenetic mechanism of tumour suppressor gene inactivation. In the case of *CDKN2A*, for example, methylation is perhaps the most prevalent mechanism whereby this gene is inactivated in tumours (Herman *et al.*, 1995; Loughran *et al.*, 1996). Therefore, we decided to look at the methylation status of *CAVEOLIN-1* in DNA from several of the cell lines previously analysed in order to determine whether methylation occurred, with what frequency, and whether it correlated with the level of expression of Caveolin.

Genomic DNA from sample cell lines digested with a combination of *Eco*R I and *Sma* I restriction enzymes was hybridised on a blot with the *CAVEOLIN-1* cDNA probe (Figure 5.9a). The recognition sequence for *Sma* I is CCCGGG; this sequence is resistant to cleavage if the third cytosine residue is methylated on the 5-position. Exon 1 of *CAVEOLIN-1* lies within a single *Sma* I fragment approximately 700 bp in size (Figure 5.7a). A fragment this size can be observed on a genomic DNA blot if the CpG island associated with exon 1 is undermethylated, but is inapparent if methylated. Figure 5.9a provides evidence for methylation of *CAVEOLIN-1* in up to 50% of the

Figure 5.9. Methylation status of *CAVEOLIN-1* in tumour-derived cell lines and its relation to expression. Southern blot analysis reveals an approximately 700bp *Sma* I-cleavable product in only a proportion of cell lines (A). Expression of *CAVEOLIN-1* in these cell lines as revealed by RT-PCR analysis (B) and western blot analysis (C). Oligonucleotide primers which amplify a region of *G3PDH* cDNA were included in the RT-PCR analysis as an internal control for amplification efficiency. The α and β in (C) refer to the two protein isoforms of Caveolin-1, which are detectable in certain of the samples (*e.g.* OVCAR5).


A)
J82
143BTK
HT1080
HELA
SUSM-1
A1698
A1698OR
COLO357
MDA-MB-231
TMSG
SUIT2
OVCAR5
ZR-75
MCF-7
CH1
OVCAR3
OVCAR4
A2780
PACA3
HS700T
700 bp
B)
bp
ΦX174/Hae III RF
J82
143BTK
HT1080
HELA
SUSM-1
A1698
A1698OR
COLO357
MDA-MB-231
TMSG
SUIT2
OVCAR5
ZR-75
MCF-7
CH1
OVCAR3
OVCAR4
A2780
Water blank
ΦX174/Hae III RF
1078
872
1353
603
310
271, 281
234 194
118
72
CAVEOLIN-1
G3PDH
C)
J82
143BTK
HT1080
HELA
SUSM-1
A1698
A1698OR
COLO357
MDA-MB-231
TMSG
SUIT2
OVCAR5
ZR-75
MCF-7
CH1
OVCAR3
OVCAR4
A2780
α
β
Caveolin-1
22 kDa

cancer derived cell lines studied, including ZR-75, MCF-7, CH1, OVCAR3, OVCAR4, A2780, PACA3, and HS700T. Although detection of methylation of exon 1 of *CAVEOLIN-1* by Southern blot analysis was perhaps sub-optimal (due largely to the probe sharing only 30 bp of homology with exon 1), the hybridisation pattern was reproducible, and did not result from incomplete restriction digestion since hybridisation with a probe derived from *G3PDH* revealed no inconsistencies between samples (data not shown). From RT-PCR analysis, it is evident that the amount of *Sma* I-cleavable product (*i.e.* methylation) correlates with the level of transcription of *CAVEOLIN-1*: levels of *CAVEOLIN-1* transcript are barely detectable in the cell lines ZR-75, MCF-7, CH1, OVCAR3, OVCAR4, and A2780, which most convincingly demonstrate methylation of the gene (Figure 5.9b). This implies that methylation is a major mechanism of transcriptional regulation for *CAVEOLIN-1*. From western analysis, there also appears to be a close correlation between the level of transcription and translation of *CAVEOLIN-1* (Figure 5.9c).

5.3.2 Loss of Caveolin-1 protein in HeLa

Despite being able to detect a *CAVEOLIN-1* transcript in HeLa cells (Figures 5.5 and 5.9b), levels of Caveolin-1 protein are disproportionately low (Figure 5.9c), suggesting that the expression of Caveolin-1 may also be post-transcriptionally regulated. It is known that the level of Caveolin-1 protein is inversely correlated with the expression of oncogenes, including v-Src (Koleske *et al.*, 1995). Indeed, Caveolin-1 was first identified as being a major phosphorylation target for v-Src (Glenney, 1989); phosphorylation may subsequently have an effect on protein stability. HeLa cells are known to be positive for HPV, and express the oncoproteins E6 and E7. One wondered whether expression of these proteins might be related to loss of Caveolin-1 in HeLa. Therefore, the level of Caveolin-1 expression was determined in a number of cell lines which are known to express E6 and E7, and compared to the levels in cell lines that do not (Figure 5.10). From western blot analysis, there is no correlation between Caveolin-1 expression and the presence of E6 or E7.

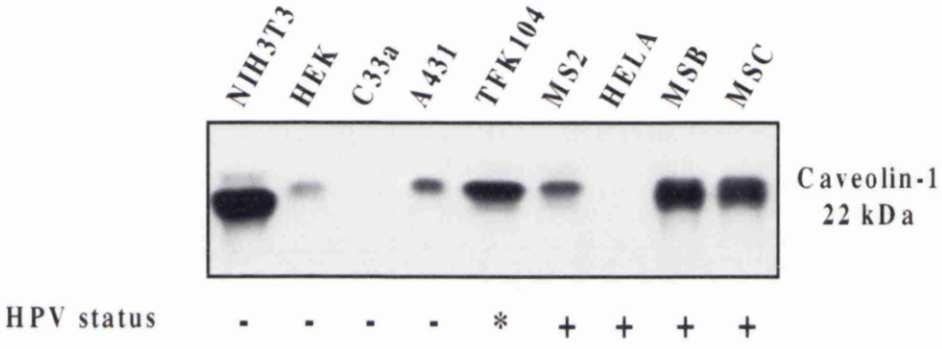


Figure 5.10. Lack of correlation between expression of HPV oncoproteins and Caveolin-1 protein. The expression of Caveolin-1 protein was determined by western blot analysis for a number of cell lines, which either do or do not express HPV oncoproteins (indicated at the bottom of the panel). A431, C33a, MS2, MSB, and MSC are cervical carcinoma-derived cell lines, while HeLa is derived from carcinoma of the vulva. HEK are a culture of human embyronic keratinocytes, while TFK104 are a strain of human embyronic keratinocytes immortalised with the E6 and E7 oncogenes of HPV16 (*). Murine NIH3T3 fibroblasts were included as a positive control for Caveolin-1 expression. (Cell pellets for the extraction of protein were provided by Drs D. Owens, A. Malliri, and E. K. Parkinson, BICR.)

CHAPTER 6

DISCUSSION

6. Discussion

From my investigations, it would appear that allele loss at the q31 region of human chromosome 7 occurs in 40% of breast carcinomas. The SCDR was between D7S522 and 17TA-5/17B-RE3 in intron 17b of the *CFTR* gene. Allele loss on the long arm of chromosome 7 was also observed in approximately half the immortal SUSM-1/*Hytk7* segregants. Losses occurred most frequently at three loci: D7S2555, D7S821, and D7S633–17TA-5/17B-RE3. Only the D7S633–17TA-5/17B-RE3 locus was also implicated in the tumour LOH study. An approximately 500 Kbp region of interstitial allele loss between 724CA and 786CA identified in SUSM-1/*Hytk7* segregant 4 is nested within the breast tumour SCDR (Figure 6.1). The overlap between the region of allele loss in SUSM-1/*Hytk7* segregant 4, the smallest region of loss defined using this model, and the SCDR in breast carcinomas provides only a modicum of genetic support for our hypothesis that the chromosome 7 tumour suppressor gene and the complementation group D replicative senescence gene, *SEND*, are one and the same. Other work from the group provides further support for this hypothesis. Mary Berrington has shown that the tumour derived cell lines MDA-MB-231, COLO357, and OVCAR5 demonstrate LOH specifically on the long arm of chromosome 7, including the q31 region, and further that mortalin in these cell lines is distributed as in other immortality complementation group D cell lines. Moreover, introduction of chromosome 7 into these cell lines by microcell mediated monochromosome transfer induces replicative senescence accompanied by reversion of mortalin distribution to that found in mortal cells (unpublished data).

That the D7S522–17TA-5/17B-RE3 interval harbours a TSG is consistent with a number of other studies of LOH performed in a broad spectrum of human tumours (Figure 6.2); a survey of this published literature suggest a median incidence of 49% for LOH on 7q. Dr Hilary Russell, a collaborator at Queen's University, Belfast, used twelve microsatellite markers from 7q21–q36, including four of our novel CA.GT dinucleotide repeat markers, to examine the incidence of 7q LOH in ovarian tumours. Overall, 48% of the tumours, benign and malignant, exhibited LOH at one or more of the markers assayed. Again, the highest rate of loss (42%) was observed at the 17TA-5/17B-RE3 marker. Moreover, seven tumours were identified with partial deletions encompassing *CFTR* and the region just proximal to this gene, exactly co-incident with the extent of allele loss observed in SUSM-1/*Hytk7* segregant 4 (unpublished data). The

Figure 6.1. Overlap between allele loss in breast carcinomas and immortal SUSM-1/*Hytk7* segregants. The shaded regions, beneath the tie line, depict the smallest common region of allele loss in breast carcinomas (lighter) and immortal SUSM-1/*Hytk7* segregants (darker). Clone coverage and STS content map of the D7S486–786CA interval are also shown. Symbols beneath the tie line represent different varieties of STS marker (polymorphic/non-polymorphic, expressed/non-expressed), while symbols just above the tie line represent genes (not drawn to scale) or regions of identity with EST sequences (from sequence data in GenEMBL).The proffered mapping position of *CAVEOLIN*-1 is inferred from STS content mapping of YAC clones.

Novel STS
Novel CA.GT repeat
Published STS
Genethon CA.GT repeat
RFLP
Trapped exon
CpG island
EST
Novel gene
7cen
7qter
sWSS2099
WI-3876
sWSS3428
AFMB316XB9
sWSS2710
sWSS843
sWSS1398
IASTS1
WI-18209
sWSS2899
IASTS4
WI-7882
715CA
sWSS305
727CA
METH
METD
D7S2460
WI-455
SHGC-10385
CAV1CA2
CAV1CA1
D7S522
WI-5336
WI-8726
D7S2543
D7S486
SHGC-8708
786CA
D7S2742
Exon20
17B-R1/17B-RE3
Exon12
sWSS376
Exon10
Exon6
Exon4
Exon3
D7S677
Exon1
sWSS915
D7S399
sWSS850
D7S633
KM19
CS.7
740CA
Xv-2c
WI-7597
sWSS1948
724CA
778CA
Cda0zb11
sWSS845
sWSS844
sL12
sWSS377
IASTS3
H11146
B1719Q
8-1-24
162-024
195-P4
198-09
162-021
A19152
L2088Q
B0964Q
F2012Q
I14270
K1448Q
CAPZA3
MET
CAVEOLIN1
RFESS1
CFTR
WNT2
Z39076
RPTIA105
Z39427

Figure 6.2. Allele loss studies in human tumours and SUSM-1 immortal segregants. Chromosome 7 is depicted as an ideogram showing cytogenetic banding. Alongside are shown the polymorphic STSs used to perform allele loss. Those in blue represent Genethon markers, in red CHLC markers, in green the novel CA.GT markers described above, and in black an RFLP within the c-*MET* locus (METH) and the microsatellite marker in intron 17b of the *CFTR* locus.

1) Ovary, 21% (Kerr et al., 1996)

2) Prostate, 30% (Takahashi et al., 1995).

3) Pancreas, 80% (Achille *et al.*, 1996)

4) Ovary, 50% (Koike *et al.*, 1997)

5) Breast, 12% (Lin *et al.*, 1996)

6) Renal cell carcinoma, 64% (Shridhar *et al.*, 1997)

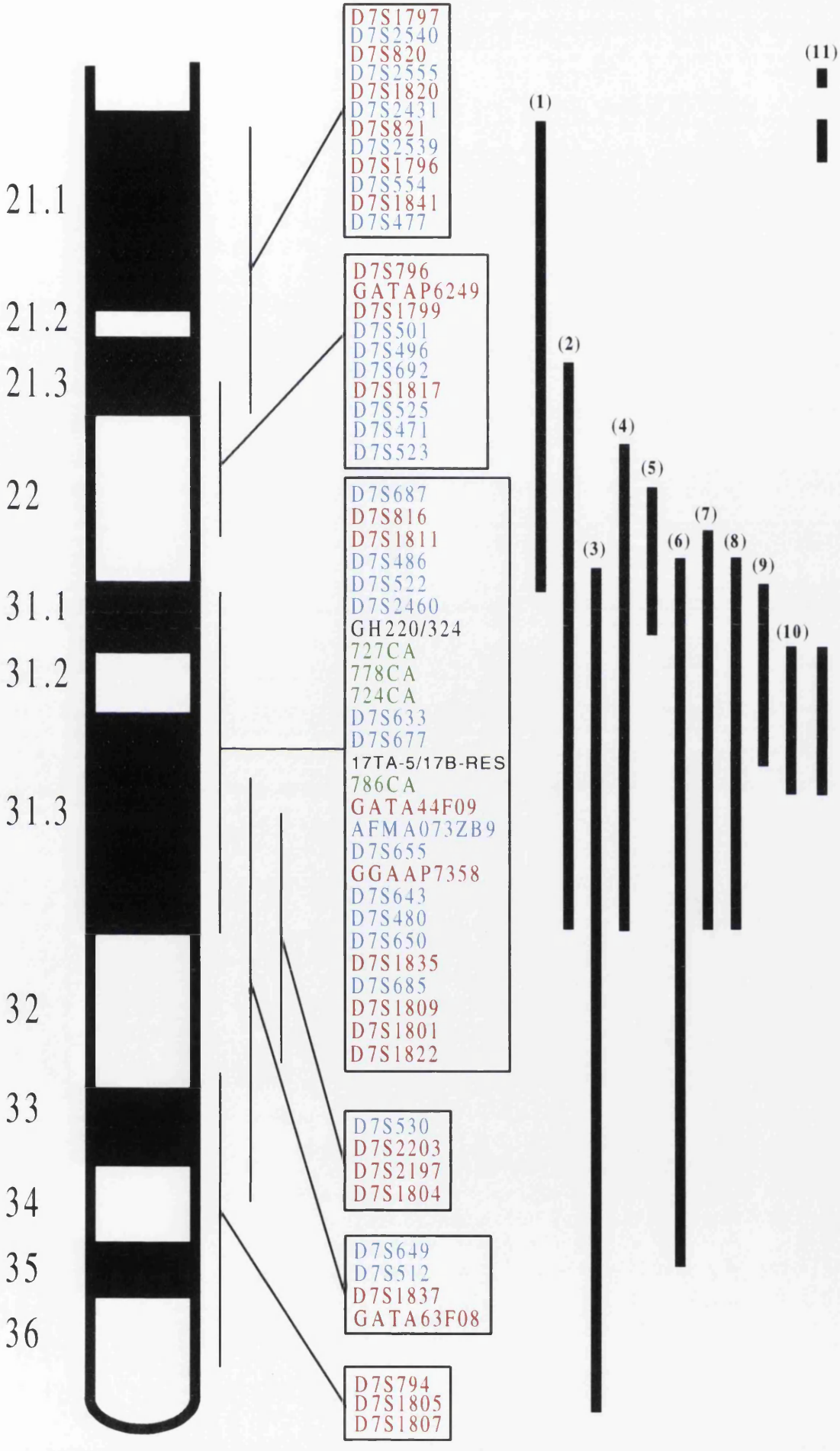
7) Ovary, 59% (Edelson *et al.*, 1997)

8) Breast, 83% (Zenklusen *et al.*, 1994)

9) Breast, 36% (Above)

10)Ovary, 48% (Martin *et al.*, in preparation)

11)Immortal SUSM-1/*Hytk7* segregants, 48% (Above)


21.1
21.2
21.3
22
31.1
31.2
31.3
32
33
34
35
36
D7S1797
D7S2540
D7S820
D7S2555
D7S1820
D7S2431
D7S821
D7S2539
D7S1796
D7S554
D7S1841
D7S477
D7S796
GATAP6249
D7S1799
D7S501
D7S496
D7S692
D7S1817
D7S525
D7S471
D7S523
D7S687
D7S816
D7S1811
D7S486
D7S522
D7S2460
GH220/324
727CA
778CA
724CA
D7S633
D7S677
17TA-5/17B-RES
786CA
GATA44F09
AFMA073ZB9
D7S655
GGAAP7358
D7S643
D7S480
D7S650
D7S1835
D7S685
D7S1809
D7S1801
D7S1822
D7S530
D7S2203
D7S2197
D7S1804
D7S649
D7S512
D7S1837
GATA63F08
D7S794
D7S1805
D7S1807
(1)
(2)
(3)
(4)
(5)
(6)
(7)
(8)
(9)
(10)
(11)

syntenic region in mouse, mChr6A2, is also frequently lost in chemically induced skin and liver tumours (Zenklusen *et al.*, 1996[b]; Zenklusen *et al.*, 1997). We find that the allele loss during skin carcinogenesis in mouse is centred on a marker D6mit236, which maps to an intron of the mouse *cftr* gene (V. O'Neill, unpublished data). Interestingly, of the seven murine squamous cell carcinoma/chromosome 7 hybrids described by Zenklusen *et al.*, (1994[a]), one of two hybrids which reverted to the malignant phenotype exhibited specific loss of a marker mapping within the *CFTR* locus on the introduced chromosome. These independent studies substantiate an association between loss of this region and carcinogenesis.

However, the cloning of TSGs by somatic cell genetics is still in its infancy, and certain of the candidates uncovered thus far (*DCC*, *FHIT*, *TSG101*) are still controversial. It is prudent, therefore, to exercise caution when drawing inferences from LOH data. It should be noted that a wide spectrum of incidences of LOH have to date been reported at 7q31, from the highest 80% (Zenklusen *et al.* 1994[b]) to less than 10% (Kerangueven *et al.*, 1995[b]) at a single marker—D7S522—and within a single tumour type—breast cancer. It is difficult to fully account for this appreciable spread in reported incidence of LOH in terms of tumour stage or grade, sample processing, and/or data analysis alone. These other studies, at the very least, call in to question the veracity of the data generated by the Zenklusen-Conte camp. In one detailed analysis by The Breast Cancer Somatic Genetics Consortium, in which 14 cancer centres compiled LOH allelotypings for three markers from 7q31-q32 in 683 breast cancer samples from 9 European countries, significant differences in self-reporting of LOH were found to exist between centres which could not be accounted for by the 12% discordancy rate between original and double-blind LOH scoring (Devilee *et al.*, 1997). The average incidence of LOH found by the Consortium for 7q31 was 19%, with the highest incidence reported being 40%. It is possible that environmental and genetic factors contribute to the difference in LOH incidence reported by these centres. It would be interesting to study the frequency of LOH at the other immortality complementation group loci between different population groups to determine whether environmental and/or genetic factors are modulating the mechanism by which tumours are becoming immortal.

Another point of inconsistency within the literature is that different studies have delineated different minimally deleted regions, although these may map only a few megabases away from each other. This might suggest the existence of genetic heterogeneity in the tumour types surveyed, implying perhaps the existence of more than

one TSG locus on chromosome 7. Indeed, evidence has been adduced for a TSG mapping to 7p12–7q11.23 that is involved in choriocarcinoma (Matsuda *et al.*, 1997), and in this study, as mentioned, I identified three hot spots for allele loss in SUSM-1/*Hytk7* segregants. Often though the boundaries of a minimally deleted region may be defined by LOH found in a single tumour, and since from the above study we can anticipate a 12% error rate in scoring LOH, such error might also therefore account for different regions being described. RFLP analysis of DNA from the two immortal fibroblast cell lines, SUSM-1 and KMST-6, revealed genomic losses at the 7q31 region in both (Ogata *et al.*, 1993). The authors claim to have identified a homozygous deletion in the SUSM-1 cell line on a southern blot using a probe pF-167.2 to the D7S252 locus (7q31.1). We find no evidence for homozygous deletion in this region on analysis of numerous microsatellite markers by PCR, indeed many markers in this region were heterozygous in SUSM-1 cells, thus highlighting a further discrepancy between my work and published reports. All these issues remain to be satisfactorily resolved.

These differences to one side, it is still difficult to determine what incidence of LOH should be taken to be significant (What background incidence of LOH might we expect to find by chance?). The incidence of LOH found in sporadic tumours for an established TSG locus such as *TP53* is between 28% and 57% (Nagai *et al.*, 1994, Niederacher *et al.*, 1997), and an incidence of LOH over 20% is commonly taken to be significant. Measured against this benchmark the frequency of LOH I find at 7q31 and that reported by several other laboratories appears to be highly significant. There are however further complications to consider, one such being that the human genome is not uniformly stable. The 7q31 chromosomal region for instance has been reported independently to harbour both recombination hot spots (Kerem *et al.*, 1989) and an aphidicolin-inducible chromosome fragile site, *FRA7G* (Berger *et al.*, 1985). Huang *et al.*, (1998) have recently mapped *FRAG7* to a 300 Kbp region within the D7S486–D7S522 interval. Like the one other common fragile site so far cloned *FRA3B*, which is also aphidicolin-inducible, *FRAG7* comprises a viral integration hot spot (HPV16 in the case of *FRA3B* and an endogenous retrovirus HERV-H in the case of *FRAG7*) as well as sequences with homology to small polydispersed circular DNAs (spcDNAs), suggesting a common mechanism underlying the fragility of both sites.

Yunis and Soreng (1984) first proposed that fragile sites could predispose chromosomes to breakage and could thus play a role in cancer development. Recombination hot spots have also been mooted to contribute to carcinogenesis, due to

their role in the duplication and deletion of genes (Oudet *et al.*, 1992; Reiter *et al.*, 1996). Further, DNA repair and meitotic recombination share common mechanisms and molecules. High frequency LOH in tumours is associated with both fragile sites and recombination hot spots elsewhere in the genome (Druck *et al.*, 1995; Buchhagen, 1996; Benitez *et al.*, 1997). Thus, the association between LOH and the fragile site/recombination hot spots at 7q31 may be non-random afterall.

The question remains, however, whether such an association has any bearing on the disease process itself. It is possible that against the background of global genomic instability prevalent within cancer cells that inherently unstable regions might preferentially undergo deletions and rearrangements. This could result in such sites becoming apparent when co-selected with other mutations conferring a selective growth advantage in clones of tumour cells, without themselves contributing to the disease process. An alternative possibility, more optimistic from our point of view, is that the most genetically labile locus in a pathway that results in tumour suppression would be expected to be inactivated more frequently than other possible loci, all other things being equal.

Forty percent of immortal cell lines are believed to map into complementation group D and yet purportedly there are at least four essential genetic targets (Pereira-Smith and Smith, 1983). Perhaps this reveals intrinsic differences in the propensities of the various possible target genes involved in immortality complementation to undergo inactivation. It is also important to note that LOH at the 7q31 chromosomal region has not been found to occur in a number of tumour types (*i.e.* bladder, cervix, and gliomas), suggesting that losses in this region are specific consequences of selection in particular cell types. Further, in a study of squamous cell carcinoma-derived cell lines, LOH on a region of chromosome 7 was found to be mutually exclusive with LOH on a region of chromosome 4 (Loughran et al., 1997[b]), again arguing for selective loss of a chromosome 7 region, which is also consistent with the idea that these chromosomal loci harbour immortality complementation group genes.

It is significant that functional evidence has been provided for a tumour suppressor/replicative senescence gene on human chromosome 7 (Zenklusen *et al.*, 1994[a]; Ogata *et al.*, 1993), providing experimental support independently of LOH findings for its existence. However, chromosome 7 is 150 Mbp in extent and represents approximately 5% of the genome. In the absence of direct evidence that sequences derived from 7q31 can mediate tumour suppression or restore replicative senescence, it

remains uncertain whether a gene/genes residing there is/are responsible for the tumour suppression/replicative senescence observed on introducing chromosome 7 into tumour/complementation group D cells. It might be appropriate to try and suppress the immortality of group D cells by transfecting them with BAC or PAC clones from the 7q31 region, while co-selecting for drug resistance. Alternatively, one might try to generate radiation hybrids containing smaller chromosome 7 fragments that could serve as donors during microcell mediated transfer. However, as stated in the introduction, trying to suppress growth in tumour cells by genetic complementation is fraught with difficulties, since tumour cells are so well adapted to evading such intervention. An added complication is that 'junk' DNA can sometimes have surprising biological effects when expressed out of context.

Of the genes so far identified and characterised which map to the D7S522-17TA-5/17B-RE3 interval, *CAVEOLIN-1* was perhaps the most interesting candidate for a multi-tissue TSG. Caveolin was first identified as a major phosphorylation substrate of v-src that localised to the striated inner surface of 50 to 100 nm invaginations of the plasma membrane termed caveolae (Glenney and Soppet, 1992; Rothberg, 1992). It was independently identified as VIP-21, a 21 kDa integral membrane component of trans-Golgi network derived vesicles (Kurzchalia *et al.*, 1992). Caveolae are believed to be present in most cell types, but are most abundant in terminally differentiated cells including adipocytes, endothelial cells, type I pneumocytes, and skeletal muscle cells (reviewed in Fan *et al.*, 1983). Caveolin is believed to be the principle structural component of caveolae: its expression within a cell correlates with the number of caveolae (Scherer *et al.*, 1994; Koleske *et al.*, 1995), while heterologous expression of caveolin induces the *de novo* formation of caveolae (Fra *et al.*, 1995), additionally, high molecular mass homo-oligomers of caveolin are able to self-associate and form caveolae-like structures (Sargiacomo *et al.*, 1995; Monier *et al.*, 1995). Recently, a family of caveolin-related proteins has been identified (Scherer *et al.*, 1996; Tang *et al.*, 1996), and caveolin has been re-named caveolin-1. Human Caveolin-2, which is 38% identical and 58% similar to Caveolin-1, is expressed in many of the same tissues as Caveolin-1 and co-localises with it in caveolae membranes (Scherer *et al.*, 1996). Human Caveolin-3 is 65% identical and 85% similar to Caveolin-1, and is the predominant caveolin isoform in skeletal and cardiac muscle where it replaces caveolin-1 (Tang *et al.*, 1996). Recessive mutations in the gene encoding Caveolin-3 are believed

to be responsible for a subset of autosomal dominant limb-girdle muscular dystrophy (Minetti *et al.*, 1998).

Caveolin has been implicated as a regulator of a diverse number of intracellular signalling pathways. Purified caveolae membranes are enriched with specific lipids (cholesterol and glycosphingolipids (Murata *et al.*, 1995; Fra *et al.*, 1995)) and a plethora of lipid-modified signalling molecules including H-ras, c-src, other src-like kinases, eNOS, plasminogen activator, as well as heterotrimeric G-proteins (Li *et al.*, 1995; Stahl and Mueller, 1995; Li *et al.*, 1996; Garcia-Cardena *et al.*, 1996; Song *et al.*, 1996). In addition, a number of membrane associated receptors, including several that are coupled to G-proteins or possess intrinsic tyrosine kinase activity, localise to or are internalised by caveolae (reviewed in Lisanti *et al.*, 1994). A twenty amino-acid membrane-proximal scaffolding domain, that is highly homologous among the three forms of caveolin, has been identified. This domain mediates both the oligomerisation of caveolin as well as its interaction with several of these signalling molecules, including wild-type c-src, H-ras and G_α subunits (Li *et al.*, 1996). Significantly, the scaffolding domains of Caveolins-1 and -3, but not -2, are sufficient to inhibit the autoactivation of src tyrosine kinases, and the GTPase activity of G_α subunits (Couet *et al.*, 1997). Caveolae may play an important role in sequestering inactive signalling molecules, and down-regulating receptor activity. Alternatively, caveolae may provide an environment for the regulated activation of these molecules and explain cross-talk between different signalling pathways.

Caveolin is one of only a few transformation-dependent v-src substrates identified. The functional significance of its phosphorylation is unknown; it may allow binding between caveolin and signalling molecules that contain an SH2-domain. In support of this suggestion, caveolin-1 has been found to co-immunoprecipitate with Shc (Wary *et al.*, 1996). Both tyrosine phosphorylation of caveolin and transformation by v-*src* depend upon membrane targeting of v-src by N-terminal myristylation (Glenney, 1989). This has prompted the suggestion that caveolin may be a critical target during cellular transformation. This hypothesis has been supported by the observation that both caveolin expression and caveolae are reduced in NIH 3T3 cells transformed by activated oncogenes other than v-*src*, including H-*ras*, v-*abl*, and *bcr-abl*. Further, the extent of reduction in the expression of caveolin correlated well with the ability of these oncogenically transformed cells to form colonies in soft-agar (Koleske *et al.*, 1995). More recently, conditional expression of *caveolin-1* in *ras*-transformed NIH 3T3 cells

was shown to abrogate anchorage-independent growth; this was accompanied by inhibition of the ras/raf/MAPK signalling pathway, and by induction of apoptosis (Engelman *et al.*, 1997); while ectopic expression of Caveolin-1 in human breast tumour-derived cell lines has also been shown to suppress *in vitro* tumour growth (Lee *et al.*, 1998). A role for caveolin-1 in anchorage-dependent growth had previously been suggested by the demonstration that it interacts with both integrins and Shc (Wary *et al.*, 1996), providing a possible link between integrin signalling and the the ras/raf/MAPK pathway.

CAVEOLIN-1 is expressed in cell lines representing various of the tumour types in which chromosome 7 LOH has been observed and, like the putative tumour suppressor gene, *CAVEOLIN-1* is also conserved in mouse. Caveolin-1 was found to be one of 26 gene products down-regulated during mammary tumourigenesis using differential display (Sager *et al.*, 1994), and to be absent in several mammary carcinoma derived cell-lines including MT-1, MCF-7, ZR-75-1, T47D, MDA-MB-361, and MDA-MB-474 (Sager *et al.*, 1994, Lee *et al.*, 1998). Caveolin-1 has been found to associate with the neurotrophin receptor, $p75^{NTR}$, which is required for sphingomyelin hydrolysis and ceramide production in neurones and glial cells (Bilderback *et al.*, 1997). In turn ceramide, which accumulates in senescent cells, is capable of inducing senescent-like growth arrest in normal human diploid fibroblasts (Venable *et al.*, 1995), providing a possible link between Caveolin-1 and replicative senescence, which would fit with our hypothesis that the TSG and *SEND* are one and the same.

However, against *CAVEOLIN-1* being a TSG is the apparent absence of mutational inactivation of *CAVEOLIN-1* as determined by SSCP-heteroduplex analysis performed on genomic DNA samples prepared from a number of tumour-derived cell lines (and also by sequencing *CAVEOLIN-1* cDNA prepared from a subset of them); coupled with the absence of any gross alterations or homozygous deletions of the gene being found by southern blot analysis (in agreement with Lee *et al.*, 1998), or of any altered gene products being detected by RT-PCR or western blot analysis. We have demonstrated, however, that the level of expression of *CAVEOLIN-1* is subject to epigenetic modification by methylation in several of these cell lines, and if *CAVEOLIN-1* were indeed a tumour suppressor gene *in vivo*, this mechanism could be an alternative means by which the gene becomes inactivated during tumourigenesis. A CpG island associated with the first two exons of *CAVEOLIN-1* is methylated in up to 50% of the tumour-derived cell lines tested here. Furthermore, the degree of methylation correlates

with the level of expression of Caveolin-1 mRNA. The most abundant levels of Caveolin-1 transcript were detected in the cell lines J82, A1698, MDA-MB-231, and OVCAR5, and these demonstrate the highest detectable levels of a *Sma* I-cleavable fragment (i.e *CAVEOLIN-1* is undermethylated in these cell lines). Little or no Caveolin transcript or protein could be detected for the breast carcinoma cell lines ZR-75-1 or MCF-7 (in accordance with the previous studies above), or for the ovarian carcinoma cell lines A2780, CH1, OVCAR3 and -4, for which no *Sma* I-cleavable fragment was apparent (i.e *CAVEOLIN-1* is methylated). These findings demonstrate an important role for methylation in regulating the expression of *CAVEOLIN-1* in tumour-derived cell lines. Cell-type factors other than methylation are, however, likely to influence the level of expression of Caveolin protein. Thus in the case of COLO357 cells there is scant transcript and protein, but only partial methylation of the *CAVEOLIN-1* gene, while mRNA expression is detectable in HeLa cells but there is little protein product, implying post-transcriptional regulation of the level of gene product in this cell line (this is independent of the expression of HPV oncoproteins). Ultimately, it would be desirable to detect methylation of the *CAVEOLIN-1* gene not only in tumour-derived cell lines but also in cancerous tissue. However, contamination of cancerous tissue by stromal cells, which express high levels of Caveolin-1, might be anticipated to antagonise this analysis.

Expression of Caveolin-1 can not be detected by immunohistochemistry in glandular epithelial cells, which are believed to be the cell type that undergoes transformation in the majority of breast carcinomas, in sections of normal breast tissue, although there is strong Caveolin-1 immunolabelling in myoepithelial and stromal cells (Figure 6.3; J. Reeves, Royal Infirmary, Glasgow, UK). It would be predicted, therefore, that in sections of breast tumours, which have a high content of epithelial cells, less overall Caveolin staining would be apparent, as is indeed evident, and this would explain why *CAVEOLIN-1* was found to be down-regulated in breast cancers (Sager *et al.*, 1994). However, it is apparent from the breast cancer derived cell line MDA-MB-231 that at least a subset of breast epithelial tumour cell lines express *CAVEOLIN-1*.

Our finding that *CAVEOLIN-1* is not expressed in normal breast glandular epithelial cells appears to contradict the findings of Lee *et al.* (1998), who demonstrate the presence of Caveolin-1 protein in early passage epithelial cells cultured from normal breast tissue, as well as in immortalised, non-tumourigenic, mammary epithelial cells. One possible explanation for this difference is that *in vitro* culture conditions may

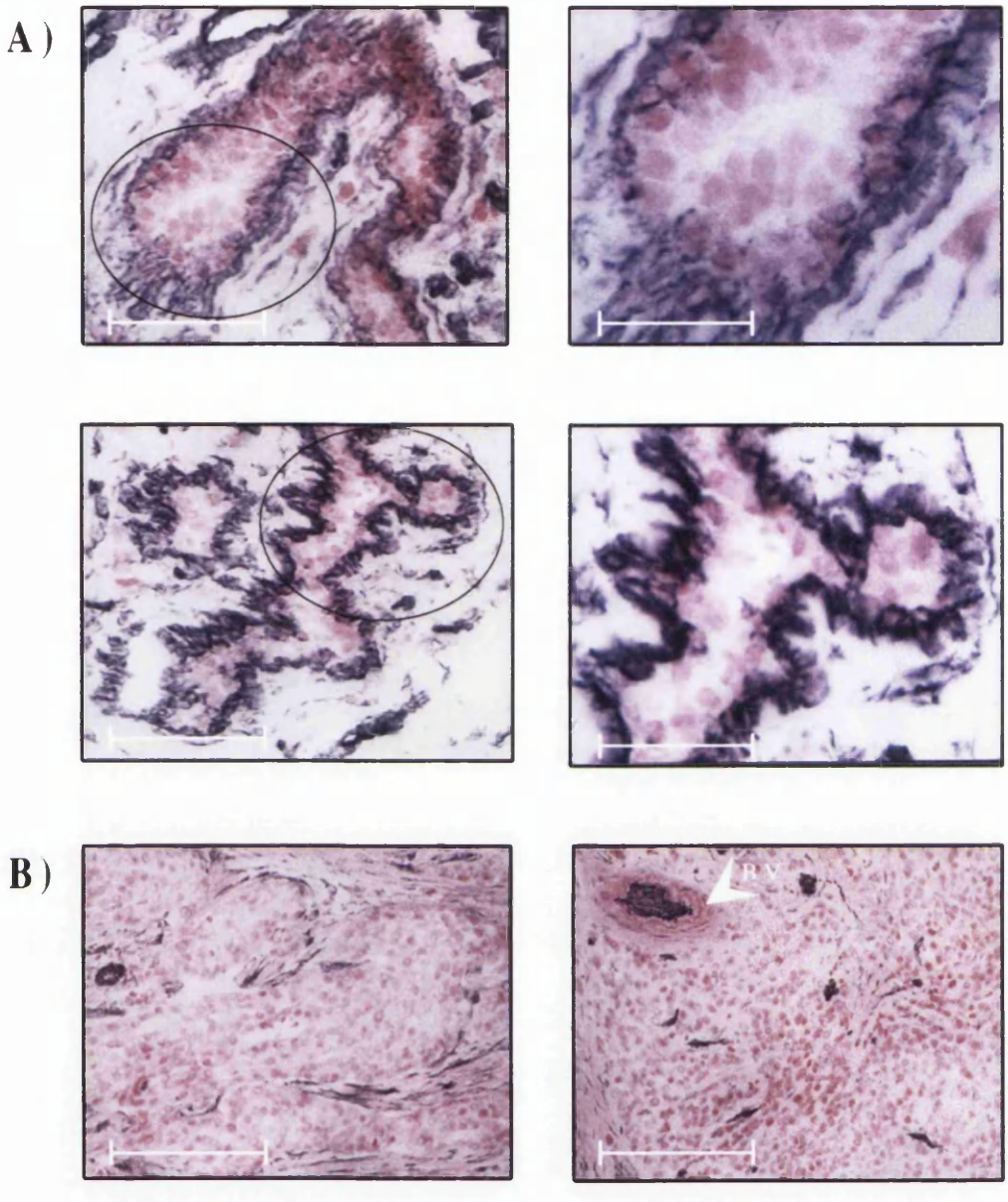


Figure 6.3. *CAVEOLIN-1* expression in normal and tumour breast tissue. (**A**) Normal breast epithelial cells contained within a duct (counter-stained lightly with safranin) do not demonstrate staining due to immunohistochemical detection of Caveolin-1, although staining is apparent in stromal cells and myoepithelial cells surrounding the duct. Examples of ducts from two different sections of breast reduction tissue are presented with higher magnification images of the circled regions shown on the right. (**B**) Caveolin-1 staining is present in primary breast tumour stromal cells and endothelial cells, but absent in the invasive carcinoma cells. Again sections from two different primary breast tumours are shown at the same magnification as sections of normal tissue (left). BV, blood vessel. (Lower magnification images, bars = 50µm; higher magnification images, bars = 20µm).

induce *CAVEOLIN-1* expression in non-transformed glandular epithelial cells, but not malignant epithelial cells. Certainly, Lee *et al.* show that the expression of the α-isoform of Caveolin-1 increases in a cell cycle dependent manner in cultured breast epithelial cells (which is in itself perhaps a surprising finding for a putative growth inhibitory molecule); thus, we might only expect to detect Caveolin-1 protein in proliferating cells, and a greater fraction of cells will be in cycle in culture compared to *in vivo*, implying that it will be correspondingly harder to detect *CAVEOLIN-1* expression in tissue sections compared to cultured cells. It might be possible to resolve this issue by examining *CAVEOLIN-1* expression in the breast ducts of pregnant mice, or in pre-malignant, hyperproliferative lesions.

An alternative explanation for our different findings is that epithelial cells in culture do not represent glandular epithelial cells, but may be derived from another cell type, perhaps myoepithelial cells which we show do express Caveolin-1 protein. In this case, the consequence of over-expressing Caveolin-1 in malignant epithelial cells, namely growth inhibition, may be less physiologically relevant, since they do not represent an appropriate target cell type. It still remains to be satisfactorily explained how re-expression of *CAVEOLIN-1* in carcinoma cells was consequential when it failed to result in caveolae formation, if endocytosis/vesicular transport associated with the function of this protein is believed to be important for its effects on growth (Koleske *et al.*, 1995; Scherer *et al.*, 1995). Perhaps an ability of Caveolin protein to modulate integrin-mediated growth signalling is more significant than its ability to induce caveolae formation. The authors also do not compare the growth rate of cultured normal mammary epithelial cells to their transformed counterparts, to give an indication of the effect of physiological levels of the protein on cell growth.

Caveolae are present in many normal cell types, including epithelial cells, and it is still possible that loss of Caveolin expression following methylation of the gene could contribute to carcinogenesis. However, there appears to be no correlation between the presence of wild-type Caveolin-1 and allele loss on chromosome 7 since the cell lines COLO357, MDA-MB-231, OVCAR5, and TMSG all demonstrate chromosome 7 LOH which encompasses the *CAVEOLIN-1* locus (unpublished data), but with the exception of COLO357 express wild-type Caveolin-1. This finding has also been extended to a series of head and neck squamous cell carcinoma derived cell lines (J. Munro, BICR, unpublished data), established by Dr K. Eddington while at the BICR, and analysed for allele loss on chromsome 7 (Loughran *et al.*, 1997). The failure to detect 'first-hit'

mutations in *CAVEOLIN-1* (either micro-scale alteration or gross deletions and re-arrangements) in any of the cancer cell lines studied here, coupled with the expression of wild-type protein in cells portraying chromosome 7 LOH suggests that this gene is not the target for inactivation by LOH (the 'second hit'), which is frequently observed at 7q31 in human solid cancers. Further, there is no correlation between the expression of *CAVEOLIN-1* and complementation group status for immortality since the cell lines A1698, A1698OR, MDA-MB-231, OVCAR5, and SUSM-1 are all in complementation group D, but nonetheless express wild-type Caveolin-1. Therefore, despite *in vitro* findings that Caveolin-1 can function as a suppressor of cellular transformation, and despite our finding modifications of the gene in tumour-derived cell lines, *CAVEOLIN-1* is unlikely to be the immortality complementation group D gene, nor does it appear to be a target for mutational inactivation during tumourigenesis. However, it can not be formally excluded that Caveolin-1 is partly responsible for suppressing cellular transformation *in vivo*.

Analysis of another possible candidate gene, *hF11A10.5*, is incomplete, although again it is possible to say that re-arrangements of the gene were not observed on Southern blots in tumours, tumour-derived cell lines, or immortal SUSM-1/*Hytk7* segregants, nor was a transcript from this gene found to be lost or altered in a number of tumour-derived cell lines that we analysed. Further, of four exons examined for the presence of mutations by SSCP-heteroduplex analysis, no alterations in electrophoretic mobility that might indicate the presence of a mutation were observed in DNA samples from tumours, tumour-derived cell lines, or immortal SUSM-1/*Hytk7* segregants. It is still necessary to examine the remaining exons of this gene for the presence of mutations in order to exclude this candidate.

There are several obvious future experiments whose performance might facilitate the cloning of the chromosome 7 multi-tissue tumour suppressor/replicative senescence gene. Presently, the smallest deleted interval where the gene potentially maps is defined by a single immortal SUSM-1/*Hytk7* segregant, segregant 4. It is therefore desirable to generate and allelotype more immortal segregants. These could originate from a different recipient cell line to SUSM-1 to see whether the same region is implicated. Both MDA-MB-231 and OVCAR5 present themselves as obvious choices. It is also desirable to continue establishing bacterial clone coverage of the D7S522-17TA-5/17B-RE3 interval. The end-clones of cosmid/PAC contigs established through STS content mapping, riboprobing, and restriction digest fingerprinting, can be used to generate

riboprobes that can then be used to screen the chromosome 7 cosmid and genomic PAC libraries. The extent of allele loss at the other two frequently altered regions in immortal SUSM-1/*Hytk*7 segregants, close to D7S2555 and D7S821 (both at 7q21), need also to be more precisely defined. This may necessitate the cloning of novel polymorphic markers. It is possible that certain of the regions identified through segregant deletion analysis may not be involved in replicative senescence but are being deleted due to different selective pressures. This issue could be resolved by performing microcell mediated monochromosome transfer of chromosome 7 into non-complementation group D cell lines, and analysing hybrids for allele loss on chromosome 7.

Clearly, the identification and analysis of candidate genes from the D7S522–17TA-5/17B-RE3 interval is incomplete. Further quarries may be unearthed by performing exon-trapping on more bacterial clones, starting with those clones from the deleted interval in SUSM-1*Hytk*7 segregant 4. YAC clone DNA or inter*Alu* PCR products generated from YAC clones can be used to perform direct cDNA selection, employing pooled cDNA from various libraries, including the senescent fibroblast cDNA library, as the starting material. Also since much of this interval has already been sequenced, probes designed from sequences with significant homology to known genes or ESTs, and/or sequences with features of putative exons could be generated and used to probe cDNA libraries. In addition, putative exons will be subjected to SSCP-heteroduplex analysis.

As an alternative to positional cloning, it may be desirable to attempt different, perhaps indirect, strategies for cloning the tumour suppressor/replicative senescence gene. These approaches might include performing subtractive hybridisation between senescing and pre-senescent cells, and identifying genes up-regulated during senescence which map to chromosome 7. 48.5% of SUSM-1/*Hytk*7 immortal segregants had no detectable allele loss. In these segregants it is possible that *SEND* was inactivated by micro-scale alterations, undetectable by examining LOH, or by epigenetic mechanisms, such as promoter silencing by methylation, or alternatively that a different replicative senescence gene was inactivated in a SUSM-1/*Hytk*7 hybrid. It might be possible to perform RDA between genomic DNA prepared from immortal SUSM-1/*Hytk*7 segregants, with no obvious losses scored by allelotyping, mixed with A92 mouse genomic DNA, and SUSM-1 DNA mixed with A92/*Hytk*7 DNA. The success of this approach will of course depend upon there existing a sufficient number of RFLPs that discriminate between endogenous and exogenous copies of chromosome 7.

Alternatively, the *Hytk*-tagged chromosome 7 could be isolated from immortal SUSM-1/*Hytk*7 segregants with no obvious losses, by fusion of segregant cells with rodent cells and selection with Hygromycin, and RDA performed between genomic DNA prepared from pooled clones of these cells and parental rodent cells. RDA could also be performed between cDNA PCR amplified from senescent segregant colonies and cDNA prepared from a comparable number of immortal segregant cells. Although more demanding, the technology exists for preparing cDNA from only a few cells. However candidate genes are cloned, ultimate proof of their tumour suppressor/senescence-inducing credentials will depend upon the ability to demonstrate inactivating mutations in DNA from tumours, complementation group D immortal cell lines, and immortal segregant cell lines. It will also be desirable to demonstrate that the gene product is capable of inducing growth arrest solely in immortality complementation group D cell lines, and not cell lines mapping to other complementation groups.

Any further analysis of the tumour suppressor/replicative senescence gene would depend upon its identity. Shared homologies with known genes might give an initial indication of the function of the gene product. A comprehensive description of the gene's function would require analysis of the expression of the gene, both within the organism as a whole, during development and in developed organisms, as well as in individual cells themselves. It will be important to determine the cellular distribution of the gene product; this may entail generating antibodies against the encoded gene product, or expressing the cDNA as an epitope-tagged molecule or as a Green Fluorescent Protein (GFP) fusion construct. It will be of interest to see whether expression of the gene is temporally regulated, *i.e.*whether expression is induced upon senescence, and whether induction is sustained or transient. Additionally, it will be important to determine whether the distribution of the gene product is cell cycle dependent, whether it undergoes any post-translational modification such as phosphorylation and if so under what conditions, and with which other molecules in the cell it interacts. Candidates for the latter would include the products of the other immortality complementation group genes. Binding partners can be identified in a number of ways, which include immunoaffinity chromotography, immunoprecipitation, or 'pull-downs' with a recombinant molecule fused to a glutathione-S-transferase moeity, followed by microsequencing or mass spectroscopy analysis of isolated proteins. An alternative method is the two hybrid screen, which can be performed in yeast, insect, or mammalian cells.

Assuming that a mouse homologue of the gene does indeed exist, as has been suggested, then it will be interesting to disrupt this gene in mice by homologous recombination, and assess the effect of this on the replicative potential or tumourigenicity of mutant cells. This analysis may help confirm the dual role of the gene. However, its biological role may not be the same in mice as in humans since senescence does not appear to be regulated in the same way in these two organisms. It will also be pertinent to determine the phenotype of cells nullizygous for the gene at the biochemical level, looking to see whether the response of such cells to DNA damage is altered, and whether they are still able to undergo cell cycle arrest in response to DNA damage as well as other treatments. These cells could be generated from mutant mice (conventional or conditional knockouts depending on post-implantation viability), or complementation group D cell lines, or cultures of normal diploid human cells in which the gene has been disrupted through homologous recombination followed by drug selection. In addition, it will be interesting to see whether treatment of mutant cells with agents such as ceramide or sodium butyrate or targeting telomeres for deletion by homologous recombination induces senescence as efficiently in these cells as in wild-type cells. This would address the issue of whether or not the gene product is involved in transducing signals from shortened telomeres or other stimuli associated with the induction of senescence. It is published that re-introduction of intact chromosome 7 into SUSM-1 cells leads to telomeric attrition (Nakabayashi *et al.*, 1997). It will be interesting to see whether ectopic expression of the chromosome 7 tumour suppressor/replicative senescence gene in young mortal cells induces the early onset of senescence, and whether it alters the rate at which telomeres erode in these cells. While in immortal cells, one would wish to determine the effect ectopic expression of the tumour suppressor/replicative senescence gene has on telomerase expression and/or activity, and/or whether it can restore M1 control.

Isolating genes targeted for mutation during carcinogenesis will provide us with new weapons to fight cancer. The identification of cancer susceptibility genes will allow us to predict and prevent familial forms of disease. Failing prevention, cancer genes provide us with markers of disease progression and potential therapeutic targets. Immortality is a prevalent and insidious feature of cancer cells. The means by which tumour cells progress to an immortal phenotype is incompletely understood. Cloning and characterising the immortality complementation group genes will no doubt throw light on the mechanisms which hold the proliferative potential of somatic cells in check,

and provide clinicians with novel pathways to manipulate in cancer therapy. Cellular senescence is also implicated in the pathology associated with old age. The ability to modulate replicative senescence could one day potentially prolong healthy life.

REFERENCES

References

Aaltonen, L.A., Peltomaki, P., Leach, F.S., Sistonen, P., Pylkkanen, L., Mecklin, J.P., Jarvinen, H., Powell, S.M., Jen, J., Hamilton, S.R., *et al.* (1993). Clues to the pathogenesis of familial colorectal cancer. *Science* **260**: 812-816.

Achille, A., Biasi, M.O., Zamboni, G., Bogina, G., Magalini, A.R., Pederzoli, P., Perucho, M., Scarpa, A. (1996). Chromosome 7q allelic losses in pancreatic carcinoma. *Cancer Res.* 56: 3808-3813.

Adams, J.M., Gerondakis, S., Webb, E., Corcoran, L.M., Cory, S. (1983). Cellular myc oncogene is altered by chromosome translocation to an immunoglobulin locus in murine plasmacytomas and is rearranged similarly in human Burkitt lymphomas. *Proc. Natl. Acad. Sci. U.S.A.* **80**: 1982-1986.

Aebi, S., Kurdi-Haidar, B., Gordon, R., Cenni, B., Zheng, H., Fink, D., Christen, R.D., Boland, C.R., Koi, M., Fishel, R., Howell, S.B. (1996). Loss of DNA mismatch repair in acquired resistance to cisplatin. *Cancer Res.* **56**: 3087-3090.

Afshari, C.A., Vojta, P.J., Annab, L.A., Futreal, P.A., Willard, T.B., Barrett, J.C. (1993). Investigation of the role of G1/S cell cycle mediators in cellular senescence. *Exp. Cell. Res.* **209**: 231-237.

Alcorta, D.A., Xiong, Y., Phelps, D., Hannon, G., Beach, D., Barrett, J.C. (1996). Involvement of the cyclin-dependent kinase inhibitor p16(INK4a) in replicative senescence of normal human fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.* **93**: 13742-13747.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. (1990). Basic local alignment search tool. *J. Mol. Biol.* **215**: 403-410.

Anzick, S.L., Kononen, J., Walker, R.L., Azorsa, D.O., Tanner, M.M., Guan, X.Y., Sauter, G., Kallioniemi, O.P., Trent, J.M., Meltzer, P.S. (1997). AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science* **277**: 965-968.

Armitage, P., Doll, R. (1954). The age distribution of cancer and a multi-stage theory of carcinogenesis. *Br. J. Cancer* **8**: 1-12.

Ashley, D.J.B. (1969). The two 'hit' theory of carcinogenesis. *Br. J. Cancer* **23**: 313-328.

Athma, P., Rappaport, R., Swift, M. (1996). Molecular genotyping shows that ataxia-telangiectasia heterozygotes are predisposed to breast cancer. *Cancer Genet. Cytogenet.* **92**: 130-134.

Bakalkin, G., Yakovleva, T., Selivanova, G., Magnusson, K.P., Szekely, L., Kiseleva, E., Klein, G., Terenius, L., Wiman, K.G. (1994). p53 binds single-stranded DNA ends and catalyzes DNA renaturation and strand transfer. *Proc. Natl. Acad. Sci. U.S.A.* **91**: 413-417.

Baker, S.J., Fearon, E.R., Nigro, J.M., Hamilton, S.R., Preisinger, A.C., Jessup, J.M., van Tuinen, P.,Ledbetter, D.H., Barker, D.F., Nakamura, Y., *et al.* (1989). Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. *Science* **244**: 217-221.

Bakhshi, A., Jensen, J.P., Goldman, P., Wright, J.J., McBride, O.W., Epstein, A.L., Korsmeyer, S.J. (1985). Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around JH on chromosome 14 and near a transcriptional unit on 18. *Cell* **41**: 899-906.

Ballester, R., Marchuk, D., Boguski, M., Saulino, A., Letcher, R., Wigler, M., Collins, F. (1990). The NF1 locus encodes a protein functionally related to mammalian GAP and yeast IRA proteins. *Cell* **63**: 851-859.

Baltimore, D. (1970). RNA-dependent DNA polymerase in virions of RNA tumour viruses. *Nature* **226**: 1209-1211.

Band, V., De Caprio, J.A., Delmolino, L., Kulesa, V., Sager, R. (1991). Loss of p53 protein in human papilloma virus type 16 E6-immortalized human mammary epithelial cells. *J. Virol.* **65**: 6671-6676.

Barlow, C., Hirotsune, S., Paylor, R., Liyanage, M., Eckhaus, M., Collins, F., Shiloh, Y., Crawley, J.N., Ried, T., Tagle, D., Wynshaw-Boris, A. (1996). Atm-deficient mice: a paradigm of ataxia telangiectasia. *Cell* **86**: 159-171.

Bayle, J.H., Elenbaas, B., Levine, A.J. (1995). The carboxyl-terminal domain of the p53 protein regulates sequence-specific DNA binding through its non specific nucleic acid-binding activity. *Proc. Natl. Acad. Sci. U.S.A.* **92**: 5729-5733.

Behrens, J., von Kries, J.P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R., Birchmeier, W. (1996). Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature* **382**: 638-642.

Benitez, J., Osorio, A., Barroso, A., Arranz, E., Diaz-Guillen, M.A., Robledo, M., Rodriguez de Cordoba, S., Heine-Suner, D. (1997). A region of allelic imbalance in 1q31-32 in primary breast cancer coincides with a recombination hotspot. *Cancer Res.* **57**: 4217-4220.

Berger, R., Bloomfield, C.D., Sutherland, G.R. (1985). Report of the Committee on Chromosome Rearrangements in Neoplasia and on Fragile Sites. *Cytogenet. Cell Genet.* **40**: 490-535.

Bieche, I., Champeme, M.H., Matifas, F., Hacene, K., Callahan, R., Lidereau, R. (1992). Loss of heterozygosity on chromosome 7q and aggressive primary breast cancer. *Lancet* **339**: 139-143.

Biegel, J.A., White, P.S., Marshall, H.N., Fujimori, M., Zackai, E.H., Scher, C.D., Brodeur, G.M., Emanuel, B.S. (1993). Constitutional 1p36 deletion in a child with neuroblastoma. *Am. J. Hum. Genet.* **52**: 176-182.

Bilderback, T.R., Grigsby, R.J., Dobrowsky, R.T. (1997). Association of p75(NTR) with caveolin and localisation of neurotrophin-induced sphingomyelin hydrolysis to caveolae. *J. Biol. Chem.* **272**: 10922-10927.

Bird, A.P. (1986). CpG-rich islands and the function of DNA methylation. *Nature* **321**: 209-213.

Bitgood, M.J., McMahon, A.P. (1995). Hedgehog and Bmp genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. *Dev. Biol.* **172**: 126-138.

Bodnar, A.G., Ouellette, M., Frolkis, M., Holt, S.E., Chiu, C.P., Morin, G.B., Harley, C.B., Shay, J.W., Lichtsteiner, S., Wright, W.E. (1998). Extension of life-span by introduction of telomerase into normal human cells. *Science* **279**: 349-352.

Bond, J., Haughton, M., Blaydes, J., Gire, V., Wynford-Thomas, D., Wyllie, F. (1996). Evidence that transcriptional activation by p53 plays a direct role in the induction of cellular senescence. *Oncogene* **13**: 2097-2104.

Bond, J.A., Blaydes, J.P., Rowson, J., Haughton, M.F., Smith, J.R., Wynford-Thomas, D., Wyllie, F.S. (1995). Mutant p53 rescues human diploid cells from senescence without inhibiting the induction of SDI1/WAF1. *Cancer Res.* **55**: 2404-2409.

Bond, J.A., Wyllie, F.S., Wynford-Thomas, D. (1994). Escape from senescence in human diploid fibroblasts induced directly by mutant p53. *Oncogene* **9**: 1885-1889.

Bonetta, L., Kuehn, S.E., Huang, A., Law, D.J., Kalikin, L.M., Koi, M., Reeve, A.E., Brownstein, B.H., Yeger, H., Williams, B.R., *et al.* (1990). Wilms tumour locus on 11p13 defined by multiple CpG island-associated transcripts. *Science* **250**: 994-997.

Bossy-Wetzel, E., Bakiri, L., Yaniv, M. (1997). Induction of apoptosis by the transcription factor c-Jun. *EMBO J.* **16**: 1695-1709.

Bouffard, G.G., Idol, J.R., Braden, V.V., Iyer, L.M., Cunningham, A.F., Weintraub, L.A., Touchman, J.W., Mohr-Tidwell, R.M., Peluso, D.C., Fulton, R.S., Ueltzen, M.S., Weissenbach, J., Magness, C.L., Green, E.D. (1997). A physical map of human chromosome 7: an integrated YAC contig map with average STS spacing of 79 kb. *Genome Res.* **7**: 673-692.

Bova, G.S., Carter, B.S., Bussemakers, M.J., Emi, M.., Fujiwara, Y., Kyprianou, N., Jacobs, S.C., Robinson, J.C., Epstein, J.I., Walsh, P.C., *et al.* (1993). Homozygous deletion and frequent allelic loss ofchromosome 8p22 loci in human prostate cancer. *Cancer Res.* **53**: 3869-3873.

Boveri, T. (1914). *Zur frage der erstehung maligner tumoren* (On the problems of the origins of human tumours). Gustav Fischer, Jena.

Bremner, R., Cohen, B.L., Sopta, M., Hamel, P.A., Ingles, C.J., Gallie, B.L., Phillips, R.A. (1995). Direct transcriptional repression by pRB and its reversal by specific cyclins. *Mol. Cell. Biol.* **15**: 3256-3265.

Brenner, S., Livak, K.J. (1989). DNA fingerprinting by sampled sequencing. *Proc. Natl. Acad. Sci USA* **86**: 8902-8906.

Brodeur, G.M., Sekhon, G., Goldstein, M.N. (1977). Chromosomal aberrations in human neuroblastomas. *Cancer* **40**: 2256-2263.

Brown, J.P., Wei, W., Sedivy, J.M. (1997). Bypass of senescence after disruption of p21CIP1/WAF1 gene in normal diploid human fibroblasts. *Science* **277**: 831-834.

Bryan, T.M., Englezou, A., Gupta, J., Bacchetti, S., Reddel, R.R. (1995). Telomere elongation in immortal human cells without detectable telomerase activity. *EMBO J.* **14**: 4240-4248.

Bryan, T.M., Reddel, R.R. (1994). SV40-induced immortalization of human cells. *Crit. Rev. Oncog.* **5**: 331-357.

Buchhagen, D.L. (1996). Frequent involvement of chromosome 3p alterations in lung carcinogenesis: allelotypes of 215 established cell lines at six chromosome 3p loci. *J. Cell. Biochem* **24 (Suppl)**: 198-209.

Buckler, A.J., Chang, D.D., Graw, S.L., Brook, J.D., Haber, D.A., Sharp, P.A., Housman, D.E. (1991). Exon amplification: a strategy to isolate mammalian genes based on RNA splicing. *Proc. Natl. Acad. Sci. U.S.A.* **88**: 4005-4009.

Bunn, C.L., Tarrant, G.M. (1980). Limited lifespan in somatic cell hybrids and cybrids. *Exp. Cell. Res.* **127**: 385-396.

Burke, D.T., Carle, G.F., Olson, D.M. (1987). Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. *Science* **236**: 806-812.

Call, K.M., Glaser, T., Ito, C.Y., Buckler, A.J., Pelletier, J., Haber, D.A., Rose, E.A., Kral, A., Yeger, H., Lewis, W.H., *et al.* (1990). Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms' tumor locus. *Cell* **60**: 509-520.

Callebaut, I., Mornon, J.P. (1997). From BRCA1 to RAP1: a widespread BRCT module closely associated with DNA repair. *FEBS Lett.* **400**: 25-30.

Campisi, J. (1996). Replicative senescence: an old lives' tale? *Cell* **23**: 497-500.

Capecchi, M.R. (1989). Altering the genome by homologous recombination. Science **244**: 1288-1292.

Cappellen, D., Gil Diez de Medina, S., Chopin, D., Thiery, J.P., Radvanyi, F. (1997). Frequent loss of heterozygosity on chromosome 10q in muscle-invasive transitional cell carcinomas of the bladder. *Oncogene* **14**: 3059-3066.

Cavenee, W.K., Dryja, T.P., Phillips, R.A., Benedict, W.F., Godbout, R., Gallie, B.L., Murphree, A.L., Strong, L.C., White, R.L. (1983). Expression of recessive alleles by chromosomal mechanisms in retinoblastoma. *Nature* **305**: 779-784.

Cawthon, R.M., Weiss, R., Xu, G.F., Viskochil, D., Culver, M., Stevens, J., Robertson, M., Dunn, D., Gesteland, R., O'Connell, P., et. al. (1990). A major segment of the neurofibromatosis type 1 gene: cDNA sequence, genomic structure, and point mutations. *Cell* **62**: 193-201.

Chan, L.C., Karhi, K.K., Rayter, S.I., Heisterkamp, N., Eridani, S., Powles, R., Lawler, S.D., Groffen, J., Foulkes, J.G., Greaves, M.F., et. al. (1987). A novel abl protein expressed in Philadelphia chromosome positive acute lymphoblastic leukaemia. *Nature* **325**: 635-637.

Chandrasekharappa, S.C., Guru, S.C., Manickam, P., Olufemi, S.E., Collins, F.S., Emmert-Buck, M.R., Debelenko, L.V., Zhuang, Z., Lubensky, I.A., Liotta, L.A., Crabtree, J.S., Wang, Y., Roe, B.A., Weisemann, J., Boguski, M.S., Agarwal, S.K., Kester, M.B., Kim, Y.S., Heppner, C., Dong, Q., Spiegel, A.M., Burns, A.L., Marx, S.J. (1997). Positional cloning of the gene for multiple endocrine neoplasia-type 1. *Science* **276**: 404-407 .

Chen, P., Lin, H.H., Weissman, B.E. (1995). A functional analysis of tumor suppressor activity for peripheral neuroepitheliomas by monochromosome transfer. *Oncogene* **10**: 577-586.

Chen, W.D., Otterson, G.A., Lipkowitz, S., Khleif, S.N., Coxon, A.B., Kaye, F.J. (1997). Apoptosis is associated with cleavage of a 5 kDa fragment from RB which mimics dephosphorylation and modulates E2F binding. *Oncogene* **14**: 1243-1248.

Chen, Y., Chen, C.F., Riley, D.J., Allred, D.C., Chen, P.L., Von Hoff, D., Osborne, C.K., Lee, W.H. (1995). Aberrant subcellular localization of BRCA1 in breast cancer. *Science* **270**: 789-791.

Chen, Y., Struhl, G. (1996). Dual roles for patched in sequestering and transducing Hedgehog. *Cell* **87**: 553-563.

Chen, Y.C., Chen, P.J., Yeh, S.H., Tien, H.F., Wang, C.H., Tang, J.L., Hong, R.L. (1990). Deletion of the human retinoblastoma gene in primary leukemias. *Blood* **76**: 2060-2064.

Chong, L., van Steensel, B., Broccoli, D., Erdjument-Bromage, H., Hanish, J., Tempst, P., de Lange, T. (1995). A human telomeric protein. *Science* **270**: 1663-1667.

Chumakov, I.M., Rigault, P., Le Gall, I., Bellanne-Chantelot, C., Billault, A., Guillou, S., Soularue, P., Guasconi, G., Poullier, E., Gros, I., *et al.* (1995). A YAC contig map of the human genome. *Nature* **377 (Suppl)**: 175-297.

Church, D.M., Stotler, C.J., Rutter, J.L., Murrell, J.R., Trofatter, J.A., Buckler, A.J. (1994). Isolation of genes from complex sources of mammalian genomic DNA using exon amplification. *Nat. Genet.* **6**: 98-105.

Clarke, A.R., Maandag, E.R., van Roon, M., van der Lugt, N.M., van der Valk, M., Hooper, M.L., Berns, A., te Riele, H. (1992). Requirement for a functional Rb-1 gene in murine development. *Nature* **359**: 328-330.

Cleary, M.L., Sklar, J. (1985). Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near a transcriptionally active locus on chromosome 18. *Proc. Natl. Acad. Sci. U.S.A.* **82**: 7439-7443.

Cobrinik, D., Lee, M.H., Hannon, G., Mulligan, G., Bronson, R.T., Dyson, N., Harlow, E., Beach, D.,Weinberg, R.A., Jacks, T. (1996). Shared role of the pRB-related p130 and p107 proteins in limb development. *Genes Dev.* **10**: 1633-1644.

Collins, J., Hohn, B. (1978). Cosmids: a type of plasmid gene-cloning vector that is packageable in vitro in bacteriophage lambda heads. *Proc. Natl. Acad. Sci. USA* **75**: 4242-4246.

Collins, S. (1995). Positional cloning moves from perditional to traditional. *Nat. Genet.* **9**: 347-350.

Collins, S., Groudine, M. (1982). Amplification of endogenous myc-related DNA sequences in a human myeloid leukaemia cell line. *Nature* **298**: 679-681.

Cossarizza, A., Ortolani, C., Forti, E., Montagnani, G., Paganelli, R., Zannotti, M., Marini, M., Monti, D., Franceschi, C. (1991). Age-related expansion of functionally inefficient cells with markers of natural killer activity in Down's syndrome. *Blood* **77**: 1263-1270.

Cotter, F.E., Hampton, G.M., Nasipuri, S., Bodmer, W.F., Young, B.D. (1990). Rapid isolation of human chromosome-specific DNA probes from a somatic cell hybrid. *Genomics* **7**: 257-263.

Couch, F.J., Farid, L.M., DeShano, M.L., Tavtigian, S.V., Calzone, K., Campeau, L., Peng, Y., Bogden, B., Chen, Q., Neuhausen, S., Shattuck-Eidens, D., Godwin, A.K., Daly, M., Radford, D.M., Sedlacek, S., Rommens, J., Simard, J., Garber, J., Merajver, S., Weber, B.L. (1996). BRCA2 germline mutations in male breast cancer cases and breast cancer families. *Nat. Genet.* **13**: 123-125.

Couet, J., Li, S., Okamoto, T., Ikezu, T., Lisanti, M.P. (1997). Identification of peptide and protein ligands for the caveolin-scaffolding domain. Implications for the interaction of caveolin with caveolae-associated proteins. *J. Biol. Chem.* **272**: 6525-6533.

Counter, C.M., Avilion, A.A., LeFeuvre, C.E., Stewart, N.G., Greider, C.W., Harley, C.B., Bacchetti, S. (1992). Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. *EMBO J.* **11**: 1921-1929.

Cross, S.M., Sanchez, C.A., Morgan, C.A., Schimke, M.K., Ramel, S., Idzerda, R.L., Raskind, W.H., Reid, B.J. (1995). A p53-dependent mouse spindle checkpoint. *Science* **267**: 1353-1356.

Cuthbert, A.P., Trott, D.A., Ekong, R.M., Jezzard, S., England, N.L., Themis, M., Todd, C.M., Newbold, R.F. (1995). Construction and characterization of a highly stable human: rodent monochromosomal hybrid panel for genetic complementation and genome mapping studies. *Cytogenet. Cell. Genet.* **71**: 68-76.

Dalla-Favera, R., Wong-Staal, F., Gallo, R.C. (1982). Onc gene amplification in promyelocytic leukaemia cell line HL-60 and primary leukaemic cells of the same patient. *Nature* **299**: 61-63.

Dausset, J., Ougen, P., Abderrahim, H., Billault, A., Sambucy, J.L., Cohen, D., Le Paslier, D. (1992). The CEPH YAC library. Behring Inst. Mitt. **91**: 13-20.

de Klein, A., van Kessel, A.G., Grosveld, G., Bartram, C.R., Hagemeijer, A., Bootsma, D., Spurr, N.K., Heisterkamp, N., Groffen, J., Stephenson, J.R. (1982). A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukaemia. *Nature* **300**: 765-767.

de Lange, T. (1996). In search of vertebrate telomeric proteins. *Semin. Cell Biol.* **7**: 23-29.

Debbas, M., White, E. (1993). Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. *Genes Dev.* **7**: 546-554.

DeChiara, T.M., Robertson, E.J., Efstratiadis, A. (1991). Parental imprinting of the mouse insulin-like growth factor II gene. *Cell* **64**: 849-859.

DeGregori, J., Kowalik, T., Nevins, J.R. (1995). Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G1/S-regulatory genes. *Mol. Cell. Biol.* **15**: 4215-4224.

Deng, C., Zhang, P., Harper, J.W., Elledge, S.J., Leder, P. (1995). Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. *Cell* **82**: 675-684.

Detloff, P.J., Lewis, J., John, S.W., Shehee, W.R., Langenbach, R., Maeda, N., Smithies, O. (1994). Deletion and replacement of the mouse adult beta-globin genes by a "plug and socket" repeated targeting strategy. *Mol. cell. biol.* **14**: 6936-6943.

Devilee, P., Hermans, J., Eyfjord, J., Boorresen, A.L., Liderau, R., Sobol, H., Borg, A., Cleton-Jansen, A.M., Olah, E., Cohen, B.B., Scherneck, S., Hamann, U., Peterlin, B., Caligo, M., Bignon, Y.J., Maugard, C. (1997). Loss of heterozygosity at 7q31 in breast cancer: results from an International Collaborative Study Group. The Breast Cancer Somatic Genetics Consortium. *Genes Chromosomes Cancer* **18**: 193-199.

Diatchenko, L., Lau, Y.F., Campbell, A.P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S. , Lukyanov, K., Gurskaya, N., Sverdlov, E.D., Siebert, P.D. (1996). Suppression subtractive hybridization: a method forgenerating differentially regulated or tissue-specific cDNA probes and libraries. *Proc. Natl. Acad. Sci. USA* **93**: 6025-6030.

Dibs, C., Faure, S., Fizames, C., Samson, D., Drouot, N., Vignal, A., Millasseau, P., Marc, S., Hazan, J., Seboun, E., Lathrop, M., Gyapay, G., Morissette, J., and Weissenbach, J. (1996). A comprehensive genetic map of the human genome based on 5,264 microsatellites. *Nature* **380**: 152-154.

Dimri, G.P., Hara, E., Campisi, J. (1994). Regulation of two E2F-related genes in presenescent and senescent human fibroblasts. *J. Biol. Chem.* **269**: 16180-16186.

Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-Smith, O., *et al.* (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **92**: 9363-9367.

Dimri, G.P., Nakanishi, M., Desprez, P.Y., Smith, J.R., Campisi, J. (1996). Inhibition of E2F activity by the cyclin-dependent protein kinase inhibitor p21 in cells expressing or lacking a functional retinoblastoma protein. *Mol. Cell. Biol.* 16: 2987-2997.

Dmitrovsky, E., Kuehl, W.M., Hollis, G.F., Kirsch, I.R., Bender, T.P., Segal, S. (1986). Expression of a transfected human c-myc oncogene inhibits differentiation of a mouse erythroleukaemia cell line. *Nature* **322**: 748-750.

Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A., Butel, J.S., Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* **356**: 215-221.

Dowdy, S.F., Hinds, P.W., Louie, K., Reed, S.I., Arnold, A., Weinberg, R.A. (1993). Physical interaction of the retinoblastoma protein with human D cyclins. *Cell* **73**: 499-511.

Druck, T., Kastury, K., Hadaczek, P., Podolski, J., Toloczko, A., Sikorski, A., Ohta, M., La Forgia, S., Lasota, J., McCue, P., *et al.* (1995). Loss of heterozygosity at the familial RCC t(3;8) locus in most clear cell renal carcinomas. *Cancer Res.* **55**: 5348-5353.

Duan, D.R., Pause, A., Burgess, W.H., Aso, T., Chen, D.Y., Garrett, K.P., Conaway, R.C., Conaway, J.W., Linehan, W.M., Klausner, R.D. (1995). Inhibition of transcription elongation by the VHL tumour suppressor protein. *Science* **269**: 1402-1406.

Dulic, V., Drullinger, L.F., Lees, E.,. Reed, S.I., Stein, G.H. (193). Altered regulation of G1 cyclins in senescent human diploid fibroblasts: accumulation of inactive cyclin E-Cdk2 and cyclin D1-Cdk2 complexes. *Proc. Natl. Acad. Sci. U.S.A.* **90**: 11034-11038.

Edington, K.G., Loughran, O.P., Berry, I.J., Parkinson, E.K. (1995). Cellular immortality: a late event in the progression of human squamous cell carcinoma of the head and neck associated with p53 alteration and a high frequency of allele loss. *Mol. Carcinog.* **13**: 254-265.

Eliyahu, D., Raz, A., Gruss, P., Givol, D., Oren, M. (1984). Participation of p53 cellular tumour antigen in transformation of normal embryonic cells. *Nature* **312**: 646-649.

Ellis, N.A., Groden, J., Ye, T.Z., Straughen, J., Lennon, D.J., Ciocci, S., Proytcheva, M., German, J. (1995). The Bloom's syndrome gene product is homologous to RecQ helicases. *Cell* **83**: 655-666.

Ellis, N.A., Roe, A.M., Kozloski, J., Proytcheva, M., Falk, C., German, J. (1994). Linkage disequilibrium between the FES, D15S127, and BLM loci in Ashkenazi Jews with Bloom syndrome. *Am. J. Hum. Genet.* **55**: 453-460.

Engelman, J.A., Wykoff, C.C., Yasuhara, S., Song, K.S., Okamoto, T., Lisanti, M.P. (1997). Recombinant expression of caveolin-1 in oncogenically transformed cells abrogates anchorage-independent growth. *J Biol Chem* **272**: 16374-16381.

England, N.L., Cuthbert, A.P., Trott, D.A., Jezzard, S., Nobori, T., Carson, D.A., Newbold, R.F. (1996). Identification of human tumour suppressor genes by monochromosome transfer: rapid growth-arrest response mapped to 9p21 is mediated solely by the cyclin-D-dependent kinase inhibitor gene, CDKN2A (p16INK4A). *Carcinogenesis* **17**: 1567-1575.

Eva, A., Aaronson, S.A. (1985). Isolation of a new human oncogene from a diffuse B cell lymphoma. *Nature* **316**: 273-275.

Evan, G.I., Wyllie, A.H., Gilbert, C.S., Littlewood, T.D., Land, H., Brooks, M., Waters, C.M., Penn, L.Z., Hancock, D.C. (1992). Induction of apoptosis in fibroblasts by c-myc protein. *Cell* **69**: 119-128.

Evans, E.P., Burtenshaw, M.D., Brown, B.B., Hennion, R., Harris, H. (1982). The analysis of malignancy by cell fusion. IX. Re-examination and clarification of the cytogenetic problem. *J. Cell Sci.* **56**: 113-130.

Ewen, M.E., Sluss, H.K., Sherr, C.J., Matsushime, H., Kato, J., Livingston, D.M. (1993). Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. *Cell* **73** : 487-497.

Fan, J.Y., Carpentier, J.L., van Obberghen, E., Grunfeld, C., Gorden, P., Orci, L. (1983). Morphological changes of the 3T3-L1 fibroblast plasma membrane upon differentiation to the adipocyte form. *J. Cell. Sci.* **61**: 219-230

Fazeli, A., Dickinson, S.L., Hermiston, M.L., Tighe, R.V., Steen, R.G., Small, C.G., Stoeckli, E.T., Keino-Masu, K., Masu, M., Rayburn, H., Simons, J., Bronson, R.T., Gordon, J.I., Tessier-Lavigne, M., Weinberg, R.A. (1997). Phenotype of mice lacking functional Deleted in colorectal cancer (Dcc) gene. *Nature* **386**: 796-804.

Fearon, E.R., Vogelstein, B. (1990). A genetic model for colorectal tumourigenesis. *Cell* **61**: 759-767.

Feaver, W.J., Svejstrup, J.Q., Bardwell, L., Bardwell, A.J., Buratowski, S., Gulyas, K.D., Donahue, T.F., Friedberg, E.C., Kornberg, R.D. (1993). Dual roles of a multiprotein complex from S. cerevisiae in transcription and DNA repair. Cell **75**: 1379-1387.

Feng, J., Funk, W.D., Wang, S.S., Weinrich, S.L., Avilion, A.A., Chiu, C.P., Adams, R.R., Chang, E., Allsopp, R.C., Yu, J., *et al.* (1995). The RNA component of human telomerase. *Science* **269**: 1236-1241.

Fero, M.L., Rivkin, M., Tasch, M., Porter, P., Carow, C.E., Firpo, E., Polyak, K., Tsai, L.H., Broudy,V., Perlmutter, R.M., Kaushansky, K., Roberts, J.M. (1996). A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice. *Cell* **85**: 733-744.

Field, S.J., Tsai, F.Y., Kuo, F., Zubiaga, A.M., Kaelin, W.G., Livingston, D.M., Orkin, S.H.,Greenberg, M.E. (1996). E2F-1 functions in mice to promote apoptosis and suppress proliferation. *Cell* **85**: 549-561.

Finlay, C.A., Hinds, P.W., Levine, A.J. (1989). The p53 proto-oncogene can act as a suppressor of transformation. *Cell* **57**: 1083-1093.

Fishel, R., Lescoe, M.K., Rao, M.R., Copeland, N.G., Jenkins, N.A., Garber, J., Kane, M., Kolodner, R. (1993). The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. *Cell* **75**: 1027-1038.

Ford, J.M., Hanawalt, P.C. (1995). Li-Fraumeni syndrome fibroblasts homozygous for p53 mutations are deficient in global DNA repair but exhibit normal transcription-coupled repair and enhanced UV resistance. *Proc. Natl. Acad. Sci. U.S.A.* **92**: 8876-8880.

Foster, S.A., Galloway, D.A. (1996). Human papillomavirus type 16 E7 alleviates a proliferation block in early passage human mammary epithelial cells. *Oncogene* **12**: 1773-1779.

Fra, A.M., Masserini, M., Palestini, P., Sonnino, S., Simons, K. (1995). A photo-reactive derivative of ganglioside GM1 specifically cross-links VIP21-caveolin on the cell surface. *FEBS Lett.* **375**: 11-14.

Fra, A.M., Williamson, E., Simons, K., Parton, R.G. De novo formation of caveolae in lymphocytes by expression of VIP21-caveolin. *Proc. Natl. Acad. Sci. USA* **92**: 8655-8659.

Friedberg, E.C., Walker, G.C., Siede, W. (1995). DNA repair and mutagenesis. ASM Press, Washington, DC

Friedlander, P., Haupt, Y., Prives, C., Oren, M. (1996). A mutant p53 that discriminates between p53-responsive genes cannot induce apoptosis. *Mol. cell. biol.* **16**: 4961-4971.

Friend, S.H., Bernards, R., Rogelj, S., Weinberg, R.A., Rapaport, J.M., Albert, D.M., Dryja, T.P. (1986). A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature* **323**: 643-646.

Fu, L., Minden, M.D., Benchimol, S. (1996). Translational regulation of human p53 gene expression. *EMBO J.* **15**: 4392-4401.

Fukasawa, K., Choi, T., Kuriyama, R., Rulong, S., Van de Woude, G.F. (1996). Abnormal centrosome amplification in the absence of p53. *Science* **271**: 1744-1747.

Fukuchi, K., Martin, G.M., Monnat, R.J. (1989). Mutator phenotype of Werner syndrome is characterized by extensive deletions. *Proc. Natl. Acad. Sci. U S A* **86**: 5893-5897.

Futreal, P.A., Liu, Q., Shattuck-Eidens, D., Cochran, C., Harshman, K., Tavtigian, S., Bennett, L.M., Haugen-Strano, A., Swensen, J., Miki, Y., *et al.* (1994). BRCA1 mutations in primary breast and ovarian carcinomas. *Science* **266**: 120-122.

Gailani, M.R., Stahle-Backdahl, M., Leffell, D.J., Glynn, M., Zaphiropoulos, P.G., Pressman, C.,Unden, A.B., Dean, M., Brash, D.E., Bale, A.E., Toftgard, R. (1996). The role of the human homologue of Drosophila patched in sporadic basal cell carcinomas. *Nat. Genet.* **14**: 78-81.

Gale, R.P., Canaani, E. (1984). An 8-kilobase abl RNA transcript in chronic myelogenous leukemia. *Proc. Natl. Acad. Sci. U.S.A.* **81**: 5648-5652.

Gallagher, W.M., Cairney, M., Schott, B., Roninson, I.B., Brown, R. (1997). Identification of p53 genetic suppressor elements which confer resistance to cisplatin. *Oncogene* **14**: 185-193.

Garcia-Cardena, G., Fan, R., Stern, D.F., Liu, J., Sessa, W.C. (1996). Endothelial nitric oxide synthase is regulated by tyrosine phosphorylation and interacts with caveolin-1. *J. Biol. Chem.* **271**: 27237-27240.

Gardiner-Garden, M., Frommer, M. (1987). CpG islands in vertebrate genomes. *J. Mol. Biol.* **196**: 261-282.

Garkavtsev, I., Grigorian, I.A., Ossovskaya, V.S., Chernov, M.V., Chumakov, P.M., Gudkov, A.V. (1998). The candidate tumour suppressor p33ING1 cooperates with p53 in cell growth control. *Nature* **391**: 295-298.

Garkavtsev, I., Kazarov, A., Gudkov, A., Riabowol, K. (1996). Suppression of the novel growth inhibitor p33ING1 promotes neoplastic transformation. *Nat. Genet.* **14**: 415-420.

Garkavtsev, I., Riabowol, K. (1997). Extension of the replicative life span of human diploid fibroblasts by inhibition of the p33ING1 candidate tumor suppressor. *Mol. cell. biol.* **17**: 2014-2019.

German, J., Roe, A.M., Leppert, M.F., Ellis, N.A. (1994). Bloom syndrome: an analysis of consanguineous families assigns the locus mutated to chromosome band 15q26.1. *Proc. Natl. Acad. Sci. U.S.A.* **91**: 6669-6673.

Gessler, M., Poustka, A., Cavenee, W., Neve, R.L., Orkin, S.H., Bruns, G.A. (1990) Homozygous deletion in Wilms' tumours of a zinc-finger gene identified by chromosome jumping. *Nature* **343**: 774-778.

Glenney, J.R. Jr (1989). Tyrosine phosphorylation of a 22-kDa protein is correlated with transformation by Rous sarcoma virus. *J. Biol. Chem.* **264**: 20163-20166.

Glenney, J.R., Soppet, D. (1992). Sequence and expression of caveolin, a protein component of caveolae plasma membrane domains phosphorylated on tyrosine in Rous sarcoma virus-transformed fibroblasts. *Proc. Natl. Acad. Sci. USA* **89**: 10517-10521.

Glover, T.W., Berger, C., Coyle, J., Echo, B. (1988). DNA polymerase alpha inhibition by aphidicolin induces gaps and breaks at common fragile sites in human chromosomes. *Hum. Genet.* **67**: 136-142.

Goddard, K.A., Yu, C.E., Oshima, J., Miki, T., Nakura, J., Piussan, C., Martin, G.M., Schellenberg, G.D., Wijsman, E.M. (1996). Toward localization of the Werner syndrome gene by linkage disequilibrium and ancestral haplotyping: lessons learned from analysis of 35 chromosome 8p11.1-21.1 markers. *Am. J. Hum. Genet.* **58**: 1286-1302.

Gollahon, L.S., Shay, J.W. (1996). Immortalization of human mammary epithelial cells transfected with mutant p53 (273his). *Oncogene* **12**: 715-725.

Gonzalez-Agosti, C., Xu, L., Pinney, D., Beauchamp, R., Hobbs, W., Gusella, J., Ramesh, V. (1996). The merlin tumor suppressor localizes preferentially in membrane ruffles. *Oncogene* **13**: 1239-1247.

Good, L., Dimri, G.P., Campisi, J., Chen, K.Y. (1996). Regulation of dihydrofolate reductase gene expression and E2F components in human diploid fibroblasts during growth and senescence. *J. Cell. Physiol.* **168**: 580-588.

Gottlieb, T.M., Oren, M. (1996). p53 in growth control and neoplasia. *Biochim. Biophys. Acta* **1287**: 77-102.

Graeber, T.G., Osmanian, C., Jacks, T., Housman, D.E., Koch, C.J., Lowe, S.W., Giaccia, A.J. (1996). Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. *Nature* **379**: 88-91.

Green, E.D., Olson, M.V. (1990). Chromosomal region of the cystic fibrosis gene in yeast artificial chromosomes: a model for human genome mapping. *Science* **250**: 94-98.

Green, M., Krontiris, T.G. (1993). Allelic variation of reporter gene activation by the HRAS1 minisatellite. *Genomics* **17**: 429-434.

Green, M.R. (1989). When the products of oncogenes and anti-oncogenes meet. *Cell* **56**: 1-3.

Greenwell, P.W., Kronmal, S.L., Porter, S.E., Gassenhuber, J., Obermaier, B., Petes, T.D. (1995). TEL1, a gene involved in controlling telomere length in S.cerevisiae, is homologous to the human ataxia telangiectasia gene. *Cell* **82**: 823-829.

Greider, C.W., Blackburn, E.H. (1985). Identification of a specific telomere terminal transferaseactivity in Tetrahymena extracts. *Cell* **43**: 405-413.

Gress, T.M., Muller-Pillasch, F., Geng, M., Zimmerhackl, F., Zehetner, G., Friess, H., Buchler, M., Adler, G., Lehrach, H. (1996). A pancreatic cancer-specific expression profile. *Oncogene* **13**: 1819-1830.

Groden, J., Thliveris, A., Samowitz, W., Carlson, M., Gelbert, L., Albertsen, H., Joslyn, G., Stevens, J., Spirio, L., Robertson, M., *et al.* (1991). Identification and characterization of the familial adenomatous polyposis coli gene. *Cell* **66**: 589-600.

Groffen, J., Stephenson, J.R., Heisterkamp, N., de Klein, A., Bartram, C.R., Grosveld, G. (1984). Philadelphia chromosomal breakpoints are clustered within a limited region, *bcr*, on chromosome 22. *Cell* **36**: 93-99.

Grompe, M. (1993). The rapid detection of unknown mutations in nucleic acids. *Nat. Genet.* **5**: 111-117.

Gu, W., Roeder, R.G. (1997). Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell* **90**: 595-606.

Gudkov, A.V., Kazarov, A.R., Thimmapaya, R., Axenovich, S.A., Mazo, I.A., Roninson, I.B. (1994). Cloning mammalian genes by expression selection of genetic suppressor elements: association of kinesin with drug resistance and cell immortalization. *Proc. Natl. Acad. Sci. U.S.A.* **91**: 3744-3748.

Gudkov, A.V., Zelnick, C.R., Kazarov, A.R., Thimmapaya, R., Suttle, D.P., Beck, W.T., Roninson, I.B. (1993). Isolation of genetic suppressor elements, inducing resistance to topoisomerase II-interactive cytotoxic drugs,from human topoisomerase II cDNA. *Proc. Natl. Acad. Sci. U.S.A.* **90**: 3231-3235.

Gumbiner, B.M. (1995). Signal transduction of beta-catenin. *Curr. Opin. Cell Biol.* **7**: 634-640.

Gupta, P.K., Sahota, A., Boyadjiev, S.A., Bye, S., Shao, C., O'Neill, J.P., Hunter, T.C., Albertini, R.J., Stambrook, P.J., Tischfield, J.A. (1997). High frequency in vivo loss of heterozygosity is primarily a consequence of mitotic recombination. *Cancer Res.* **57**: 1188-1193.

Gustafson, C.E., Wilson, P.J., Lukeis, R., Baker, E., Woollatt, E., Annab, L., Hawke, L., Barrett, J.C., Chenevix-Trench, G. (1996). Functional evidence for a colorectal cancer tumor suppressor gene at chromosome 8p22-23 by monochromosome transfer. *Cancer Res.* **56**: 5238-5245.

Gyapay, G., Schmitt, K., Fizames, C., Jones, H., Vega-Czarny, N., Spillett, D., Muselet, D.,Prud'Homme, J.F., Dib, C., Auffray, C., Morissette, J., Weissenbach, J., Goodfellow, P.N. (1996). A radiation hybrid map of the human genome. *Hum. Mol. Genet.* **5**: 339-346.

Gyapay, G., Morissette, J., Vignal, A., Dib, C., Fizames, C., Millasseau, P., Marc, S., Bernardi, G., Lathrop, M., Weissenbach, J. (1994). The 1993-94 Genethon human genetic linkage map. *Nat. Genet.* **7** (2 Spec No): 246-339.

Haber, D., Harlow, E. (1997). Tumour-suppressor genes: evolving definitions in the genomic age. *Nat. Genet.* **16**: 320-322.

Habets, G.G., Scholtes, E.H., Zuydgeest, D., van der Kammen, R.A., Stam, J.C., Berns, A., Collard, J.G. (1994). Identification of an invasion-inducing gene, Tiam-1, that encodes a protein with homology to GDP-GTP exchangers for Rho-like proteins. *Cell* **77**: 537-549.

Hahn, H., Wicking, C., Zaphiropoulous, P.G., Gailani, M.R., Shanley, S., Chidambaram, A.,Vorechovsky, I., Holmberg, E., Unden, A.B., Gillies, S., Negus, K., Smyth, I., Pressman, C., Leffell, D.J., Gerrard, B., Goldstein, A.M., Dean, M., Toftgard, R., Chenevix-Trench, G., Wainwright, B., Bale, A.E. (1996)[a]. Mutations of the human

homolog of Drosophila patched in the nevoid basal cell carcinoma syndrome. *Cell* **85**: 841-851.

Hahn, S.A., Schutte, M., Hoque, A.T., Moskaluk, C.A., da Costa, L.T., Rozenblum, E., Weinstein, C.L., Fischer, A., Yeo, C.J., Hruban, R.H., Kern, S.E. (1996)[b]. DPC4, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science* **271**: 350-353.

Hall, M., Peters, G. (1996). Genetic alterations of cyclins, cyclin-dependent kinases,and Cdk inhibitors in human cancer. *Adv. Cancer Res.* **68**: 67-108.

Hanson, K.D., Sedivy, J.M. (1995). Analysis of biological selections for high-efficiency gene targeting. *Mol. Cell. Biol.* **15**: 45-51.

Hara, E., Smith, R., Parry, D., Tahara, H., Stone, S., Peters, G. (1996). Regulation of p16CDKN2 expression and its implications for cell immortalization and senescence. *Mol. Cell. Biol.* **16**: 859-867.

Hara, E., Yamaguchi, T., Nojima, H., Ide, T., Campisi, J., Okayama, H., Oda, K. (1994). Id-related genes encoding helix-loop-helix proteins are required for G1 progression and are repressed in senescent human fibroblasts. *J. Biol. Chem.* **269**: 2139-2145.

Harbour, J.W., Lai, S.L., Whang-Peng, J., Gazdar, A.F., Minna, J.D., Kaye, F.J. (1988). Abnormalities in structure and expression of the human retinoblastoma gene in SCLC. *Science* **241**: 353-357.

Harley, C.B., Futcher, A.B., Greider, C.W. (1990) Telomeres shorten during ageing of human fibroblasts *Nature* **345**: 458-460.

Harrington, L., McPhail, T., Mar, V., Zhou, W., Oulton, R., Bass, M.B., Arruda, I., Robinson, M.O. (1997). A mammalian telomerase-associated protein. *Science* **275**: 973-977.

Harris, H., Miller, O.J., Klein, G., Worst, P., Tachibana, T (1969). Suppression of malignancy by cell fusion. *Nature* **223**: 363-368.

Hastie, N.D. (1994). The genetics of Wilms' tumour—a case of disrupted development. *Annu. Rev. Genet.* **28**: 523-558.

Hastie, N.D., Dempster, M., Dunlop, M.G., Thompson, A.M., Green, D.K., Allshire, R.C. (1990). Telomere reduction in human colorectal carcinoma and with ageing. *Nature* **346**: 866-868.

Hatada, I. and Mukai, T. (1995). Genomic imprinting of $p57^{KIP2}$, a cyclin-dependent kinase inhibitor, in mouse. *Nat. Genet.* **11**: 204-206.

Hatada, I., Ohashi, H., Fukushima, Y., Kaneko, Y., Inoue, M., Komoto, Y., Okada, A., Ohishi, S., Nabetani, A., Morisaki, H., Nakayama, M., Niikawa, N., Mukai, T. (1996). An imprinted gene p57KIP2 is mutated in Beckwith-Wiedemann syndrome. *Nat. Genet.* **14**: 171-173.

Hayflick, L. (1965). The limited in vitro lifetime of human diploid cell strains. *Exp. Cell Res.* **37**: 614-636.

Hayward, W.S., Neel, B.G., Astrin, S.M. (1981). Activation of a cellular onc gene by promoter insertion in ALV-induced lymphoid leukosis. *Nature* **290**: 475-480.

Hedrick, L., Cho, K.R., Fearon, E.R., Wu, T.C., Kinzler, K.W., Vogelstein, B. (1994). The DCC gene product in cellular differentiation and colorectal tumorigenesis. *Genes Dev.* **8**: 1174-1183.

Henry, I., Bonaiti-Pellie, C., Chehensse, V., Beldjord, C., Schwartz, C., Utermann, G., Junien, C. (1991). Uniparental paternal disomy in a genetic cancer-predisposing syndrome. *Nature* **351**: 665-667.

Hensler, P.J., Annab, L.A., Barrett, J.C., Pereira-Smith, O.M. (1994). A gene involved in control of human cellular senescence on human chromosome 1q. *Mol. Cell. Biol.* **14**: 2291-2297.

Herman, J.G., Merlo, A., Mao, L., Lapidus, R.G., Issa, J.P., Davidson, N.E., Sidransky, D., Baylin, S.B. (1995). Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. *Cancer Res.* **55**: 4525-4530.

Herskowitz, I. (1989). Functional inactivation of genes by dominant negative mutations. *Nature* **329**: 219-222.

Hesketh, R. (1995). *The oncogenes factsbook.* Academic Press, London

Hinds, P., Finlay, C., Levine, A.J. (1989). Mutation is required to activate the p53 gene for cooperation with the ras oncogene and transformation. *J. Virol.* **63**: 739-746.

Hoeijmakers, J. (1993). Nucleotide excision repair II: From yeast to mammals. *Trends Genet.* **9**: 211-217.

Hofstra, R.M., Landsvater, R.M., Ceccherini, I., Stulp, R.P., Stelwagen, T., Luo, Y., Pasini, B., Hoppener, J.W., van Amstel, H.K., Romeo, G., *et al.* (1994). A mutation in the *RET* proto-oncogene associated with multiple endocrine neoplasia type 2B and sporadic medullary thyroid carcinoma. *Nature* **367**: 375-376.

Hollstein, M., Shomer, B., Greenblatt, M., Soussi, T., Hovig, E., Montesano, R., Harris, C.C. (1996). Somatic point mutations in the gene of human tumours and cell lines: updated compilation. *Nucleic Acids Res.* **24**: 141-146.

Holt, J.T., Thompson, M.E., Szabo, C., Robinson-Benion, C., Arteaga, C.L., King, M.C., Jensen, R.A. (1996). Growth retardation and tumour inhibition by BRCA1. *Nat. Genet.* **12**: 298-302.

Holt, S.E, Shay, J.W., Wright, W.E.(1996). Refining the telomere-telomerase hypothesis of aging and cancer. *Nature Biotech.* **14**: 834-837.

Holzmayer, T.A., Pestov, D.G., Roninson, I.B. (1992). Isolation of dominant negative mutants and inhibitoryantisense RNA sequences by expression selection ofrandom DNA fragments. *Nucleic Acids Res.* **20**: 711-717.

Horn, G.T., Richards, B., Merrill, J.J., Klinger, K.W. (1990). Characterization and rapid diagnostic analysis of DNA polymorphisms closely linked to the cystic fibrosis locus. *Clin. Chem.* **36**: 1614-1619..

Howe, J.R., Roth, S., Ringold, J.C., Summers, R.W., Jarvinen, H.J., Sistonen, P., Tomlinson, I.P.M., Houlston, R.S., Bevan, S., Mitros, F.A., Stone, E.M., Aaltonen, L.A. (1998). Mutations in the SMAD4/DPC4 gene in juvenile polyposis. *Science* **280**: 1086-1088.

Huang, H.J., Qian, J., Proffit, J., Wilber, K., Jenkins, R., Smith, D.I. (1998). FRAG7 extends over a broad region: coincidence of human endogenous retroviral sequences (HERV-H) and small polydispersed circular DNAs (spcDNA) and fragile sites.

Huang, H.J., Yee, J.K., Shew, J.Y., Chen, P.L., Bookstein, R., Friedmann, T., Lee, E.Y., Lee, W.H. (1988). Suppression of the neoplastic phenotype by replacement of the RB gene in human cancer cells. *Science* **242**: 1563-1566.

Hudson, T.J., Stein, L.D., Gerety, S.S., Ma, J., Castle, A.B., Silva, J., Slonim, D.K., Baptista, R., Kruglyak, L., Xu, S.H., *et al.* (1995). An STS-based map of the human genome. *Science* **270**: 1945-1954.

Hughson, M.D., Meloni, A., Dougherty, S., Silva, F.G., Sandberg, A.A. (1996). Analysis of 3p allelic loss in papillary and nonpapillary renal cell carcinomas. Correlation with tumor karyotypes. *Cancer Genet. Cytogenet.* **87**: 133-139.

Hunter, T., Pines, J. (1994). Cyclins and cancer. II: Cyclin D and CDK inhibitors come of age. *Cell* **79**: 573-582.

Hussussian, C.J., Struewing, J.P., Goldstein, A.M., Higgins, P.A., Ally, D.S., Sheahan, M.D., Clark, W.H., Tucker, M.A., Dracopoli, N.C. (1994). Germline p16 mutations in familial melanoma. *Nat. Genet.* **8**: 15-21.

Huynh, D.P., Pulst, S.M. (1996). Neurofibromatosis 2 antisense oligodeoxynucleotides induce reversible inhibition of schwannomin synthesis and cell adhesion in STS26T and T98G cells. *Oncogene* **13**: 73-84.

Iliopoulos, O., Kibel, A., Gray, S., Kaelin, W.G. (1995). Tumour suppression by the human von Hippel-Lindau gene product. *Nat. Med.* **1**: 822-826.

Ioannou, P.A., Amemiya, C.T., Garnes, J., Kroisel, P.M., Shizuya, H., Chen, C., Batzer, M.A., deJong, P.J. (1994). A new bacteriophage P1-derived vector for the propagation of large human DNA fragments. *Nat. Genet.* **6**: 84-89.

Ionov, Y., Peinado, M.A., Malkhosyan, S., Shibata, D., Perucho, M. (1993). Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature* **363**: 558-561.

Iosbe, M., Emanuel, B.S., Givol, D., Oren, M., Croce, C.M. (1986). Localization of gene for human p53 tumour antigen to band 17p13. *Nature* **320**: 84-85.

Iseki, S., Araga, A., Ohuchi, H., Nohno, T., Yoshioka, H., Hayashi, F., Noji, S. (1996). Sonic hedgehog is expressed in epithelial cells during development of whisker, hair, and tooth. *Biochem. Biophys. Res. Commun.* **218**: 688-693.

Ittmann, M. (1996). Allelic loss on chromosome 10 in prostate adenocarcinoma. *Cancer Res.* **56**:2143-2147.

Jacks, T., Fazeli, A., Schmitt, E.M., Bronson, R.T., Goodell, M.A., Weinberg, R.A. (1992). Effects of an Rb mutation in the mouse. *Nature* **359**: 295-300.

Janicke, R.U., Walker, P.A., Lin, X.Y., Porter, A.G. (1996). Specific cleavage of the retinoblastoma protein by an ICE-like protease in apoptosis. *EMBO J.* **15**: 6969-6978.

Jayaraman, J., Prives, C. (1995). Activation of p53 sequence-specific DNA binding by short single strands of DNA requires the p53 C-terminus. *Cell* **81**: 1021-1029.

Jazwinski, S.M. (1993). The genetics of aging in the yeast Saccharomyces cerevisiae. *Genetica* **91**: 35-51.

Jensen, R.A., Thompson, M.E., Jetton, T.L., Szabo, C.I., van der Meer, R., Helou, B., Tronick, S.R., Page, D.L., King, M.C., Holt, J.T. (1996). BRCA1 is secreted and exhibits properties of a granin. *Nat. Genet* **12**: 303-308.

Jensen, D.E., Proctor, M., Marquis, S.T., Gardner, H.P., Ha, S.I., Chodosh, L.A., Ishov, A.M., Tommerup, N., Vissing, H., Sekido, Y., Minna, J., Borodovsky, A., Schultz, D.C., Wilkinson, K.D., Maul, G.G., Barlev, N., Berger, S.L., Prendergast, G.C., Rauscher, F.J. (1998). BAP1: a novel ubiquitin hydrolase which binds to the BRCA1 RING finger and enhances BRCA1-mediated cell growth suppression. *Oncogene* **16**: 1097-1112.

Johnson, D.G., Schwarz, J.K., Cress, W.D., Nevins, J.R. (1993). Expression of transcription factor E2F1 induces quiescent cells to enter S phase. *Nature* **365**: 349-352.

Johnson, R.L., Rothman, A.L., Xie, J., Goodrich, L.V., Bare, J.W., Bonifas, J.M., Quinn, A.G., Myers, R.M., Cox, D.R., Epstein, E.H., Scott, M.P. (1996). Human homolog of patched, a candidate gene for the basal cell nevus syndrome. *Science* **272**: 1668-1671.

Jonasson, J., Povey, S., Harris, H. (1977). The analysis of malignancy by cell fusion. VII. Cytogenetic analysis of hybrids between malignant and diploid cells and of tumours derived from them. *J. Cell Sci.* **24**: 217-254.

Jost, C.A.,. Marin, M.C., Kaelin, W.G. (1997). p73 is a human p53-related protein that can induce apoptosis. *Nature* **389**: 191-194.

Kagan, J., Stein, J., Babaian, R.J., Joe, Y.S., Pisters, L.L., Glassman, A.B., von Eschenbach, A.C., Troncoso, P. (1995). Homozygous deletions at 8p22 and 8p21 in

prostate cancer implicate these regions as the sites for candidate tumor suppressor genes. *Oncogene* **11**: 2121-2126.

Kamb, A., Shattuck-Eidens, D., Eeles, R., Liu, Q., Gruis, N.A., Ding, W., Hussey, C., Tran, T., Miki, Y., Weaver-Feldhaus, J., *et al.* (1994). Analysis of the p16 gene (CDKN2) as a candidate for the chromosome 9p melanoma susceptibility locus. *Nat. Genet.* **8**: 23-26.

Kariya, Y., Kato, K., Hayashizaki, Y., Himeno, S., Tarui, S., Matsubara, K. (1987). Revision of consensus sequence of human Alu repeats—a review. *Gene* **53**: 1-10.

Karlsson, C., Stenman, G., Vojta, P.J., Bongcam-Rudloff, E., Barrett, J.C., Westermark, B., Paulsson, Y. (1996). Escape from senescence in hybrid cell clones involves deletions of two regions located on human chromosome 1q. *Cancer Res.* **56**: 241-245.

Karran, P., Marinus, M.G. (1982). Mismatch correction at O6-methylguanine residues in E. coli DNA. *Nature* **296**: 868-869.

Kastan, M.B., Onyekwere, O., Sidransky, D., Vogelstein, B., Craig, R.W. (1991). Participation of p53 protein in the cellular response to DNA damage. *Cancer Res.* **51**: 6304-6311.

Kastan, M.B., Zhan, Q., el-Deiry, W.S., Carrier, F., Jacks, T., Walsh, W.V., Plunkett, B.S., Vogelstein, B., Fornace, A.J. (1992). A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell* **71**: 587-597.

Kato, J., Matsushime, H., Hiebert, S.W., Ewen, M.E., Sherr, C.J. (1993). Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclinD-dependent kinase CDK4. *Genes Dev.* **7**: 331-342.

Katzav, S., Martin-Zanca, D., Barbacid, M. (1989) vav, a novel human oncogene derived from a locus ubiquitously expressed in hematopoietic cells. *EMBO J.* 8: 2283-2290.

Kaghad, M., Bonnet, H., Yang, A., Creancier, L., Biscan, J.C., Valent, A., Minty, A., Chalon, P., Lelias, J.M., Dumont, X., Ferrara, P., McKeon, F., Caput, D. (1997). Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. *Cell* **90**: 809-819.

Kemp, C.J., Wheldon, T., Balmain, A. (1994). p53-deficient mice are extremely susceptible toradiation-induced tumorigenesis. *Nat. Genet.* **8**: 66-69.

Kerangueven, F., Essioux, L., Dib, A., Noguchi, T., Allione, F., Geneix, J., Longy, M., Lidereau, R.,Eisinger, F., Pebusque, M.J., *et al.* (1995)[a]. Loss of heterozygosity and linkage analysis in breastcarcinoma: indication for a putative third susceptibility gene on the short arm of chromosome 8. *Oncogene* **10**:1023-1026.

Kerangueven, F., Noguchi, T., Adelaide, J., Jacquemier, J., Sobol, H., Birnbaum, D. (1995)[b]. Allelic loss at markers of chromosomal band 7q31 is not a frequent event in human breast cancer. *Oncol. Rep.* **2**: 89-90.

Kerem, B., Rommens, J.M., Buchanan, J.A., Markiewicz, D., Cox, T.K., Chakravarti, A., Buchwald, M., Tsui, L.C. (1989). Identification of the cystic fibrosis gene: genetic analysis. *Science* **245**: 1073-1080.

Kerr, J., Leary, J.A., Hurst, T., Shih, Y.C., Antalis, T.M., Friedlander, M., Crawford, E., Khoo, S.K., Ward, B., Chenevix-Trench, G. (1996). Allelic loss on chromosome 7q in ovarian adenocarcinomas: two critical regions and a rearrangement of the PLANH1 locus. *Oncogene* **13**: 1815-1818.

Kerr, J.F., Wyllie, A.H., Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* **26**: 239-257.

Kim, N.W. (1997). Clinical implications of telomerase in cancer. *Eur J. Cancer* **33**: 781-786.

Kim, N.W., Piatyszek, M.A., Prowse, K.R., Harley, C.B., West, M.D., Ho, P.L., Coviello, G.M., Wright, W.E., Weinrich, S.L., Shay, J.W. (1994). Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**: 2011-2015.

Kim, S., Villeponteau, B., Jazwinski, S.M. (1996). Effect of replicative age on transcriptional silencing near telomeres in Saccharomyces cerevisiae. *Biochem. Biophys. Res. Commun.* **219**: 370-376.

Kingsley, D.M. The TGF-beta superfamily: new members, new receptors,and new genetic tests of function in different organisms. *Genes Dev.***8**: 133-146.

Kinzler, K.W, Vogelstein, B. (1997). Gatekeepers and caretakers. *Nature* **386**: 761-763.

Kinzler, K.W., Nilbert, M.C., Vogelstein, B., Bryan, T.M., Levy, D.B., Smith, K.J., Preisinger, A.C., Hamilton, S.R., Hedge, P., Markham, A., *et al.* (1991)[a]. Identification of a gene located at chromosome 5q21 that is mutated in colorectal cancers. *Science* **251**: 1366-1370.

Kinzler, K.W., Nilbert, M.C., Vogelstein, B., Bryan, T.M., Levy, D.B., Smith, K.J., Preisinger, A.C., Hamilton, S.R., Hedge, P., Markham, A., *et al.* (1991)[b]. Identification of a gene located at chromosome 5q21 that is mutated in colorectal cancers. *Science* **251**: 1366-1370.

Kiyokawa, H., Kineman, R.D., Manova-Todorova, K.O., Soares, V.C., Hoffman, E.S., Ono, M., Khanam, D., Hayday, A.C., Frohman, L.A., Koff, A. (1996). Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27(Kip1). *Cell* **85**: 721-732.

Klein, G. (1987). The approaching era of the tumor suppressor genes. *Science* **238**: 1539.1545

Klinger, H.P. (1980). Suppression of tumorigenicity in somatic cell hybrids. I. Suppression and reexpression of tumorigenicity in diploid human X D98AH2 hybrids and independent segregation of tumorigenicity from other cell phenotypes. *Cytogenet. Cell Genet.* **27**: 254-266.

Knudson, A.G. (1971). Mutation and cancer: statistical study of retinoblastoma. *Proc. Natl. Acad. Sci. U.S.A.* **68**: 820-823.

Knudson, A.G. (1978). Retinoblastoma: a prototypic hereditary neoplasm. *Semin. Oncol.* **5**: 7-60.

Ko, L.J., Prives, C. (1996). p53: puzzle and paradigm. *Genes Dev.* **10**: 1054-1072.

Koi, M., Johnson, L.A., Kalikin, L.M., Little, P.F., Nakamura, Y., Feinberg, A.P. (1993). Tumor cell growth arrest caused by subchromosomal transferable DNA fragments from chromosome 11. *Science* **260**: 361-364.

Koike, M., Takeuchi, S., Yokota, J., Park, S., Hatta, Y., Miller, C.W., Tsuruoka, N., Koeffler, H.P. (1997). Frequent loss of heterozygosity in the region of the D7S523 locus in advanced ovarian cancer. *Genes Chromosomes Cancer* **19**: 1-5.

Kok, K,. Naylor, S.L., Buys, C.H. (1997). Deletions of the short arm of chromosome 3 in solid tumors and the search for suppressor genes. *Adv. Cancer Res.* **71**: 27-92.

Koleske, A.J., Baltimore, D., Lisanti, M.P. (1995). Reduction of caveolin and caveolae in oncogenically transformed cells. *Proc. Natl. Acad. Sci. U.S.A.* **92**: 1381-1385.

Koonin, E.V., Altschul, S.F., Bork, P. (1996). BRCA1 protein products ... Functional motifs... *Nat. Genet.* **13**: 266-268.

Korinek, V., Barker, N., Morin, P.J., van Wichen, D., de Weger, R., Kinzler, K.W., Vogelstein, B., Clevers, H. (1997). Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. *Science* **275**: 1784-1787.

Koufos, A., Grundy, P., Morgan, K., Aleck, K.A., Hadro, T., Lampkin, B.C., Kalbakji, A., Cavenee,W.K. (1989). Familial Wiedemann-Beckwith syndrome and a second Wilms tumor locus both map to 11p15.5. *Am. J. Hum. Genet.* **44**: 711-719.

Kozma, R., Ahmed, S., Best, A., Lim, L. (1995). The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia inSwiss 3T3 fibroblasts. *Mol. Cell. Biol.* **15**: 1942-1952.

Krontiris, T.G., Devlin, B., Karp, D.D., Robert, N.J., Risch, N. (1993). An association between the risk of cancer and mutations in the HRAS1 minisatellite locus. *N. Engl. J. Med.* **329**: 517-523.

Krontiris, T.G., DiMartino, N.A., Colb, M., Parkinson, D.R. (1985). Unique allelic restriction fragments of the human Ha-ras locus in leukocyte and tumour DNAs of cancer patients. *Nature* **313**: 369-374.

Krueger, N.X. and Saito, H. (1992). A human transmembrane protein-tyrosine-phosphatase, PTP zeta, is expressed in brain and has an N-terminal receptor domain homologous to carbonic anhydrases. *Proc. Natl. Acad. Sci. U.S.A.*. **89**: 7417-7421.

Kruk, P.A., Rampino, N.J., Bohr, V.A. (1995). DNA damage and repair in telomeres: relation to aging. *Proc. Natl. Acad. Sci. U.S.A.* **92**: 258-262.

Kuniyasu, H., Yasui, W., Yokozaki, H., Akagi, M., Akama, Y., Kitahara, K., Fujii, K., Tahara, E. (1994). Frequent loss of heterozygosity of the long arm of chromosome 7 is closely associated with progression of human gastric carcinomas. *Int. J. Cancer* **59**: 597-600.

Kurzchalia, T.V., Dupree, P., Parton, R.G., Kellner, R., Virta, H., Lehnert, M., Simons, K. (1992). VIP21, a 21-kD membrane protein is an integral component of trans-Golgi-network-derived transport vesicles. *J. Cell. Biol.* **118**: 1003-1014.

Kurzrock, R., Shtalrid, M., Romero, P., Kloetzer, W.S., Talpas, M., Trujillo, J.M., Blick, M., Beran, M., Gutterman, J.U. A novel c-abl protein product in Philadelphia-positive acute lymphoblastic leukaemia. *Nature* **325**: 631-635.

Lamb, R.F, Ozanne, B.W., Roy, C., McGarry, L., Stipp, C., Mangeat, P., Jay, D.G. (1997). Essential functions of ezrin in maintenance of cell shape and lamellipodial extension in normal and transformed fibroblasts. *Current Biology* **7**: 682-688.

Laird PW, Zijderveld A, Linders K, Rudnicki MA, Jaenisch R, Berns A. (1991). Simplified mammalian DNA isolation procedure. *Nucleic Acids Res.* **19**: 4293.

Lancaster, J.M., Wooster, R., Mangion, J., Phelan, C.M., Cochran, C., Gumbs, C., Seal, S., Barfoot, R., Collins, N., Bignell, G., Patel, S., Hamoudi, R., Larsson, C., Wiseman, R.W., Berchuck, A., Iglehart, J.D., Marks, J.R., Ashworth, A., Stratton, M.R., Futreal, P.A. 1996 BRCA2 mutations in primary breast and ovarian cancers. *Nat. Genet.* **13**: 238-240.

Larsson, S.H., Charlieu, J.P., Miyagawa, K., Engelkamp, D., Rassoulzadegan, M., Ross, A., Cuzin, F., van Heyningen, V., Hastie, N.D. (1995). Subnuclear localization of WT1 in splicing or transcription factor domains is regulated by alternative splicing. *Cell* **81**: 391-401.

Latif, F., Tory, K., Gnarra, J., Yao, M., Duh, F.M., Orcutt, M.L., Stackhouse, T., Kuzmin, I., Modi, W., Geil, L., *et al.* (1993). Identification of the von Hippel-Lindau disease tumour suppressor gene. *Science* **260**: 1317-1320.

Laureys, G., Speleman, F., Opdenakker, G., Benoit, Y., Leroy, J. (1990). Constitutional translocation t(1;17) (p36;q12-21) in a patient with neuroblastoma. *Genes Chromosomes Cancer* **2**: 252-254.

Lavery, D.J., Lopez-Molina, L., Fleury-Olela, F., Schibler, U. (1997). Selective amplification via biotin- and restriction-mediated enrichment (SABRE), a novel selective amplification procedure for detection of differentially expressed mRNAs. *Proc. Natl. Acad. Sci. U.S.A.* **94**: 6831-6836.

Lee, E.Y., Chang, C.Y., Hu, N., Wang, Y.C., Lai, C.C., Herrup, K., Lee, W.H., Bradley, A. (1992). Mice deficient for Rb are nonviable and show defects in neurogenesis and haematopoiesis. *Nature* **359**: 288-294.

Lee, E.Y., To, H., Shew, J.Y., Bookstein, R., Scully, P., Lee, W.H. (1988). Inactivation of the retinoblastoma susceptibility gene in human breast cancers. *Science* **241**: 218-221.

Lee, M.H., Williams, B.O., Mulligan, G., Mukai, S., Bronson, R.T., Dyson, N., Harlow, E., Jacks, T. (1996).Targeted disruption of p107: functional overlap betweenp107 and Rb. *Genes Dev.* **10**: 1621-1632.

Lee, S., Elenbaas, B., Levine, A., Griffith, J. (1995). p53 and its 14 kDa C-terminal domain recognize primary DNA damage in the form of insertion/deletion mismatches. *Cell* **81**: 1013-1020.

Lee, M.P., Feinberg, A.P. (1997). Aberrant splicing but not mutations of TSG101 in human breast cancer. *Cancer Res.* **57**: 3131-3134.

Lee, S.W., Reimer, C.L., Oh, P., Campbell, D.B., Schnitzer, J.E. (1998). Tumor cell growth inhibition by caveolin re-expression in human breast cancer cells. *Oncogene* **16**: 1391-1397.

Lee, W.H., Bookstein, R., Hong, F., Young, L.J., Shew, J.Y., Lee, E.Y. (1987). Human retinoblastoma susceptibility gene: cloning, identification, and sequence. *Science* **235**: 1394-1399.

Lehrach, H. *et al.* (1990). In Davies, K.E. & Tilghman, S.M. (eds.), Genome Analysis Volume 1: Genetic and Physical Mapping. Cold Spring Harbor Laboratory Press, Cold Spring Harbor , pp. 39-81.

Levis, R.W., Ganesan, R., Houtchens, K., Tolar, L.A., Sheen, F.M. (1993). Transposons in place of telomeric repeats at a Drosophila telomere. *Cell* **75**: 1083-1093.

Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S.I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S.H., Giovanella, B.C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M.H., Parsons, R. (1997). PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* **275**: 1943-1947

Li, L., Li, X., Francke, U., Cohen, S.N. (1996). The TSG101 tumour susceptibility gene is located in chromosome 11 band p15 and is mutated in human breast cancer. *Cell* **88**: 143-154.

Li, S., Couet, J., Lisanti, M.P. (1996). Src tyrosine kinases, G alpha subunits, and H-Ras share a common membrane-anchored scaffolding protein, caveolin. Caveolin binding negatively regulates the auto-activation of Src tyrosine kinases. *J. Biol. Chem.* **271**: 29182-29190.

Li, S., Okamoto, T., Chun, M., Sargiacomo, M., Casanova, J.E., Hansen, S.H., Nishimoto, I., Lisanti, M.P. (1995). Evidence for a regulated interaction between heterotrimeric G proteins and caveolin. *J. Biol. Chem.* **270**: 15693-15701.

Li, S., Song, K.S., Lisanti, M.P. (1996). Expression and characterization of recombinant caveolin. Purification by polyhistidine tagging and cholesterol-dependent incorporation into defined lipid membranes. *J. Biol. Chem.* **271**: 568-573.

Liang, P., Pardee, A.B. (1992). Differential display of eukaryotic messenger RNA bymeans of the polymerase chain reaction. *Science* **257**: 967-971.

Liaw, D., Marsh, D.J., Li, J., Dahia, P.L., Wang, S.I., Zheng, Z., Bose, S., Call, K.M., Tsou, H.C., Peacocke, M., Eng, C., Parsons, R. (1997). Germline mutations of the PTEN gene in Cowden disease,an inherited breast and thyroid cancer syndrome. *Nat. Genet.* **16**: 64-67.

Lin, J.C., Scherer, S.W., Tougas, L., Traverso, G., Tsui, L.C., Andrulis, I.L., Jothy, S., Park, M. (1996). Detailed deletion mapping with a refined physical map of 7q31 localizes a putative tumor suppressor gene for breast cancer in the region of MET. *Oncogene* **13**: 2001-2008.

Lingner, J., Hughes, T.R., Shevchenko, A., Mann, M., Lundblad, V., Cech, T.R. (1997). Reverse transcriptase motifs in the catalytic subunit of telomerase. *Science* **276**: 561-567.

Lingner, J., Cech, T.R., Hughes, T.R., Lundblad, V. (1997). Three Ever Shorter Telomere (EST) genes are dispensable for in vitro yeast telomerase activity. *Proc. Natl. Acad. Sci.* USA **94**: 11190-11195.

Linke, S.P., Clarkin, K.C., Di Leonardo, A., Tsou, A., Wahl, G.M. (1996). A reversible, p53-dependent G0/G1 cell cycle arrest induced by ribonucleotide depletion in the absence of detectable DNA damage. *Genes Dev.* **10**: 934-947.

Lisanti, M.P., Scherer, P.E., Vidugiriene, J., Tang, Z., Hermanowski-Vosatka, A., Tu, Y.H., Cook, R.F., Sargiacomo, M. (1994). Characterization of caveolin-rich membrane domains isolated from an endothelial-rich source: implications for human disease. *J. Cell. Biol.* **126**: 111-126.

Lisitsyn, N., Lisitsyn, N., Wigler, M. (1993). Cloning the differences between two complex genomes. *Science* **259**: 946-951.

Lisitsyn, N.A., Segre, J.A., Kusumi, K., Lisitsyn, N.M., Nadeau, J.H., Frankel, W.N., Wigler, M.H., Lander, E.S. (1994). Direct isolation of polymorphic markers linked to a trait by genetically directed representational difference analysis. *Nat. Genet.* **6**: 57-63.

Liu, B., Nicolaides, N.C., Markowitz, S., Willson, J.K., Parsons, R.E., Jen, J., Papadopolous, N., Peltomaki, P., de la Chapelle, A., Hamilton, S.R., *et al.* (1995). Mismatch repair gene defects in sporadic colorectal cancers with microsatellite instability. *Nat. Genet.* **9**: 48-55.

Liu, B., Parsons, R., Papadopoulos, N., Nicolaides, N.C., Lynch, H.T., Watson, P., Jass, J.R., Dunlop, M., Wyllie, A., Peltomaki, P., de la Chapelle, A., Hamilton, S.R., Vogelstein, B., Kinzler, K.W. (1996). Analysis of mismatch repair genes in hereditary non-polyposis colorectal cancer patients. *Nat. Med.* **2**: 169-174.

Livingstone, L.R., White, A., Sprouse, J., Livanos, E., Jacks, T., Tlsty, T.D. (1992). Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. *Cell* **70**: 923-935.

Loughran, O., Clark, L.J., Bond, J., Baker, A., Berry, I.J., Edington, K.G., Ly, I.S., Simmons, R., Haw, R., Black, D.M., Newbold, R.F., Parkinson, E.K. (1997)[b]. Evidence for the inactivation of multiple replicative lifespan genes in immortal human squamous cell carcinoma keratinocytes. *Oncogene* **14**: 1955-1964.

Loughran, O., Malliri, A., Owens, D., Gallimore, P.H., Stanley, M.A., Ozanne, B., Frame, M.C., Parkinson, E.K. (1996)[a]. Association of CDKN2A/p16INK4A with human head and neck keratinocyte replicative senescence: relationship of dysfunction to immortality and neoplasia. *Oncogene* **13**: 561-568.

Lowe, S.W., Ruley, H.E. (1993). Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. *Genes Dev.* **7**: 535-545.

Lowe, S.W., Schmitt, E.M., Smith, S.W., Osborne, B.A., Jacks, T. (1993). p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature* **362**: 847-849.

Luan, X., Shi, G., Zohouri, M., Paradee, W., Smith, D.I., Decker, H.J., Cannizzaro, L.A. (1997). The FHIT gene is alternatively spliced in normal kidneyand renal cell carcinoma. *Oncogene* **15**: 79-86.

Ludwig, R.L., Bates, S., Vousden, K.H. (1996). Differential activation of target cellular promoters by p53 mutants with impaired apoptotic function. *Mol. Cell. Biol.* **16**: 4952-4960.

Lugo, T.G., Pendergast, A.M., Muller, A.J., Witte, O.N. (1990). Tyrosine kinase activity and transformation potency of bcr-abl oncogene products. *Science* **247**: 1079-1082.

Lundblad, V., Blackburn, E.H. (1993). An alternative pathway for yeast telomere maintenance rescues est1- senescence. *Cell* **73**: 347-360.

Macoska, J.A., Trybus, T.M., Benson, P.D., Sakr, W.A., Grignon, D.J., Wojno, K.D., Pietruk, T., Powell, I.J. (1995). Evidence for three tumor suppressor gene loci on chromosome 8p in human prostate cancer. *Cancer Res.* **55**: 5390-5395.

Madden, S.L., Cook, D.M., Morris, J.F., Gashler, A., Sukhatme, V.P., Rauscher, F.J. (1991). Transcriptional repression mediated by the WT1 Wilms' tumor gene product. *Science* **253**: 1550-1553.

Maheswaran, S., Englert, C., Bennett, P., Heinrich, G., Haber, D.A. (1995). The WT1 gene product stabilizes p53 and inhibits p53-mediated apoptosis. *Genes Dev.* **9**: 2143-2156.

Malkin, D., Li, F.P., Strong, L.C., Fraumeni, J.F., Nelson, C.E., Kim, D.H., Kassel, J., Gryka, M.A., Bischoff, F.Z., Tainsky, M.A., *et al.* (1990). Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. *Science* **250**: 1233-1238.

Maniatis, T., Fritsch, E.F., Sambrook, J. (1989). Molecular Cloning: A Laboratory Manual (2nd Ed.). Cold Spring Harbour Laboratory Press.

Marcand, S., Buck, S.W., Moretti, P., Gilson, E., Shore, D. (1996). Silencing of genes at non telomeric sites in yeast is controlled by sequestration of silencing factors at telomeres by Rap 1 protein. *Genes Dev.* **10**: 1297-1309.

Marcus, J.N., Watson, P., Page, D.L., Narod, S.A., Lenoir, G.M., Tonin, P., Linder-Stephenson, L., Salerno, G., Conway, T.A., Lynch, H.T. (1996). Hereditary breast cancer: pathobiology, prognosis, and BRCA1 and BRCA2 gene linkage. *Cancer* **77**: 697-709.

Matsuda, T., Sasaki, M., Kato, H., Yamada, H., Cohen, M., Barrett, J.C., Oshimura, M., Wake, N. (1997). Human chromosome 7 carries a putative tumor suppressor gene(s) involved in choriocarcinoma. *Oncogene* **15**: 2773-2781.

Matsumoto, T., Imamura, O., Yamabe, Y., Kuromitsu, J., Tokutake, Y., Shimamoto, A., Suzuki, N., Satoh, M., Kitao, S., Ichikawa, K., Kataoka, H., Sugawara, K., Thomas, W., Mason, B., Tsuchihashi, Z., Drayna, D., Sugawara, M., Sugimoto, M., Furuichi, Y., Goto, M. (1997). Mutation and haplotype analyses of the Werner's syndrome gene based on its genomic structure: genetic epidemiology in the Japanese population. *Hum. Genet.* **100**: 123-130.

Matsuoka, S., Thompson, J.S., Edwards, M.C., Bartletta, J.M., Grundy, P., Kalikin, L.M., Harper, J.W., Elledge, S.J., Feinberg, A.P. (1996). Imprinting of the gene encoding a human cyclin-dependent kinase inhibitor, $p57^{KIP2}$, on chromosome 11p15. *Proc. Natl. Acad. Sci. U.S.A.* **93**: 3026-3030.

Maw, M.A., Grundy, P.E., Millow, L.J., Eccles, M.R., Dunn, R.S., Smith, P.J., Feinberg, A.P., Law, D.J., Paterson, M.C., Telzerow, P.E., *et al.* (1992). A third Wilms' tumor locus on chromosome 16q. *Cancer Res.* **52**: 3094-3098.

McKay, I.A., Paterson, H., Brown, R., Toksoz, D., Marshall, C.J., Hall, A. (1986). N-ras and human cancer. *Anticancer Res.* **6(3 Pt B)**: 483-490.

Mellon, I., Champe, G.N. (1996). Products of DNA mismatch repair genes mutS and mutL are required for transcription-coupled nucleotide-excision repair of the lactose operon in Escherichia coli. *Proc. Natl. Acad. Sci. U.S.A.* **93**: 1292-1297.

Mellon, I., Rajpal, D.K., Koi, M., Boland, C.R., Champe, G.N. (1996). Transcription-coupled repair deficiency and mutations in human mismatch repair genes. *Science* **272**: 557-560.

Merajver, S.D., Pham, T.M., Caduff, R.F., Chen, M., Poy, E.L., Cooney, K.A., Weber, B.L.O., Collins, F.S., Johnston, C., Frank, T.S. (1995). Somatic mutations in the BRCA1 gene in sporadic ovarian tumours. *Nat. Genet.* **9**: 439-443.

Merlo, A., Herman, J.G., Mao, L., Lee, D.J., Gabrielson, E., Burger, P.C., Baylin, S.B., Sidransky, D. (1995). 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. *Nat. Med.* **1**: 686-692.

Metcalfe, J.A., Parkhill, J., Campbell, L., Stacey, M., Biggs, P., Byrd, P.J., Taylor, A.M. (1996). Accelerated telomere shortening in ataxia telangiectasia. *Nat. Genet.* **13**: 350-353.

Meyerson, M., Counter, C.M., Eaton, E.N., Ellisen, L.W., Steiner, P., Caddle, S.D., Ziaugra, L., Beijersbergen, R.L., Davidoff, M.J., Liu, Q., Bacchetti, S., Haber, D.A., Weinberg, R.A. (1997). hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. *Cell* **90**: 785-795.

Miki, T., Smith, C.L., Long, J.E., Eva, A., Fleming, T.P. Oncogene ect2 is related to regulators of small GTP-binding proteins. *Nature* **362**: 462-465.

Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P.A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L.M., Ding, W., *et al.* (1994). A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* **266**: 66-71.

Minetti, C., Sotgia, F., Bruno, C., Scartezzini, P., Broda, P., Bado, M., Masetti, E., Mazzocco, M., Egeo, A., Donati, M.A., Volonte, D., Galbiati, F., Cordone, G., Bricarelli, F.D., Lisanti, M.P., Zara, F. (1998). Mutations in the caveolin-3 gene cause autosomal dominant limb-girdle muscular dystrophy. *Nat. Genet.* **18**: 365-368.

Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O., Clevers, H. (1996). XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos. *Cell* **86**: 391-399.

Moll, U.M., Ostermeyer, A.G., Haladay, R., Winkfield, B., Frazier, M., Zambetti, G. (1996). Cytoplasmic sequestration of wild-type p53 protein impairs the G1 checkpoint after DNA damage. *Mol. cell. biol.* **16**: 1126-1137.

Monier, S., Parton, R.G., Vogel, F., Behlke, J., Henske, A., Kurzchalia, T.V. (1995). VIP21-caveolin, a membrane protein constituent of the caveolar coat, oligomerizes in vivo and in vitro. *Mol. Biol. Cell* **6**: 911-927.

Morgenbesser, S.D., Williams, B.O., Jacks, T., DePinho, R.A. (1994). p53-dependent apoptosis produced by Rb-deficiency in the developing mouse lens. *Nature* **371**: 72-74.

Morin, P.J., Sparks, A.B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., Kinzler, K.W. (1997). Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science* **275**: 1787-1790.

Moslein, G., Tester, D.J., Lindor, N.M., Honchel, R., Cunningham, J.M., French, A.J., Halling, K.C., Schwab, M., Goretzki, P., Thibodeau, S.N. (1996). Microsatellite instability and mutation analysis of hMSH2 and hMLH1 in patients with sporadic, familial and hereditary colorectal cancer. *Hum. Mol. Genet.* **5**: 1245-1252.

Mosner, J., Mummenbrauer, T., Bauer, C., Sczakiel, G., Grosse, F., Deppert, W. (1995). Negative feedback regulation of wild-type p53 biosynthesis. *EMBO J.* **14**: 4442-4449.

Motokura, T., Bloom, T., Kim, H.G., Juppner, H., Ruderman, J.V., Kronenberg, H.M., Arnold, A. (1991). A novel cyclin encoded by a bcl1-linked candidate oncogene. *Nature* **350**: 512-515.

Muchmore, S.W., Sattler, M., Liang, H., Meadows, R.P., Harlan, J.E., Yoon, H.S., Nettesheim, D.,Chang, B.S., Thompson, C.B., Wong, S.L., Ng, S.L., Fesik, S.W. (1996). X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. *Nature* **381**: 335-341.

Muggleton-Harris, A.L., DeSimone, D.W. (1980). Replicative potentials of various fusion products between WI-38 and SV40 transformed WI-38 cells and their components. *Somatic Cell Genet.* **6**: 689-698.

Mulligan, L.M., Kwok, J.B., Healey, C.S., Elsdon, M.J., Eng, C., Gardner, E., Love, D.R., Mole, S.E., Moore, J.K., Papi, L., *et al.* (1993). Germ-line mutations of the *RET* proto-oncogene in multiple endocrine neoplasia type 2A. *Nature* **363**: 458-460.

Mummenbrauer, T., Janus, F., Muller, B., Wiesmuller, L., Deppert, W., Grosse, F. (1996). p53 protein exhibits 3'-to-5' exonuclease activity. *Cell* **85**: 1089-1099.

Murata, M., Peranen, J., Schreiner, R., Wieland, F., Kurzchalia, T.V., Simons, K. (1995). VIP21/caveolin is a cholesterol-binding protein. *Proc. Natl. Acad. Sci. U.S.A.* **92**: 10339-10343.

Murnane, J.P., Sabatier, L., Marder, B.A., Morgan, W.F. (1994). Telomere dynamics in an immortal human cell line. *EMBO J.* **13**: 4953-4962.

Murray, J.C., Buetow, K.H., Weber, J.L., Ludwigsen, S., Scherpbier-Heddema, T., Manion, F., Quillen, J., Sheffield, V.C., Sunden, S., Duyk, G.M., *et al.* (1994). A comprehensive human linkage map with centimorgan density. Cooperative Human Linkage Center (CHLC). *Science* **265**: 2049-2054.

Myers, M.P., Stolarov, J.P., Eng, C., Li, J., Wang, S.I., Wigler, M.H., Parsons, R., Tonks, N.K. (1997). P-TEN, the tumor suppressor from human chromosome10q23, is a dual-specificity phosphatase. *Proc. Natl. Acad. Sci. U.S.A.* **94**: 9052-9057.

Nagai, M.A., Pacheco, M.M., Brentani, M.M., Marques, L.A., Brentani, R.R., Ponder, B.A., Mulligan, L.M. (1994). Allelic loss on distal chromosome 17p is associated with poor prognosis in a group of Brazilian breast cancer patients. *Br. J. Cancer* **69**: 754-758.

Nagase, H., Bryson, S., Cordell, H., Kemp, C.J., Fee, F., Balmain, A. (1995). Distinct genetic loci control development of benign and malignant skin tumours in mice. *Nat. Genet.* **10**: 424-429.

Nakabayashi, K., Ogata, T., Fujii, H, Tahara, H., Ide, T., Wadhwa, R., Kaul, S.C., Mitsui, Y., Ayusawa, D. (1997). Decrease in amplified telomeric sequences and induction of senescence markers by introduction of human chromosome 7 or its segments in SUSM-1. *Experimental Cell Res.* **235**: 345-353.

Nakamura, T.M., Morin, G.B., Chapman, K.B., Weinrich, S.L., Andrews, W.H., Lingner, J., Harley, C.B., Cech, T.R. (1997). Telomerase catalytic subunit homologs from fission yeast and human. *Science* **277**: 955-959.

Nakayama, K., Ishida, N., Shirane, M., Inomata, A., Inoue, T., Shishido, N., Horii, I., Loh, D.Y., Nakayama, K. (1996). Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell* **85**: 707-720.

Nau, M.M., Brooks, B.J., Battey, J., Sausville, E., Gazdar, A.F., Kirsch, I.R., McBride, O.W., Bertness, V., Hollis, G.F., Minna, J.D. (1985). L-myc, a new myc-related gene amplified and expressed in human small cell lung cancer. *Nature* **318**: 69-73.

Nelson, D.L., Ledbetter, S.A., Corbo, L., Victoria, M.F., Ramirez-Solis, R., Webster, T.D., Ledbetter, D.H., Caskey, C.T. (1989). Alu polymerase chain reaction: a method for rapid isolation of human-specific sequences from complex DNAsources. *Proc. Natl. Acad. Sci. U.S.A.* **86**: 6686-6690.

Nelson, S.F.,. McCusker, J.H., Sander, M.A., Kee, Y., Modrich, P., Brown, P.O. (1993). Genomic mismatch scanning: a new approach to genetic linkage mapping. *Nat. Genet.* **4**: 11-18.

Niederacher, D., Picard, F., van Roeyen, C., An, H.X., Bender, H.G., Beckmann, M.W. (1997). Patterns of allelic loss on chromosome 17 in sporadic breast carcinomas detected by fluorescent-labeled microsatellite analysis. *Genes Chromosomes Cancer* **18**: 181-192.

Nigro, J.M., Baker, S.J., Preisinger, A.C., Jessup, J.M., Hostetter, R., Cleary, K., Bigner, S.H., Davidson, N., Baylin, S., Devilee, P., *et al.* (1989). Mutations in the p53 gene occur in diverse human tumour types. *Nature* **342**: 705-708.

Ning, Y., Weber, J.L., Killary, A.M., Ledbetter, D.H., Smith, J.R., Pereira-Smith, O.M. (1991). Genetic analysis of indefinite division in human cells: evidence for a cell senescence-related gene(s) on human chromosome 4. *Proc. Natl. Acad. Sci. U.S.A.* **88**: 5635-5639.

Nishisho, I., Nakamura, Y., Miyoshi, Y., Miki, Y., Ando, H., Horii, A., Koyama, K., Utsunomiya, J., Baba, S., Hedge, P. (1991). Mutations of chromosome 5q21 genes in FAP and colorectal cancer patients. *Science* **253**: 665-669.

Nizetic, D., Monard, S., Young, B., Cotter, F., Zehetner, G., Lehrach, H. (1994). Construction of cosmid libraries from flow-sorted human chromosomes 1, 6, 7, 11, 13, and 18 for reference library resources. *Mamm. Genome* **5**: 801-802.

Nobes, C.D., Hall, A. (1995). Rho, rac and cdc42 regulate the assembly of multi-molecular focal complexes associated with actin stress fibres, lamellipodia and filopodia. *Cell* **81**: 53-62.

Noble, J.R., Rogan, E.M., Neumann, A.A., Maclean, K., Bryan, T.M., Reddel, R.R. (1996). Association of extended in vitro proliferative potentialwith loss of p16INK4 expression. *Oncogene* **13**: 1259-1268.

Noda, A., Ning, Y., Venable, S.F., Pereira-Smith, O.M., Smith, J.R. (1994). Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. *Exp. Cell. Res.* **211**: 90-98.

Northrup, H., Rosenbloom, C., O'Brien, W.E., Beaudet, A.L. (1989). Additional polymorphism for D7S8 linked to cystic fibrosis including detection by DNA amplification. *Nucleic Acids Res.* **17**: 1784.

Nowell, P.C., Hungerford, D.A. (1960). A minute chromosome in human granulocytic leukaemia. *Science* **132**: 1497.

Nunes, V., Chillon, M., Lench, N., Ramsay, M., Estivill, X. (1990). PCR detection of the pKM.19/ScrfI RFLP (D7S23), a marker closely linked to the cystic fibrosis mutation. *Nucleic Acids Res.* **18**: 4957.

Nunez, G., London, L., Hockenbery, D., Alexander, M., McKearn, J.P., Korsmeyer, S.J. (1990). Deregulated Bcl-2 gene expression selectively prolongs survival of growth factor-deprived haemopoietic cell lines. *J. Immunol.* **144**: 3602-3610.

Nusse, R., Varmus, H.E. (1982). Many tumours induced by the mouse mammary tumour virus contain a provirus integrated in the same region of the host genome. *Cell* **31**: 99-109.

Obata, M., Lee, G.H., Kanda, H., Kitagawa, T., Ogawa, K. (1997) Loss of heterozygosity at loci on chromosome 4, a common genetic event during the spontaneous immortalization of mouse embryonic fibroblasts. *Mol. Carcinog.* **19**: 17-24.

Ogata, T., Ayusawa, D., Namba, M., Takahashi, E., Oshimura, M., Oishi, M. (1993). Chromosome 7 suppresses indefinite division of nontumorigenic immortalized human fibroblast cell lines KMST-6 and SUSM-1. *Mol. Cell. Biol.* **13**: 6036-6043.

Ogata, T., Oshimura, M., Namba, M., Fujii, M., Oishi, M., Ayusawa, D. (1995). Genetic complementation of the immortal phenotype in group D cell lines by introduction of chromosome 7. *Jpn. J. Cancer Res.* **86**: 35-40.

Ogawa, O., Eccles, M.R., Szeto, J., McNoe, L.A., Yun, K., Maw, M.A., Smith, P.J., Reeve, A.E. (1993). Relaxation of insulin-like growth factor II gene imprinting implicated in Wilms' tumour. *Nature* **362**: 749-751.

Ohmura, H., Tahara, H., Suzuki, M., Ide, T., Shimizu, M., Yoshida, M.A., Tahara, E., Shay, J.W., Barrett, J.C., Oshimura, M. (1995). Restoration of the cellular senescence program and repression of telomerase by human chromosome 3. *Jpn. J. Cancer Res.* **86**: 899-904.

Ohta, M., Inoue, H., Cotticelli, M.G., Kastury, K., Baffa, R., Palazzo, J., Siprashvili, Z., Mori, M., McCue, P., Druck, T., *et al.* (1996). The FHIT gene, spanning the chromosome 3p14.2 fragile site and renal carcinoma-associated t(3;8) breakpoint, is abnormal in digestive tract cancers. *Cell* **84**: 587-597.

Olovnikov, A.M. (1973). A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. J. *Theor. Biol.* **41**: 181-190.

Oltavi, Z.N., Korsmeyer, S.J. (1994). Checkpoints of duelling dimers foil death wishes. *Cell* **79**:189-194.
Oro, A.E., Higgins, K.M., Hu, Z., Bonifas, J.M., Epstein, E.H., Scott, M.P. (1997). Basal cell carcinomas in mice overexpressing sonic hedgehog. *Science* **276**: 817-821.

Oudet, C., Hanauer, A., Clemens, P., Caskey, T., Mandel, J.L. (1992). Two hot spots of recombination in the DMD genecorrelate with the deletion prone regions. *Hum. Mol. Genet.* **1**: 599-603.

Panagopoulos, I., Thelin, S., Mertens, F., Mitelman, F., Aman, P. (1997). Variable FHIT transcripts in non-neoplastic tissues. *Genes Chromosomes Cancer* **19**: 215-219.

Pardee, A.B. (1989). G1 events and regulation of cell proliferation. *Science* **246**: 603-608.

Pereira-Smith, O.M., Smith, J.R. (1981). Expression of SV40 T antigen in finite life-span hybrids of normal and SV40-transformed fibroblasts. *Somatic Cell Genet.* 7: 411-421.

Pereira-Smith, O.M., Smith, J.R. (1983). Evidence for the recessive nature of cellular immortality. *Science* **221**: 964-966.

Pereira-Smith, O.M., Smith, J.R. (1988). Genetic analysis of indefinite division in human cells:identification of four complementation groups. *Proc. Natl. Acad. Sci. U.S.A.* **85**: 6042-6046.

Phelan, C.M., Rebbeck, T.R., Weber, B.L., Devilee, P., Ruttledge, M.H., Lynch, H.T., Lenoir, G.M., Stratton, M.R., Easton, D.F., Ponder, B.A., Cannon-Albright, L., Larsson, C., Goldgar, D.E., Narod, S.A. (1996).Ovarian cancer risk in BRCA1 carriers is modified by the HRAS1 variable number of tandem repeat (VNTR) locus. *Nat. Genet.* **12**: 309-311.

Ping, A.J., Reeve, A.E., Law, D.J., Young, M.R., Boehnke, M., Feinberg, A.P. (1989). Genetic linkage of Beckwith-Wiedemann syndrome to11p15. *Am. J. Hum. Genet.* **44**: 720-723

Polakis, P. (1997) The adenomatous polyposis coli (APC) tumour suppressor. *Biochim. Biophys. Acta* **1332**: 127-147.

Pritchard-Jones, K., Fleming, S., Davidson, D., Bickmore, W., Porteous, D., Gosden, C., Bard, J., Buckler, A., Pelletier, J., Housman, D., *et al.* (1990). The candidate Wilms' tumour gene is involved ing enitourinary development. *Nature* **346**: 194-197.

Prochownik, E.V., Kukowska, J. (1986). Deregulated expression of c-myc by murine erythroleukaemia cells prevents differentiation. *Nature* **322**: 848-850.

Qin, X.Q., Livingston, D.M., Kaelin, W.G., Adams, P.D. (1994). Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. *Proc. Natl. Acad. Sci. U.S.A.* **91**: 10918-10922.

Qiu, R.G., Chen, J., Kirn, D., McCormick, F., Symons, M. (1995) An essential role for Rac in Ras transformation. *Nature* **374**: 457-459.

Radna, R.L., Caton, Y., Jha, K.K., Kaplan, P., Li, G., Traganos, F., Ozer, H.L. (1989). Growth of immortal simian virus 40 tsA-transformed human fibroblasts is temperature dependent. *Mol. Cell. Biol.* **9**: 3093-3096.

Rahman, N., Arbour, L., Tonin, P., Baruchel, S., Pritchard-Jones, K., Narod, S.A., Stratton, M.R. (1997). The familial Wilms' tumour susceptibility gene, FWT1,may not be a tumour suppressor gene. *Oncogene* **14**: 3099-3102.

Rahman, N., Arbour, L., Tonin, P., Renshaw, J., Pelletier, J., Baruchel, S., Pritchard-Jones, K., Stratton, M.R., Narod, S.A. (1996). Evidence for a familial Wilms' tumour gene (FWT1) on chromosome 17q12-q21. *Nat. Genet.* **13**: 461-463.

Rajan, J.V., Wang, M., Marquis, S.T., Chodosh, L.A. (1996). Brca2 is coordinately regulated with Brca1 during proliferation and differentiation in mammary epithelial cells. *Proc. Natl. Acad. Sci. U.S.A.* **93**: 13078-13083.

Rauscher, F.J., Morris, J.F., Tournay, O.E., Cook, D.M., Curran, T. (1990). Binding of the Wilms' tumor locus zinc finger protein to the EGR-1 consensus sequence. *Science* **250**: 1259-1262.

Reed, A.L., Califano, J., Cairns, P., Westra, W.H., Jones, R.M., Koch, W., Ahrendt, S., Eby, Y., Sewell, D., Nawroz, H., Bartek, J., Sidransky, D. (1996). High frequency of p16 (CDKN2/MTS-1/INK4A) inactivation in head and neck squamous cell carcinoma. *Cancer Res.* **56**: 3630-3633.

Reese, M.G., Harris, N.L. and Eeckman, F.H. Large Scale Sequencing: Specific Neural Networks for Promoter and Splice Site Recognition. Proceedings of the Pacific Symposium on Biocomputing.Editor: Larry Hunter and Terri Klein, Kona, Hawaii, 1996.

Reeve, A.E., Sih, S.A., Raizis, A.M., Feinberg, A.P. (1989). Loss of allelic heterozygosity at a second locus on chromosome 11 in sporadic Wilms' tumor cells. *Mol. Cell. Biol.* **9**: 1799-1803.

Reiter, L.T., Murakami, T., Koeuth, T., Pentao, L., Muzny, D.M., Gibbs, R.A., Lupski, J.R. (1996). A recombination hotspot responsible for two inherited peripheral neuropathies is located near a mariner transposon-like element. *Nat. Genet.* **12**: 288-297.

Reznikoff, C.A., Yeager, T.R., Belair, C.D., Savelieva, E., Puthenveettil, J.A., Stadler, W.M. (1996). Elevated p16 at senescence and loss of p16 at immortalization in human papillomavirus 16 E6, but not E7, transformed human uroepithelial cells. *Cancer Res.* **56**: 2886-2890.

Rheinwald, J.G., Green, H. (1975). Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* **6**: 331-343.

Richards, B., Reeves, C., Horn, G.T. (1991). Dinucleotide repeat polymorphism closely linked to the cystic fibrosis (CFTR) gene. *Nucleic Acids Res.* **19**: 5798.

Ridley, A.J. Hall, A. (1992). The small GTP-binding protein rho regulates the assembly of focl adhesions and actin stress fibres in response to growth factors. *Cell* **70**: 389-399.

Ridley, A.J., Paterson, H.F., Johnston, C.L., Diekmann, D., Hall, A. (1992). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* **70**: 401-410.

Rittling, S.R., Brooks, K.M., Cristofalo, V.J., Baserga, R. (1986). Expression of cell cycle-dependent genes in young and senescent WI-38 fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.* **83**: 3316-3320.

Rodenhiser, D., Chakraborty, P., Andrews, J., Ainsworth, P., Mancini, D., Lopes, E., Singh, S. (1996). Heterogenous point mutations in the BRCA1 breast cancer susceptibility gene occur in high frequency at the site of homonucleotide tracts, short repeats and methylatable CpG/CpNpG motifs. *Oncogene* **12**: 2623-2629.

Rogan, E.M., Bryan, T.M., Hukku, B., Maclean, K., Chang, A.C., Moy, E.L., Englezou, A.,Warneford, S.G., Dalla-Pozza, L., Reddel, R.R. (1995). Alterations in p53 and p16INK4 expression and telomere length during spontaneous immortalization of Li-Fraumeni syndrome fibroblasts. *Mol. Cell. Biol.* **15**: 4745-4753.

Rommens, J.M., Iannuzzi, M.C., Kerem, B., Drumm, M.L., Melmer, G., Dean, M., Rozmahel, R., Cole, J.L., Kennedy, D., Hidaka, N., *et al.* (1989). Identification of the cystic fibrosis gene: chromosome walking and jumping. *Science* **245**: 1059-1065.

Rothberg, K.G., Heuser, J.E., Donzell, W.C., Ying, Y.S., Glenney, J.R., Anderson, R.G. (1992). Caveolin, a protein component of caveolae membrane coats. *Cell* **68**: 673-682.

Rouleau, G.A., Merel, P., Lutchman, M., Sanson, M., Zucman, J., Marineau, C., Hoang-Xuan, K., Demczuk, S., Desmaze, C., Plougastel, B., *et al.* (1993). Alteration in a new gene encoding a putative membrane-organizing protein causes neuro-fibromatosistype 2. *Nature* **363**: 515-521.

Rubin, S.C., Benjamin, I., Behbakht, K., Takahashi, H., Morgan, M.A., LiVolsi, V.A., Berchuck, A., Muto, M.G., Garber, J.E., Weber, B.L., Lynch, H.T., Boyd, J. (1996). Clinical and pathological features of ovarian cancer in women with germ-line mutations of BRCA1. *N. Engl. J. Med.* **335**: 1413-1416.

Rubinfeld, B., Robbins, P., El-Gamil, M., Albert, I., Porfiri, E., Polakis, P. (1997). Stabilization of beta-catenin by genetic defects in melanoma cell lines. *Science* **275**: 1790-1792.

Ruffner, H., Verma ,I.M. (1997). BRCA1 is a cell cycle-regulated nuclear phosphoprotein. *Proc. Natl. Acad. Sci. U.S.A.* **94**: 7138-7143.

Ruoslahti, E., Reed, J.C. (1994). Anchorage dependence, integrins, and apoptosis. *Cell* **77**: 477-478.

Sandell, L.L., Zakian, V.A. (1993). Loss of a yeast telomere: arrest, recovery, and chromosome loss. *Cell* **75**: 729-739.

Sandhu, A.K., Hubbard, K., Kaur, G.P., Jha, K.K., Ozer, H.L., Athwal, R.S. (1994). Senescence of immortal human fibroblasts by the introduction of normal human chromosome 6. *Proc. Natl. Acad. Sci. U.S.A.* **91**: 5498-5502.

Sager, R., Sheng, S., Anisowicz, A., Sotiropoulou, G., Zou, Z., Stenman, G., Swisshelm, K., Chen, Z., Hendrix, M.J., Pemberton, P., *et al.* (1994). RNA genetics of breast cancer: maspin as paradigm. *Cold Spring Harb. Symp. Quant. Biol.* **59**:537-546.

Santos, E., Tronick, S.R., Aaronson, S.A., Pulciani, S., Barbacid, M. (1982). T24 human bladder carcinoma oncogene is an activated form of the normal human homologue of BALB- and Harvey-MSV transforming genes. *Nature* **298**: 343-347.

Sargiacomo, M., Scherer, P.E., Tang, Z., Kubler, E., Song, K.S., Sanders, M.C., Lisanti, M.P. (1995). Oligomeric structure of caveolin: implications for caveolae membrane organization. *Proc. Natl. Acad. Sci. USA* **92**: 9407-9411.

Savitsky, K., Bar-Shira, A., Gilad, S., Rotman, G., Ziv, Y., Vanagaite, L., Tagle, D.A., Smith, S., Uziel, T., Sfez, S., *et al.* (1995). A single ataxia telangiectasia gene with a product similar to PI-3 kinase. *Science* **268**: 1749-1753.

Schaeffer, L., Roy, R., Humbert, S., Moncollin, V., Vermeulen, W., Hoeijmakers, J.H., Chambon, P., Egly, J.M. DNA repair helicase: a component of BTF2 (TFIIH) basic transcription factor. *Science* **260**: 58-63.

Scherer, P.E., Lisanti, M.P., Baldini, G., Sargiacomo, M., Mastick, C.C., Lodish, H.F. (1994). Induction of caveolin during adipogenesis and association of GLUT4 with caveolin-rich vesicles. *J. Cell. Biol.* **127**: 1233-1243.

Scherer, P.E., Okamoto, T.,. Chun, M., Nishimoto, I., Lodish, H.F., Lisanti, M.P. (1996). Identification, sequence, and expression of caveolin-2 defines a caveolin gene family. *Proc. Natl. Acad. Sci. U.S.A.*. **93**: 131-135.

Schmidt, L., Duh, F.M., Chen, F., Kishida, T., Glenn, G., Choyke, P., Scherer, S.W., Zhuang, Z., Lubensky, I., Dean, M., Allikmets, R., Chidambaram, A., Bergerheim, U.R., Feltis, J.T., Casadevall, C., Zamarron, A., Bernues, M., Richard, S., Lips, C.J., Walther, M.M., Tsui, L.C., Geil, L., Orcutt, M.L., Stackhouse, T., Zbar, B. *et al.* (1997). Germline and somatic mutations in the tyrosine kinase domain of the *MET* proto-oncogene in papillary renal carcinomas. *Nat. Genet.* **16**: 68-73.

Shridhar, V., Sun, Q.C., Miller, O.J., Kalemkerian, G.P., Petros, J., Smith, D.I. (1997). Loss of heterozygosity on the long arm of human chromosome 7 in sporadic renal cell carcinomas. *Oncogene* **15**: 2727-2733.

Scherer, P.E., Tang, Z., Chun, M., Sargiacomo, M., Lodish, H.F., Lisanti, M.P. (1995) Caveolin isoforms differ in their N-terminal protein sequence and subcellular distribution. Identification and epitope mapping of an isoform-specific monoclonal antibody probe. *J. Biol. Chem.* **270**: 16395-16401.

Schuler, G.D., Boguski, M.S., Stewart, E.A., Stein, L.D., Gyapay, G., Rice, K., White, R.E., Rodriguez-Tome, P., Aggarwal, A., Bajorek, E., Bentolila, S., Birren, B.B., Butler,

A., Castle, A.B., Chiannilkulchai, N., Chu, A., Clee, C., Cowles, S., Day, P.J., Dibling, T., Drouot, N., Dunham, I., Duprat, S., East, C., Hudson, T.J., *et al.* A gene map of the human genome. *Science* **274**: 540-546.

Schutte, M., da Costa, L.T., Hahn, S.A., Moskaluk, C., Hoque, A.T., Rozenblum, E., Weinstein, C.L., Bittner, M., Meltzer, P.S., Trent, J.M., *et al.* (1995). Identification by representational difference analysis of a homozygous deletion in pancreatic carcinoma that lies within the BRCA2 region. *Proc. Natl. Acad. Sci. U.S.A.* 92: 5950-5954.

Schwab, M., Alitalo, K., Klempnauer, K.H., Varmus, H.E., Bishop, J.M., Gilbert, F., Brodeur, G., Goldstein, M., Trent, J. (1983). Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. *Nature* **305**: 245-248.

Scully, R., Chen, J., Ochs, R.L., Keegan, K., Hoekstra, M., Feunteun, J., Livingston, D.M. (1997). Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage. *Cell* **90**: 425-435.

Scully, R., Chen, J., Plug, A., Xiao, Y., Weaver, D., Feunteun, J., Ashley, T., Livingston, D.M. (1997)[a]. Association of BRCA1 with Rad51 in mitotic and meiotic cells. *Cell* **88**: 265-275.

Seitz, S., Rohde, K., Bender, E., Nothnagel, A., Kolble, K., Schlag, P.M., Scherneck, S. (1997). Strong indication for a breast cancer susceptibility gene on chromosome 8p12-p22: linkage analysis in German breast cancer families. *Oncogene* **14**: 741-743.

Serrano, M., Lee, H., Chin, L., Cordon-Cardo, C., Beach, D., DePinho, R.A. (1996). Role of the INK4a locus in tumor suppression and cell mortality. *Cell* **85**: 27-37.

Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D., Lowe, S.W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* **88**: 593-602.

Seshadri, T., Campisi, J. (1990). Repression of c-fos transcription and an altered genetic program in senescent human fibroblasts. *Science* **247**: 205-209.

Shan, B., Durfee, T., Lee, W.H. (1996). Disruption of RB/E2F-1 interaction by single point mutations in E2F-1 enhances S-phase entry and apoptosis. *Proc. Natl. Acad. Sci. U.S.A.* **93**: 679-684.

Shan, B., Lee, W.H. (1994). Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis. *Mol. Cell. Biol.* **14**: 8166-8173.

Sharan, S.K., Morimatsu, M., Albrecht, U., Lim, D.S., Regel, E., Dinh, C., Sands, A., Eichele, G., Hasty, P., Bradley, A. (1997). Embryonic lethality and radiation hypersensitivity mediated by Rad51 in mice lacking Brca2. *Nature* **386**: 804-810.

Shay, J.W., Wright, W.E. (1996). Telomerase activity in human cancer. *Curr. Opin. Oncol.* **8**: 66-71.

Shay, J.W., Wright, W.E., Brasiskyte, D., Van, der Haegen, B.A. (1993). E6 of human papillomavirus type 16 can overcome the M1 stage of immortalization in human mammary epithelialcells but not in human fibroblasts. *Oncogene* **8**: 1407-1413.

Sheffield, V.C., Weber, J.L., Buetow, K.H., Murray, J.C., Even, D.A., Wiles, K., Gastier, J.M., Pulido, J.C., Yandava, C., Sunden, S.L., *et al.* (1995). A collection of tri- and tetranucleotide repeat markers used to generate high quality, high resolution human genome-wide linkage maps. *Hum. Mol. Genet.* **4**: 1837-1844.

Sherr, C.J. (1993). Mammalian G1 cyclins. *Cell* **73**: 1059-1065.

Sherr, C.J. (1994). G1 phase progression: cycling on cue. *Cell* **79**: 551-555.

Sherr, C.J. (1996). Cancer cell cycles. *Science* **274**: 1672-1677.

Sherr, C.J. and Roberts, J.M. (1995). Inhibitors of mammalian G1 cyclin-dependent kinases. *Genes Dev.* **9**: 1149-1163.

Shih, C., Padhy, L.C., Murray, M., Weinberg, R.A. (1981). Transforming genes of carcinomas and neuroblastomas introduced into mouse fibroblasts. *Nature* **290**: 261-264.

Siegel, E.C. and Bryson, V. (1967) Mutator gene of *Eschericia coli B. J Bacteriol* **94**: 38-47.

Siemeister, G., Weindel, K., Mohrs, K., Barleon, B., Martiny-Baron, G., Marme, D. (1996). Reversion of deregulated expression of vascular endothelial growth factor in human renal carcinoma cells by von Hippel-Lindau tumor suppressor protein. *Cancer Res.* **56**: 2299-2301.

Smith, G., Stanley, L.A., Sim, E., Strange, R.C., Wolf, C.R. (1995). Metabolic polymorphisms and cancer susceptibility. *Cancer Surv.* **25**: 27-65.

Smith, J.R., Freije, D., Carpten, J.D., Gronberg, H., Xu, J., Isaacs, S.D., Brownstein, M.J., Bova, G.S., Guo, H., Bujnovszky, P., Nusskern, D.R., Damber, J.E., Bergh, A., Emanuelsson, M., Kallioniemi, O.P., Walker-Daniels, J., Bailey-Wilson, J.E., Beaty, T.H., Meyers, D.A., Walsh, P.C., Collins, F.S., Trent, J.M., Isaacs, W.B. Major susceptibility locus for prostate cancer on chromosome 1 suggested by a genome-wide search. *Science* **274**: 1371-1374.

Soder, A.I., Hoare, S.F., Muir, S., Going, J.J., Parkinson, E.K., Keith, W.N. (1997). Amplification, increased dosage and in situ expression of the telomerase RNA gene in human cancer. *Oncogene* **14**: 1013-1021.

Somasundaram, K., Zhang, H., Zeng, Y.X., Houvras, Y., Peng, Y., Zhang, H., Wu, G.S., Licht, J.D., Weber, B.L., El-Deiry, W.S. (1997). Arrest of the cell cycle by the tumour-suppressor BRCA1 requires the CDK-inhibitor p21WAF1/CiP1. *Nature* **389**: 187-190.

Song, S.K., Li Shengwen, Okamoto, T., Quilliam, L.A., Sargiacomo, M., Lisanti, M.P. (1996). Co-purification and direct interaction of Ras with caveolin, an integral

membrane protein of caveolae microdomains. Detergent-free purification of caveolae microdomains. *J. Biol. Chem.* **271**: 9690-9697.

Srivastava, S., Zou, Z.Q., Pirollo, K., Blattner, W., Chang, E.H. (1990). Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. *Nature* **348**: 747-749.

Stanbridge, E.J. (1976). Suppression of malignancy in human cells. *Nature* **260**: 17-20.

Stanbridge, E.J. (1988). Genetic analysis of human malignancy using somatic cell hybrids and monochromosome transfer. *Cancer Surv.* **7**: 317-324.

Steck, P.A., Pershouse, M.A., Jasser, S.A., Yung, W.K., Lin, H., Ligon, A.H., Langford, L.A., Baumgard, M.L., Hattier, T., Davis, T., Frye, C., Hu, R., Swedlund, B., Teng, D.H., Tavtigian, S.V. (1997). Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat. Genet.* **15**: 356-362.

Steeg, P.S., Bevilacqua, G., Kopper, L., Thorgeirsson, U.P., Talmadge, J.E., Liotta, L.A., Sobel, M.E. (1988). Evidence for a novel gene associated with low tumor metastatic potential. *J. Natl. Cancer Inst.* **80**: 200-204.

Stehelin, D., Varmus, H.E., Bishop, J.M., Vogt, P.K. (1976). DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA *Nature* **260**: 170-173.

Stein, G.H., Beeson, M., Gordon, L. (1990). Failure to phosphorylate the retinoblastoma gene product in senescent human fibroblasts. *Science* **249**: 666-669.

Steiner, P, Barnes D.M., Harris, W.H., Weinberg, R.A. (1997). Absence of rearrangements in the tumour susceptibility gene TSG101 in human breast cancer. *Nat. Genet.* **16**: 332-333.

Stone, D.M., Hynes, M., Armanini, M., Swanson, T.A., Gu, Q., Johnson, R.L., Scott, M.P., Pennica, D., Goddard, A., Phillips, H., Noll, M., Hooper, J.E., de Sauvage, F., Rosenthal, A. (1996). The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog. *Nature* **384**: 129-134.

Sturzbecher, H.W., Donzelmann, B., Henning, W., Knippschild, U., Buchhop, S. (1996). p53 is linked directly to homologous recombination processes via RAD51/RecA protein interaction. *EMBO J.* **15**: 1992-2002.

Sun, X., Shimizu, H., Yamamoto, K. (1995). Identification of a novel p53 promoter element involved in genotoxic stress-inducible p53 gene expression. *Mol. Cell. Biol.* **15**: 4489-4496.

Suzuki, H., Emi, M., Komiya, A., Fujiwara, Y., Yatani, R., Nakamura, Y., Shimazaki, J. (1995). Localization of a tumor suppressor gene associated with progression of human prostate cancer within a 1.2 Mb region of 8p22-p21.3. *Genes Chromosomes Cancer* **13**: 168-174.

Tabin, C.J., Bradley, S.M., Bargmann, C.I., Weinberg, R.A., Papageorge, A.G., Scolnick, E.M., Dhar, R., Lowy, D.R., Chang, E.H. (1982). Mechanism of activation of a human oncogene. *Nature* **300**: 143-149.

Takahashi, S., Shan, A.L., Ritland, S.R., Delacey, K.A., Bostwick, D.G., Lieber, M.M., Thibodeau, S.N., Jenkins, R.B. (1995). Frequent loss of heterozygosity at 7q31.1 in primary prostate cancer is associated with tumor aggressiveness and progression. *Cancer Res.* **55**: 4114-4119.

Takaku, K., Oshima, M., Miyoshi, H., Matsui, M., Seldin, M.F., Taketo, M.M. (1998). Intestinal tumorigenesis in compound mutant mice of both Dpc4 (Smad4) and Apc genes. *Cell* 92: 645-656.

Takeshima, H., Izawa, I., Lee, P.S., Safdar, N., Levin, V.A., Saya, H. (1994). Detection of cellular proteins that interact with the NF2 tumor suppressor gene product. *Oncogene* **9**: 2135-2144.

Tang, Z., Scherer, P.E., Okamoto, T., Song, K., Chu, C., Kohtz, D.S., Nishimoto, I., Lodish, H.F., Lisanti, M.P. Molecular cloning of caveolin-3, a novel member of the caveolin gene family expressed predominantly in muscle. *J. Biol. Chem.* **271**: 2255-2261.

Taparowsky, E., Suard, Y., Fasano, O., Shimizu, K., Goldfarb, M., Wigler, M. (1982). Activation of the T24 bladder carcinoma transforming gene is linked to a single amino acid change. *Nature* **300**: 762-765.

Tavtigian, S.V., Simard, J., Rommens, J., Couch, F., Shattuck-Eidens, D., Neuhausen, S., Merajver, S., Thorlacius, S., Offit, K., Stoppa-Lyonnet, D., Belanger, C., Bell, R., Berry, S., Bogden, R., Chen, Q., Davis, T., Dumont, M., Frye, C., Hattier, T., Jammulapati, S., Janecki, T., Jiang, P., Kehrer, R., Leblanc, J.F., Goldgar, D.E., *et al.* (1996). The complete BRCA2 gene and mutations in chromosome13q-linked kindreds. *Nat. Genet.* **12**: 333-337.

te Riele, H., Maandag, E.R., Clarke, A., Hooper, M., Berns, A. (1990). Consecutive inactivation of both alleles of the pim-1 proto-oncogene by homologous recombination in embryonic stem cells. *Nature* **348**: 649-651.

Temin, H.M., Mizutani, S. (1970). RNA-dependent DNA polymerase in virions of Rous sarcoma virus. *Nature* **226**: 1211-1213.

Teng, D.H., Bogden, R., Mitchell, J., Baumgard, M., Bell, R., Berry, S., Davis, T., Ha, P.C., Kehrer, R., Jammulapati, S., Chen, Q., Offit, K., Skolnick, M.H., Tavtigian, S.V., Jhanwar, S., Swedlund, B.,Wong, A.K., Kamb, A. (1996). Low incidence of BRCA2 mutations in breast carcinoma and other cancers. *Nat. Genet.* **13**: 241-244.

The European chromosome 16 tuberous sclerosis consortium (1993). Identification and characterisation of the tuberous sclerosis gene on chromosome 16. *Cell* **75**: 1305-1315.

Thibodeau, S.N., Bren, G., Schaid, D. (1993). Microsatellite instability in cancer of the proximal colon. *Science* **260**: 816-819.

Thompson, J.S., Johnson, L.M., Grunstein, M. (1994). Specific repression of the yeast silent mating locus HMR by an adjacent telomere. *Mol. Cell. Biol.* 14: 446-455.

Thompson, M.E., Jensen, R.A., Obermiller, P.S., Page, D.L., Holt, J.T. (1995). Decreased expression of BRCA1 accelerates growth and is often present during sporadic breast cancer progression. *Nat. Genet.* **9**: 444-450.

Thorlacius, S., Olafsdottir, G., Tryggvadottir, L., Neuhausen, S., Jonasson, J.G., Tavtigian, S.V., Tulinius, H., Ogmundsdottir, H.M., Eyfjord, J.E. (1996). A single BRCA2 mutation in male and female breast cancer families from Iceland with varied cancer phenotypes. *Nat. Genet.* **13**: 117-119.

Tirkkonen, M., Johannsson, O., Agnarsson, B.A., Olsson, H., Ingvarsson, S., Karhu, R., Tanner, M., Isola, J., Barkardottir, R.B., Borg, A., Kallioniemi, O.P. (1997). Distinct somatic genetic changes associated with tumor progression in carriers of BRCA1 and BRCA2 germ-line mutations. *Cancer Res.* **57**: 1222-1227.

Tishler, R.B., Calderwood, S.K., Coleman, C.N., Price, B.D. (1993). Increases in sequence specific DNA binding by p53 following treatment with chemotherapeutic and DNA damaging agents. *Cancer Res.* **53(Suppl)**: 2212-2216.

Todd, S., Franklin, W.A., Varella-Garcia, M., Kennedy, T., Hilliker, C.E., Hahner, L., Anderson, M., Wiest, J.S., Drabkin, H.A., Gemmill, R.M. (1997). Homozygous deletions of human chromosome 3p in lung tumors. *Cancer Res.* **57**: 1344-1352.

Toguchida, J., Ishizaki, K., Sasaki, M.S., Ikenaga, M., Sugimoto, M., Kotoura, Y., Yamamuro, T. (1988). Chromosomal reorganization for the expression of recessive mutation of retinoblastoma susceptibility gene in the development of osteosarcoma. *Cancer Res.* **48**: 3939-3943.

Toksoz, D., Williams, D.A. Novel human oncogene lbc detected by transfection with distinct homology regions to signal transduction products. *Oncogene* **9**: 621-628.

Trepicchio, W.L., Krontiris, T.G. (1992). Members of the rel/NF-kappa B family of transcriptional regulatory proteins bind the HRAS1 minisatellite DNA sequence. *Nucleic Acids Res.* **20**: 2427-2434.

Trofatter, J.A., MacCollin, M.M., Rutter, J.L., Murrell, J.R., Duyao, M.P., Parry, D.M., Eldridge, R., Kley, N., Menon, A.G., Pulaski, K., *et al.* (1993). A novel moesin-, ezrin-, radixin-like gene is a candidate for the neurofibromatosis 2 tumor suppressor. *Cell* **72**: 791-800.

Tsujimoto, Y., Cossman, J., Jaffe, E., Croce, C.M. (1985). Involvement of the bcl-2 gene in human follicular lymphoma. *Science* **228**: 1440-1443.

Uberbacher, E.C., Mural, R.J. (1991). Locating protein-coding regions in human DNA sequences by a multiple sensor-neural network approach. *Proc. Natl. Acad. Sci. U.S.A.*. **88**: 11261-11265.

Uejima, H., Mitsuya, K., Kugoh, H., Horikawa, I., Oshimura, M. (1995). Normal human chromosome 2 induces cellular senescence in the human cervical carcinoma cell line SiHa. *Genes Chromosomes Cancer* **14**: 120-127.

Uhrbom, L., Nister, M., Westermark, B. (1997). Induction of senescence in human malignant glioma cells by p16INK4A. *Oncogene* **15**: 505-514.

van Slegtenhorst, M., de Hoogt, R., Hermans, C., Nellist, M., Janssen, B., Verhoef, S., Lindhout, D., van den Ouweland, A., Halley, D., Young, J., Burley, M., Jeremiah, S., Woodward, K., Nahmias, J., Fox, M., Ekong, R., Osborne, J., Wolfe, J., Povey, S., Snell, R.G., Cheadle, J.P., Jones, A.C., Tachataki, M., Ravine, D., Sampson, J.R., *et al.* (1997). Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34. *Science* **277**: 805-808.

Varmus, H.E., Weiss, R.A., Friis, R.R., Levinson, W., Bishop, J.M. Detection of avian tumor virus-specific nucleotidesequences in avian cell DNAs (reassociation kinetics-RNA tumor viruses-gas antigen-Rous sarcoma virus, chick cells). *Proc. Natl. Acad. Sci. U.S.A.* **69**: 20-24.

Vaughn, J.P., Cirisano, F.D., Huper, G., Berchuck, A., Futreal, P.A., Marks, J.R., Iglehart, J.D. (1996). Cell cycle control of BRCA2. *Cancer Res.* **56**: 4590-4594.

Vaux, D.L., Cory, S., Adams, J.M. (1988). Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. *Nature* **335**: 440-442.

Vaziri, H., Benchimol, S. (1998). Reconstitution of telomerase activity in normal human cells leads to elongation of telomeres and extended replicative life span. *Curr. Biol.* **8**: 279-282.

Vaziri, H., Dragowska, W., Allsopp, R.C., Thomas, T.E., Harley, C.B., Lansdorp, P.M. (1994). Evidence for a mitotic clock in human hematopoietic stem cells: loss of telomeric DNA with age. *Proc. Natl. Acad. Sci. U.S.A.* **91**: 9857-9860.

Vaziri, H., Schachter, F., Uchida, I., Wei, L., Zhu, X., Effros, R., Cohen, D., Harley, C.B. (1993). Loss of telomeric DNA during aging of normal andtrisomy 21 human lymphocytes. *Am. J. Hum. Genet.* **52**: 661-667.

Venable, M.E., Lee, J.Y., Smyth, M.J., Bielawska, A., Obeid, L.M. (1995). Role of ceramide in cellular senescence. *J. Biol. Chem.* **270**: 30701-30708.

Viskochil, D., Buchberg, A.M., Xu, G., Cawthon, R.M., Stevens, J., Wolff, R.K., Culver, M., Carey, J.C., Copeland, N.G., Jenkins, N.A., *et al.* (1990). Deletions and a translocation interrupt a cloned gene at the neurofibromatosis type 1 locus. *Cell* **62**: 187-192.

Vocke, C.D., Pozzatti, R.O., Bostwick, D.G., Florence, C.D., Jennings, S.B., Strup, S.E., Duray, P.H., Liotta, L.A., Emmert-Buck, M.R., Linehan, W.M. (1996). Analysis of 99 microdissected prostate carcinomas reveals a high frequency of allelic loss on chromosome 8p12-21. *Cancer Res.* **56**: 2411-2416.

von Stein, O.D., Thies, W.G., Hofmann, M. (1997). A high throughput screening for rarely transcribed differentially expressed genes. *Nucleic Acids Res.* **25**: 2598-2602.

Wadhwa, R., Pereira-Smith, O.M., Reddel, R.R., Sugimoto, Y., Mitsui, Y., Kaul, S.C. (1995). Correlation between complementation group for immortality and the cellular distribution of mortalin. *Exp. Cell. Res.* **216**: 101-106.

Wainwright, L.J., Middleton, P.G., Rees, J.L. (1995). Changes in mean telomere length in basal cell carcinomas of the skin. *Genes Chromosomes Cancer* **12**: 45-49.

Wallace, M.R., Marchuk, D.A., Andersen, L.B., Letcher, R., Odeh, H.M., Saulino, A.M., Fountain, J.W., Brereton, A., Nicholson, J., Mitchell, A.L., *et al.* (1990). Type 1 neurofibromatosis gene: identification of a large transcript disrupted in three NF1 patients. *Science* **249**: 181-186.

Wang, E. (1995). Senescent human fibroblasts resist programmed cell death, and failure to suppress bcl2 is involved. *Cancer Res.* **55**: 2284-2292.

Wang, N., Perkins, K.L. (1984). Involvement of band 3p14 in t(3;8) hereditary renal carcinoma. *Cancer Genet. Cytogenet.* **11**: 479-481.

Wang, X.W., Vermeulen, W., Coursen, J.D., Gibson, M., Lupold, S.E., Forrester, K., Xu, G., Elmore, L., Yeh, H., Hoeijmakers, J.H., Harris, C.C. (1996). The XPB and XPD DNA helicases are components of the p53-mediated apoptosis pathway. *Genes Dev.* **10**: 1219-1232.

Wang, X.W., Yeh, H., Schaeffer, L., Roy, R., Moncollin, V., Egly, J.M., Wang, Z., Freidberg, E.C., Evans, M.K., Taffe, B.G., *et al.* (1995). p53 modulation of TFIIH-associated nucleotide excision repair activity. *Nat. Genet.* **10**: 188-195.

Warren, S.T., Schultz, R.A., Chang, C.C., Wade, M.H., Trosko, J.E. (1981). Elevated spontaneous mutation rate in Bloom syndrome fibroblasts. *Proc. Natl. Acad. Sci. U.S.A.*. **78**: 3133-3137.

Wary, K.K., Mainiero, F., Isakoff, S.J., Marcantonio, E.E., Giancotti, F.G. (1996). The adaptor protein Shc couples a class of integrins to the control of cell cycle progression. *Cell* **87**: 733-743.

Watson, J.D. (1972). Origin of concatemeric T7 DNA. *Nat. New. Biol.* **239**: 197-201.

Watson, K.L., Justice, R.W., Bryant, P.J. (1994). Drosophila in cancer research: the first fifty tumor suppressor genes. *J. Cell Sci.* **18 (Suppl)**: 19-33.

Wazer, D.E., Liu, X.L., Chu, Q., Gao, Q., Band, V. (1995). Immortalization of distinct human mammary epithelial cell types by human papilloma virus 16 E6 or E7. *Proc. Natl. Acad. Sci. U.S.A.* **92**: 3687-3691.

Weinberg, R.A. (1995). The retinoblastoma protein and cell cycle control. *Cell* **81**: 323-330.

Weinberg, R.A. (1996). E2F and cell proliferation: a world turned upside down. *Cell* **85**: 457-459.

Weintraub, S.J., Chow, K.N., Luo, R.X., Zhang, S.H., He, S., Dean, D.C. (1995). Mechanism of active transcriptional repression by the retinoblastoma protein. *Nature* **375**: 812-815.

Weintraub, S.J., Prater, C.A., Dean, D.C. (1992). Retinoblastoma protein switches the E2F site from positive to negative element. *Nature* **358**: 259-261.

Weissenbach, J., Gyapay, G., Dib, C., Vignal, A., Morissette, J., Millasseau, P., Vaysseix, G., Lathrop, M. (1992). A second-generation linkage map of the human genome. *Nature* **359**:.794-801.

Weith, A., Martinsson, T., Cziepluch, C., Bruderlein, S., Amler, L.C., Berthold, F., Schwab, M. (1989). Neuroblastoma consensus deletion maps to 1p36.1-2. *Genes Chromosomes Cancer* **1**: 159-166.

Weksberg, R., Shen, D.R., Fei, Y.L., Song, Q.L., Squire, J. (1993). Disruption of insulin-like growth factor 2 imprinting in Beckwith-Wiedemann syndrome. *Nat. Genet.* **5**:143-150.

Wellinger, R.J., Sen, D. (1997). The DNA structures at the ends of eukaryotic chromosomes. *Eur. J. Cancer* **33**: 735-749.

Westphal, C.H., Rowan, S., Schmaltz, C., Elson, A., Fisher, D.E., Leder, P. (1997) atm and p53 cooperate in apoptosis and suppression of tumorigenesis, but not in resistance to acute radiation toxicity. *Nat. Genet.* **16**: 397-401.

Whitaker, N.J., Bryan, T.M., Bonnefin, P., Chang, A.C., Musgrove, E.A., Braithwaite, A.W., Reddel, R.R. (1995). Involvement of RB-1, p53, p16INK4 and telomerase in immortalisation of human cells. *Oncogene* **11**: 971-976.

White, E (1996). Life, death, and the pursuit of apoptosis. *Genes Dev.* **10**: 1-15.

White, E., Cipriani, R., Sabbatini, P., Denton, A. (1991). Adenovirus E1B 19-kilodalton protein overcomes the cytotoxicity of E1A proteins. *J. Virol.* **65**: 2968-2978.

White, P.S., Maris, J.M., Beltinger, C., Sulman, E., Marshall, H.N., Fujimori, M., Kaufman, B.A., Biegel, J.A., Allen, C., Hilliard, C., *et al.* (1995). A region of consistent deletion in neuroblastoma maps within human chromosome 1p36.2-36.3. *Proc. Natl. Acad. Sci. U.S.A.* **92**: 5520-5524.

Williams, B.O., Remington, L., Albert, D.M., Mukai, S., Bronson, R.T., Jacks, T. (1994). Co-operative tumorigenic effects of germ line mutations in Rb and p53. *Nat. Genet.* **7**: 480-484.

Williams, C., Williamson, R., Coutelle, C., Loeffler, F., Smith, J., Ivinson, A. (1988). Same-day, first-trimester antenatal diagnosis for cystic fibrosis by gene amplification. *Lancet* **2**: 102-103.

Wong, A.J., Ruppert, J.M., Eggleston, J., Hamilton, S.R., Baylin, S.B., Vogelstein, B. (1986). Gene amplification of c-myc and N-myc in small cell carcinoma of the lung. *Science* **233**: 461-464.

Wooster, R., Bignell, G., Lancaster, J., Swift, S., Seal, S., Mangion, J., Collins, N., Gregory, S., Gumbs, C., Micklem, G. (1995). Identification of the breast cancer susceptibility gene BRCA2. *Nature* **378**: 789-792.

Wooster, R., Mangion, J., Eeles, R., Smith, S., Dowsett, M., Averill, D., Barrett-Lee, P., Easton, D.F., Ponder, B.A., Stratton, M.R. (1992). A germline mutation in the androgen receptor gene in two brothers with breast cancer and Reifenstein syndrome. *Nat. Genet.* **2**: 132-134.

Wright, W.E., Brasiskyte, D., Piatyszek, M.A., Shay, J.W. (1996)[b]. Experimental elongation of telomeres extends the lifespan of immortal x normal cell hybrids. *EMBO J.* **15**: 1734-1741.

Wright, W.E., Pereira-Smith, O.M., Shay, J.W. (1989). Reversible cellular senescence: implications for immortalization of normal human diploid fibroblasts. *Mol. Cell. Biol.* **9**: 3088-3092.

Wright, W.E., Piatyszek, M.A., Rainey, W.E., Byrd, W., Shay, J.W. (1996)[a]. Telomerase activity in human germline and embryonic tissues and cells. *Dev. Genet.* **18**: 173-179.

Wright, W.E., Shay, J.W. (1992). Telomere positional effects and the regulation of cellular senescence. *Trends Genet.* **8**: 193-197.

Wu, H., Liu, X., Jaenisch, R. (1994). Double replacement: strategy for efficient introduction of subtle mutations into the murine Col1a-1 gene by homologous recombination in embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* **91**: 2819-2823.

Wu, L.C., Wang, Z.W., Tsan, J.T., Spillman, M.A., Phung, A., Xu, X.L., Yang, M.C., Hwang, L.Y., Bowcock, A.M., Baer, R. (1996). Identification of a RING protein that can interact in vivo with the BRCA1 gene product. *Nat. Genet.* **14**: 430-440.

Wu, X., Levine, A.J. (1994). p53 and E2F-1 cooperate to mediate apoptosis. *Proc. Natl. Acad. Sci. U.S.A.* **91**: 3602-3606.

Xu, G.F., Lin, B., Tanaka, K., Dunn, D., Wood, D., Gesteland, R., White, R., Weiss, R., Tamanoi, F. (1990). The catalytic domain of the neurofibromatosis type 1 gene product stimulates ras GTPase and complements ira mutants of S. cerevisiae. *Cell* **63**: 835-841.

Xu, H., Zhou, Y., Ji, W., Perng, G., Kruzelock, R., Kong, C., Bast, R.C., Mills, G.B., Li, J., Xu, S. (1997). Reexpression of the retinoblastoma protein in tumour cells induces senescence and telomerase inhibition. *Oncogene* **15**: 2589-2596.

Xu, Y., Ashley, T., Brainerd, E.E., Bronson, R.T., Meyn, M.S., Baltimore, D. (1996)[b]. Targeted disruption of ATM leads to growth retardation, chromosomal fragmentation during meiosis, immune defects, and thymic lymphoma. *Genes Dev.* **10**: 2411-2422.

Xu, Y., Baltimore, D. (1996)[a]. Dual roles of ATM in the cellular response to radiation and in cell growth control. *Genes Dev.* **10**: 2401-2410

Yamada, H., Wake, N., Fujimoto, S., Barrett, J.C., Oshimura, M. (1990). Multiple chromosomes carrying tumor suppressor activity for a uterine endometrial carcinoma cell line identified by microcell-mediated chromosome transfer. *Oncogene* **5**: 1141-1147.

Yamasaki, L., Jacks, T., Bronson, R., Goillot, E., Harlow, E., Dyson, N.J. (1996). Tumor induction and tissue atrophy in mice lacking E2F-1. *Cell* **85**: 537-548.

Yin, Y., Tainsky, M.A., Bischoff, F.Z., Strong, L.C., Wahl, G.M. (1992). Wild-type p53 restores cell cycle control and inhibits gene amplification in cells with mutant p53 alleles. *Cell* **70**: 937-948.

Yoakum, G.H., Lechner, J.F., Gabrielson, E.W., Korba, B.E., Malan-Shibley, L., Willey, J.C., Valerio, M.G., Shamsuddin, A.M., Trump, B.F., Harris, C.C. (1985). Transformation of human bronchial epithelial cells transfected by Harvey ras oncogene. *Science* **227**:1174-1179.

Yu, C.E., Oshima, J., Fu, Y.H., Wijsman, E.M., Hisama, F., Alisch, R., Matthews, S., Nakura, J., Miki, T., Ouais, S., Martin, G.M., Mulligan, J., Schellenberg, G.D. (1996). Positional cloning of the Werner's syndrome gene. *Science* **272**: 258-262.

Yunis, J.J., Ramsay, N. (1978). Retinoblastoma and subband deletion of chromosome 13q14. Am. J. Dis. Child. **132**: 161-163.

Yunis, J.J., Soreng, A.L. (1984). Constitutive fragile sites and cancer. *Science* **226**: 1199-1204.

Yuspa, S.H., Kilkenny, A.E., Stanley, J., Lichti, U. (1985). Keratinocytes blocked in phorbol ester-responsive early stage of terminal differentiation by sarcoma viruses. *Nature* **314**: 459-462.

Zacksenhaus, E., Jiang, Z., Chung, D., Marth, J.D., Phillips, R.A, Gallie, B.L. (1996). pRb controls proliferation, differentiation, and death ofskeletal muscle cells and other lineages during embryogenesis. *Genes Dev.* **10**: 3051-3064.

Zenklusen, J.C., Bieche, I., Lidereau, R., Conti, C.J. (1994)[b]. (C-A)n microsatellite repeat D7S522 is the most commonly deleted region in human primary breast cancer. *Proc. Natl. Acad. Sci. U.S.A.* **91**: 12155-12158.

Zenklusen, J.C., Conti, C.J. (1996)[a]. Cytogenetic, molecular and functional evidence for novel tumor suppressor genes on the long arm of human chromosome 7. *Mol. Carcinog.* 15: 167-175.

Zenklusen, J.C., Hodges, L.C., Conti, C.J. (1997). Loss of heterozygosity on murine chromosome 6 in two-stage carcinogenesis: evidence for a conserved tumor suppressor gene. *Oncogene* **14**: 109-114.

Zenklusen, J.C., Oshimura, M., Barrett, J.C., Conti, C.J. (1994)[a]. Inhibition of tumorigenicity of a murine squamous cell carcinoma (SCC) cell line by a putative tumor suppressor gene on human chromosome 7. *Oncogene* **9**: 2817-2825.

Zenklusen, J.C., Rodriguez, L.V., LaCava, M., Wang, Z., Goldstein, L.S., Conti, C.J. (1996)[b]. Novel susceptibility locus for mouse hepatomas: evidence for a conserved tumor suppressor gene. *Genome Res.* **6**: 1070-1076.

Zenklusen, J.C., Thompson, J.C., Klein-Szanto, A.J., Conti, C.J. (1995)[a]. Frequent loss of heterozygosity in human primary squamous cell and colon carcinomas at 7q31.1: evidence for a broad range tumor suppressor gene. *Cancer Res.* **55**: 1347-1350.

Zenklusen, J.C., Thompson, J.C., Troncoso, P., Kagan, J., Conti, C.J. (1994)[c]. Loss of heterozygosity in human primary prostate carcinomas: a possible tumor suppressor gene at 7q31.1. *Cancer Res.* **54**: 6370-6373.

Zenklusen, J.C., Weitzel, J.N., Ball, H.G., Conti, C.J. (1995)[b]. Allelic loss at 7q31.1 in human primary ovarian carcinomas suggests the existence of a tumor suppressor gene. *Oncogene* **11**: 359-363.

Zhang, P., Liegeois, N.J., Wong, C., Finegold, M., Hou, H., Thompson, J.C., Silverman, A., Harper, J.W., DePinho, R.A., Elledge, S.J. (1997). Altered cell differentiation and proliferation in mice lacking p57KIP2 indicates a role in Beckwith-Wiedemann syndrome. *Nature* **387**: 151-158.

Zhang, Y., Musci, T., Derynck, R. (1997). The tumor suppressor Smad4/DPC 4 as a central mediatorof Smad function. *Curr. Biol.* **7**: 270-276.

Zhong, Q., Chen, C.F., Chen, Y., Chen, P.L., Lee, W.H. (1997). Identification of cellular TSG101 protein in multiple human breast cancer cell lines. *Cancer Res.* **57**:4225-4228.

Zielenski, J., Markiewicz, D., Rininsland, F., Rommens, J., Tsui, L.C. (1991). A cluster of highly polymorphic dinucleotide repeats in intron 17b of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. *Am. J. Hum. Genet.* **49**: 1256-1262.

Zielenski, J., Rozmahel, R., Bozon, D., Kerem, B., Grzelczak, Z., Riordan, J.R., Rommens, J., Tsui, L.C. (1991). Genomic DNA sequence of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. *Genomics* **10**: 214-228.

Zou, T.T., Lei, J., Shi, Y.Q., Yin, J., Wang, S., Souza, R.F., Kong, D., Shimada, Y., Smolinski, K.N., Greenwald, B.D., Abraham, J.M., Harpaz, N., Meltzer, S.J. (1997). FHIT gene alterations in esophageal cancer and ulcerativecolitis (UC). *Oncogene* **15**: 101-105.

Zuo, L., Weger, J., Yang, Q., Goldstein, A.M., Tucker, M.A., Walker, G.J., Hayward, N., Dracopoli, N.C. (1996). Germline mutations in the $p16^{INK4a}$ binding domain of CDK4 in familial melanoma. *Nat. Genet.* **12**: 97-99.

Zuppan, P., Hall, J.M., Lee, M.K., Ponglikitmongkol, M., King, M.C. (1991). Possible linkage of the estrogen receptor gene to breast cancer in a family with late-onset disease. *Am. J. Hum. Genet.* **48**: 1065-1068.

Zwicker, J., Liu, N., Engeland, K., Lucibello, F.C., Muller, R. (1996). Cell cycle regulation of E2F site occupation in vivo. *Science* **271**: 1595-1597.

APPENDIX

Table A1. Human chromosome 7 polymorphic STSs used in the analysis of LOH in breast tumour samples and SUSM-1 segregants. 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. [d] Listed markers derived by the CHLC are exclusively tetranucleotide repeats (Murray *et al.*, 1994), whereas listed markers derived by Genethon are exclusively CA.GT dinucleotide repeats. (Weissenbach *et al.*, 1992; Gyapay *et al.*, 1994). The amplimers GH220/GH324, GH390/GH391, CS.7L/CS.7R, GH295/GH296, MIV1/VIM2, GH333/GH334, and pJG3CL/pJG3CR allow detection of RFLPs when restricted with *Msp* I, *Taq* I, *Hha* I, *Pst* I, *ScrF* I, *Msp* I, and *Pst* I respectively. The amplimers GH425/GH426 and 17B-TA5/17B-RE3 contain CA.GT dinucleotide repeats. 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	Source[d]
D7S2490	AFMB299XG5	Z53428	87.6 cM		L: TGTAAGCCACCGCACCT R: AGCACCCGGCATAGATGT	Genethon
D7S2455	AFMA290XA5	Z52804	90.3 cM	202	L: CTTGAGCCTGGGAGTACGAGAC R: TCAGAAATTGCACCAAGTGCTA	Genethon
D7S2517	AFMC014ZD9	Z54004	90.3 cM	240	L: AGTGAGGCTGCTGCTCAA R: ATTCCCCTGGGCATAAAC	Genethon
D7S675	AFM295YG9	Z24161	90.3 cM 419.45 cR	206	L: GGAATNAAAGTATTACCGTATAGGA R: TTGCTGTCCAGGTGCC	Genethon
D7S2421	AFMA128YE1	Z52184	90.3 cM		L: TGCCACTCATTCCATC R: TGTGAGGCTAATGTACCG	Genethon
D7S669	AFM286XF9	Z24049	90.9 cM 415.12 cR	132	L: ATGCAACCTACCCTCAAATG R: TACGGNTTACCCACATTGCTAT	Genethon
D7S2204	CHLC.GATA73D10	G08635	91.0 cM 418.71 cR	228	L: TCATGACAAAACAGAAATTAAGTG R: AGTAAATGGAATTGCTTGTTACC	CHLC
D7S660	AFM277VD5	Z23973	94.4 cM 429.55 cR	188	L: TAGGCCAACACTGGGG R: AGCTTGATAGTGGGAATCATTT	Genethon
D7S1797	CHLC.GATA21D08	G08599	98.4 cM 429.55 cR	229	L: TTCAAGAGCTAATCCATGCC R: AAATTGAGATCGCAGCTGAC	CHLC
D7S440		X54566		175	L: ACATTCTAAGACTTTCCCAAT R: AGAGCATGCACCCTGAATTG	Weber J.L
D7S820	CHLC.GATA3F01.511	G08616	98.4 cM	222	L: TGTCATAGTTTAGAACGAACTAACG R: CTGAGGTATCAAAAACTCAGAGG	CHLC
D7S2540	AFMA043TB1	Z51567	98.4 cM	209	L: TGATTACAGGTTAAAGTAGAAGCA R: ACTGGCAGGAGGTCGT	Genethon

D7S2417	AFMA113YG1	Z52088	98.6 cM	170	L: CAGAAAGTCAAAATCGTTAGAGTC R: GGTGGGAGAGTCAATCCT	Genethon
D7S524	AFM248TA5	Z17104	98.0 cM 438.43 cR	239	L: AAGTAATGCAAAACAGCCTTGA R: ACCCACTGAAAAGATTTGTGTC	Genethon
D7S2537	AFMA037YE5	Z51543	98.2 cM	109	L: TCCACTCCATGACCTCC R: TCAANTCACACTGTACTGCAAT	Genethon
D7S2555	AFMA064WG1	Z51811	98.1 cM	122	L AGTTAGCCCATGCCAC R: GCCAATCAGTTTCAATCTC	Genethon
D7S644	AFM234XC7	Z23719	98.3 cM 448.79 cR	203	L: AGGTGAGAGACCCTCAGCAATAGTG R: CCTCCAAAGTTAGGGGTGGAAG	Genethon
D7S2481	AFMB072WB5	Z53262	98.6 cM	217	L: TTTGACATTATTATCCAGCAGG R: ATCATTGTAGACTCCCACCAGTAG	Genethon
D7S630	AFM165YH12	Z23423	98.7 cM	215	L: TCCATTCTGAGGTTTGATGT R: CCATGGTCTTTTCAATGAAC	Genethon
D7S492	AFM158XA1	Z16704	100.5 cM 444.80 cR	148	L: ATCTTGGATTTAGGGTTGGC R: GGCTCTGCTCCATCTTCATA	Genethon
D7S2409	AFMA202XA5	Z52456	104.7 cM	221	L: TAAGTNCTGGGGATCTAATG R: TACATATTTATGGGGTAACTGATGA	Genethon
D7S646	AFM240VE3	Z23758	106.7 cM 462.77 cR	180	L: CTGTCTGCTTATCCTATTCCC R: GTTGAGTGTCCACCTGAGATTTAT	Genethon
D7S689	AFM333WF5	Z24437	105.2 cM	130	L: CCTCAACCTGAATCTCACATC R: CAATGGAGCCAGACTCTGT	Genethon
D7S657	AFM263YD9	Z23869	105.2 cM 461.26 cR	245	L: GTCACAGCACAGTTTTTGG R: GTCAAGTAGAGATTGAGATTCC	Genethon
D7S652	AFM254XD5	Z23826	105.2 cM 462.87 cR	274	L: GGGCTTGTTTCATTACACGTTG R: CATGATTTTTGGCACAGAATGTTAG	Genethon
D7S2430	AFMA156TB1	Z52325	106.2 cM	149	L: TGCAATGAGCCATGTCC R: GAGTGCAGTTTAATCCCCATAG	Genethon

D7S1820	CHLC.GATA26D09	G08606		246	L: TGAATGACTTTGGTGAGTATGC R: ACCTCAAGCAGAACACTTGC	CHLC
D7S2431	AFMA163TE5	Z52343	109.0 cM	126	L: TCCACATTGCATGAGCC R: TTAGAAATCCGAGCCAATAGAA	Genethon
D7S527	AFM248VD9	Z17113	109.0 cM	292	L: CATTGCAAACTCAGGAGATA R: TAACAGAGGCATGAAAACCA	Genethon
D7S821	CHLC.GATA5D08. 584	G08626	109.1 cM 465.72 cR	264	L: ACAAAACCCCAAGTACGTGA R: TATGACAGGCATCTGGGAGT	CHLC
D7S2539	AFM088YE9	Z50943	109.0 cM	242	L: ACCAGTTGATTCAGCAATCC R: GGAGGGTATTCCATTGTCTG	Genethon
DS479	AFM036XG5	Z16460	109.5 cM	118	L: CTGGGTGACAGAGCACCAGA R: = CCTTCTTGATAGATATTAGGGGTTT	Genethon
D7S1796	CHLC.GATA21C11	G08600	109.1 cM	291	L: CCAGGAGTTTGAGGCTACAA R: TCAAGGATGGTGTCCAAGTT	CHLC
D7S491	AFM151XF10	Z16679	109.5 cM	114	L: AGCTCCAAAACCTAACTCCA R: TCAAAATTATTTGACTTCTTGATTT	Genethon
D7S554	AFM248TE5	Z17270	110.0 cM	260	L: GTCTAATTACCCACATTTCCCT R: GTTCCATATTTAAAAGAC	Genethon
D7S651	AFM249ZA5	Z23820	111.6 cM 472.30 cR	174	L: GGCTGCCTTCAAAAACTC R: AGCCTGGCATGTGGAT	Genethon
D7S647	AFM263YD9	Z23869	105.2 cM 461.26 cR	245	L: GTCACAGCACAGTTTTTGG R: GTCAAGTAGAGATTGAGATTCC	Genethon
D7S1841	CHLC.GGAA6A12	G09451	483.91 cR	206	L: TCAAGTTTGATCATGAGATTGC R: GAAAAAAAGAAAGTTGGGGG	CHLC
D7S662	AFM280VH9	Z23992	112.2 cM	219	L: GTTGACAGACAAGCACAGAC R: AGCTGTTTCCCATTTCCA	Genethon
D7S477	AFM030XB4	Z23297	112.3 cM	180	L: TTGCACCACTGTCTCCAGTC R: TTTGGGTATCCCCTGTTCAC	Genethon

D7S501	AFM199VB2	Z16867	112.8 cM	170	L: CACCGTTGTGATGGCAGAG R: ATTTCTTACCAGGCAGACTGCT	Genethon
D7S515	AFM220XC11	Z16999	112.9 cM 485.74 cR	189	L: GGGAGTTACTACCCTCACTTAATG R: GGACTGGGCAGCAAAG	Genethon
D7S666	AFM283ZA9	Z24028	112.9 cM 487.95 cR	166	L: GCCTTCTCAAGCAAATTGAT R: CCTGATATGTGAGGTAATGAAAGAG	Genethon
D7S518	AFM225XG9	Z17029	112.9 cM 487.95 cR	180	L: CAGTAGGCAGGGGTGG R: GGGTGTGTCTGTGTGACAAC	Genethon
D7S2509	AFM273VG5	Z51237	113.4 cM	276	L: GTGGGAAACAGTATGATTGATT R: CCTTTTTAAGGCTGAATAATGTGT	Genethon
D7S796	CHLC.GATA4E02.122	G08623	113.4 cM 491.10 cR	178	L: TTTTGGTATTGGCCATCCTA R: GAAAGGAACAGAGAGACAGGG	CHLC
D7S2504	AFMB348ZB5	Z53797	113.9 cM	217	L: TGTGGTACAATTTCAGACACATAA R: CTGGAAACCAGTGTTTTCACTT	Genethon
D7S658	AFM269ZG1	Z23925	114.4 cM 491.00 cR	263	L: CATCACACACCAGGGC R: AACAGAAGGACTGAACTTCATC	Genethon
D7S2446	AFMA226YE1	Z52609	114.6 cM	206	L: TTTGAGTCTTCACAGCAGTTG R: GGGAGGTTGATTTCCACAGT	Genethon
D7S1799	CHLC.GATA23F05	G09385		181	L: ATGGTATTAGGAGATGGGGC R: TTGCATAAGCCAATTTCCAT	CHLC
D7S2545	AFMA052YA5	Z51681	114.5 cM	129	L: TTTAGTATCTGGNCTTACGG R: ACCGTTAAACAGCAGTTTCTA	Genethon
D7S496	AFM172XA1	Z16766	120.7 cM	132	L: AACAACAGTCAACCCACAAT R: GCTATAACCTCATAANAAACCAAAA	Genethon
D7S692	AFM357TE1	Z24606	121.5 cM	166	L: CTGATGATTGCTATAGATATTCATC R: TGTAAACACTTTTGTAGAAGAACCT	Genethon
D7S1817	CHLC.GATA21H01	G08603	494.67 cR	121	L: CAAATTAATGGCAAAAACTGC R: CCCCCCATTGAGGTTATTAC	CHLC

D7S525	AFM248TC5	Z17106	122.3 cM 484.11 cR	222	L: GTTAGCCGAGATTGCCC R: CTTGCTGTTTAAGTACCACAAGTTC	Genethon
D7S471			107.2 cM	185	L: AGCAGCTATTATGGAATTGC R: CAACATATGCAAGGTGCCTA	WICGR
D7S523	AFM242YE3	Z17102	123.9 cM	223	L: CTGATTCATAGCAGCACTTG R: AAAACATTTCCATTACCACTG	Genethon
D7S687	AFM323YG5	Z24392	123.4 cM 496.89 cR	237	L: AAAATATTACACATGCCTGAGTG R: ACAGTGAAGCGACACCATC	Genethon
D7S816	CHLC.ATC2H06	G09255	497.66 cR	163	L: TCCTTGGCTTATACATTGTGC R: CTGAAAACACTGGATGTGACC	CHLC
D7S1811	CHLC.ATA17F10	G08583		187	L: TGCCATATTTGGGGATTCTA R: ACTTACCTGCAATGTGCACA	CHLC
D7S486	AFM098XG9	Z16567	125.3 cM 497.83 cR	145	L: AAAGGCCAATGGTATATCCC R: GCCCAGGTGATTGATAGTGC	Genethon
D7S522	AFM242YC3	Z17100	125.1 cM	220	L: GCCAAACTGCCACTTCTC R: ACGTGTTATGCCACTCCC	Genethon
D7S2460	AFM197XF10	Z51113	125.1 cM	193	L: CACATCCACTGTGTCTCATTT R: TATCTGGGACTTNACGCTTC	Genethon
MET	GH220/GH324			203	L: CCATGTAGGAGAGCCTTAGTC R: GTCTAAGGACACACCTTGC	Horn *et al.*, 1990
	AFMB316XB9	Z67602		122	L: TTGCATAATTTCTTCATTCAGTGG R: CCTCCTAAGGATTGTAGTTGAGGC	Genethon
D7S23	GH390/GH391			291	L: CAGGCGCATTCATGAGGCATT R: CCTCCCAGGTTCACGCTAT	Horn *et al.*, 1990
D7S23	CS.7L/CS.7R			330	L: CCCAGCTTCAGGGAGAGAAGCGAAGCAATG R: AAACGCGGGGTTTTAGACACGGGTGCATGA	Williams *et al.*, 1988
D7S23	GH425/GH426	X60680		120	L: GACGTGCTAGCCTGGTCTCCAGCTCT R: GATGGGGGAGGCGGTTGTAGTTTTCAAGGT	Richards *et al.*, 1991

D7S23	GH295/GH296				L: GGCAGGGCTAAATTGCAAC R: CATCCCCTTCTCACACTTC	Horn *et al.*, 1990
D7S23	MIV1/VIM2			412	L: CCTTCTAGGCTGTGTGGCT R: GTGGCTCAGAGATTCTGCC	Nunes, *et al.*, 1990
D7S633	AFM200WE1	Z23505	125.2 cM	175	L: TGAGCCTCGCATCACTG R: TCTGGGGAGTCCTTTAACAGTA	Genethon
D7S399	GH333/GH334			373	L: GCAACAATTCACCCAATTGCT R: GGTTAGGTCAGAGAACAAAG	Horn *et al.*, 1990
	NEWCAREP2	AC002465		151	L: CGGAAGTAGTTACATGAAGC R: CATTGGTATGACCCATACGG	
D7S677	AFM303VH9	Z24230	125.2 cM 501.19 cR	278	L: ATCATTCACTATGGGATAGC R: GAATTACAAGTCACTCTATACAAAA	Genethon
	NEWCAREP1	AC000111		161	L: CTGCATATTAGTCAAGGTTCTCC R: TGCCAGCTGACCCATACTGC	
CFTR	17TA-5/17B-RE3			350	L: ATAATTTCCTTGAAATCGGA R: GCTGCATTCTATAGGTTATC	Zielenski *et al.*, 1991
	NEWCAREP3	AC002431		148	L: GAGGGGTTGAATAGCAGAGG R: TATTCCACTCTGCAGTCACC	
	NEWCAREP4	AC003084		197	L: ATGATGACAGGACTCTAGGG R:TAAATTCCCACCCCTATGGC	
	NEWCAREP5	AC003084		209	L: CAAACATGCATGTATTTGGCC R: GCAGGCTGGTAAACTGATCC	
D7S8	pJG3CL/pJG3CR			380	L: CCACTGATACTGTGAGAC R: GTTGTTTCAAGTCACTGC	Northrup *et al.*, 1989
	AFMA073XA5	Z67222		165	L: CCAGAGCAAACACAATG R: CCCACAATATGAAAACCA	Genethon
D7S655	AFM263WG9	Z23863	126.5 cM 504.41 cR	264	L: CAAAATAGTGGGGTATTGGTAAA R: CCAAGTTAATCTNTGTGAAAGTGTA	Genethon

D7S2847	CHLC.GATA44F09	G08621	125.2 cM	195	L: TCACCTTCAGAAAGTATTGCC R: TGAGGTGTTTCTCCAAGCTC	CHLC
	CHLC.GGAA2G12	G12391	502.61 cR	286	L: TCTCTGAAGTAAGTCAAACAGAGG R: CTGCACTTCCTGAAAATAGAA	CHLC
D7S643	AFM224ZF10	Z23691	126.3 cM	267	L: AGCTAATATTGCTGCCTTTT R: CAATCTCTTGCTAGATGCCA	Genethon
D7S480	AFM042XH10	Z16469	127.2 cM 506.41 cR	200	L: CTTGGGGACTGAACCATCTT R: AGCTACCATAGGGCTGGAGG	Genethon
D7S650	AFM240ZH10	Z23782	128.4 cM	274	L: AGGCTGCTTAGCCATAATC R: CCACTGGTATAAGTACATCAGAAA	Genethon
D7S685	AFM317YC5	Z24334	129.4 cM 506.31 cR	181	L: AAGACCTGGCAACAGTTCTTACTA R: CGCTCATCAAGGATATTGG	Genethon
D7S1809	CHLC.GGAA9C07	G08644	127.8 cM	202	L: AGGCAAGAGCAGTAGCAAGA R: TCCACTTTAAATCAGCAGCC	CHLC
D7S1835	CHLC.GATA64H06	G08630	510.47 cR	260	L: GAGCCAATTGTACTGGATTACC R: CAGGACATGTCAATTGGACA	CHLC
D7S1801	CHLC.GATA31D01	G08612		227	L: TTAGGGGGATCTTGCTCTCT R: AGCAGGGAATAGGCTGAGTT	CHLC
D7S2203	CHLC.GATA67A05	G08633	528.30 cR	232	L: GAGCCAATTGTACTGGATTACC R: TACCTTGTTCAGAAGCCCTG	CHLC
D7S530	AFM249XF9	Z17136	136.4 cM 554.78 cR	111	L: TGCATTTTAGTGGAGCACAG R: CAGGCATTGGGAACTTTG	Genethon
D7S2197	CHLC.GATA113H05			244	L: TTAGATCATTGACACAAGGGC R: CAGGAAACCCGTGATTGTAC	CHLC
D7S1804	CHLC.GATA43C11	G08619	137.0 cM 584.31 cR	258	L: TTCAAGTGGTTGGGTTCACT R: TGGGTCTAGTCCAGTGGTGT	CHLC
D7S649	AFM240XE9	Z23771	138.0 cM 570.21 cR	276	L: ATTTTGATCCCCAGCA R: GCTTTATTATGTCTGTTGTATGA	Genethon

D7S681	AFM310YF9	Z24296	140.5 cM	254	L: TCATGTATGGACATTTGGC R: ACTCATATTGCTTAGGTTAAGGAA	Genethon
D7S631	AFM183XE11	Z23439	139.7 cM 583.28 cR	117	L: CTCAACCACATGCCAGTTTC R: TTTCTCGCCGGGTAGTAGTT	Genethon
D7S1837	CHLC.GATA65F01	G08634	598.53 cR	276	L: TTGTGTGAGCATCCTAGGGT R: CTCAGCTTGCAGACAGCTTA	CHLC
D7S2202	CHLC.GATA63F08	G08628	149.9 cM 613.95 cR	155	L: TCTCTTACCCTTTGGGACCT R: CTTGCAGATGGCCTAATTGT	CHLC
D7S794	CHLC.GATA2C04. 755	G08607	630.79 cR	168	L: GCCAATTCTCCTAACAAATCC R: TATGCCCATGTGTTAGGGTT	CHLC
D7S512	AFM214YB2	Z16976	137.9 cM 580.86 cR	176	L: TGAGAGGCTTTGCAGG R: CAGTGCTGGAGGCTGTG	Genethon
D7S1805	CHLC.GATA4H10		647.70cR	197	L: CCTGCTTTGGCTTACCTGTA R: CCCACTTCTCTGCTATTACATAT	CHLC
D7S1807	CHLC.GGAA2B12	G08640	657.00 cR	236	L: TCCTTTTCCTTTTCCCTTTC R: ATTAATAGGTTTGTCACGATTAACC	CHLC

Table A2. Human chromosome 7 non-polymorphic STSs. [a] The accession numbers are given for the sequences of the random genomic clones from which non-expressed STS sequences are derived, or for the cDNAs or ESTs from which expressed STS sequences are derived. [b] Physical distances from the top of chromosome 7 are given in centiRays (cR; 1 cR = 270 Kbp) and based on radiation hybrid mapping of the Stanford G3 and Genebridge 4 RH mapping panels (Schuler *et al.*, 1996). [c] Information for STSs derived at the NHGRI is available on-line through a site on the World Wide Web at http://www.nhgri.nih.gov/DIR/GTB/CHR7/; a summary of the integrated chromsome 7 YAC contig map produced by the NHGRI is published in Bouffard *et al.* (1997). The RHMC comprises genome mapping centres or groups at the Whitehead Institute for Biomedical Research, the Sanger Centre, Genethon, Stanford University, Oxford University, the University of Colorado Health Sciences Centre, and informatics groups at the National Centre for Biotechnology Information and the European Bioinformatics Institute. The first report of this consortium is published in Schuler *et al.* (1996), and is available in electronic form through a site on the World Wide Web at http://www.ncbi.nlm.nih.gov/SCIENCE96/. NHGRI, National Human Genome Research Institute; RHMC, radiation-hybrid mapping consortium; WICGR, Whitehead Institute Centre for Genome Research.

Locus	STS	GenBank identifier[a]	Map position (cR)[b]	Size (Bp)	Primers	Source[c]
Non-expressed						
D7S2502	sWSS2529	Z53757		61	L: GGAAACTGGAATTGTCTG R: CAAATGACTAAAACCACTG	NHGRI
	sWSS2431	G12875		84	L: ACAAAGTGCTGGGATTAC R: TTCCAACCATGAGCATAC	NHGRI
D7S2543	sWSS3040	Z51586		89	L: AAGTACAATAGGCACATC R: TCCCAGTATGTTTTCCTC	NHGRI
D7S2814	WI-5336	G04884		112	L: ATAAGACCAGATCAAGGACAGACC R: AACAATACACAAGCCCCTGC	WICGR
	sWSS305	L23672		175	L: ACACAGAGTTGGAAGGAG R: AGAAGATGGAGAGAAGGG	NHGRI
D7S1764	WI-455	G03007		249	L: CCTCTGATCATCTTCTGATCC R: GTAAAATGGATAGATTCGGACC	WICGR
	sWSS1398	G00166		159	L: GGCATTGAAACTCATATAC R: ACTGGGTTGAAATCTTGG	NHGRI
	sWSS843	G31652		165	L: GCTCTGCCATTGCTAATGATG R: CTGATTCTCCCTGTGCTAG	NHGRI
	sWSS2899	G16156		75	L: TTTCAGCCATTTCAGTCC R: ACAGTGTCTTCTTTCCTC	NHGRI
	sWSS2710	G16144		92	L: AATTATACCCTCCCAGAC R: TCATTGACTAAGGAAACAAC	NHGRI
	sWSS2099	G12756		82	L: CAACCAATTAACTGAGTG R: GTTGTATGGATGTTGTTATG	NHGRI
	sWSS377	L23698		218	L: TCCTAAAAATGGCTGTCC R: CCTACGCATAGAAACATAC	NHGRI

	sWSS844	A21831		194	L: TGGCTCAGGCCTGTATCATTTACAG R: ACCAGCAACTCAGTAGACGTCTCC	NHGRI
	sWSS845	L23744		141	L: AGCCAGGATAAAAACGGAGGTGGTC R: GATTGTGGCTAATAACAACAACAAG	NHGRI
D7S2347	WI-3876	G04662		203	L: GATTTCCCTAGAGCCTGGCT R: GAGAGAGACCTTTCAAAATGTTCC	WICGR
	sL12			140	L: AGCCAGGATAAAAACGGAGGTGGTC R: GATTGTGGCTAATAACAACAACAAG	Green and Olson, 1990
	sWSS1948	G12686		77	L: GTCATCTGATGGAAAATC R: TTTTGACATCGCTTCTGG	
	sWSS850	G31658		838	L: CAATGTGATTGGTGAAACTA R: CTTCTCCTCCTAGACACCTGCAT	NHGRI
	sWSS915	G00146		219	L: TTTTGCCATCACTTCATC R: GAAATCTCATTGTTTTAGGG	NHGRI
	sWSS376	L23697		172	L: CCTGACTAGAATCATCTAAG R: CTATCTTTTCAACCCTGAG	NHGRI
	sWSS859	L23745		119	L: CCACAACCAACTAATTTTGG R: GCTTAGGATTCTTACCCTC	NHGRI
	sWSS858	X16414		402	L: CCTGTATACTAGTAAAGGAGTGAG R: TTTAATCCCTAAGGGCCTGGAGAC	NHGRI
	sWSS357	G00015		120	L: TTAGCAGATCATCAGTATCC R: CCAGTAAGAAAACATACAGGTG	NHGRI
Expressed						
D7S2064E	SHGC-8678	Z25131	455.50	93	L: GTTTTCCTTGGCTGGGTTAATG R: TAAGTTTCCTCAGGACATGGC	RHMC
D7S2056E	SHGC-8668	Z19472	474.60	80	L: TCGATGCTCCTTCTACTCTTG R: AAGGCTTCTTTGTGGCGGTG	RHMC

D7S2117E	A001X05/ Cda1bd01/ SHGC-8711	G19737		108	L: TACTTTCAGTGATGTACTTTTTC R: CTATGTGAGGAATTACTTACAGA	RHMC
	A004D18	Z43678		135	L: GAAATCACTCAGTCATTACTTC R: AACTATTAAATTGCTCCTATAGC	RHMC
	A005X13	G20568		153	L: GGTCTGAAATAATACTAACA R: CTAGGAATATTTGAAGGAAT	RHMC
D7S2885	SHGC-13594	G14815	480.70	143	L: ATGCTACCTCCCACCACTTG R: AGGCCTCCTGTCACATTCAC	RHMC
D7S2036E/ *PPP1R3*	SHGC-12021/ SHGC-8664	G19433	483.20	104	L: ATTTGGATTGGTGAATGGGA R: ATATGAAGGAGCAAACTTATTCGC	RHMC
D7S2036E	SHGC-8664	F00032	483.20		L: CACCAATCCAAATGTTTGGGG R: GGAAGAGGGTAGACAAAAGAG	RHMC
D11S4645	SHGC-13610	G14826		134	L: CCATAGTTACAAATTCTTGGCTTC R: TGGCCTTGCCTGTGTGTAT	RHMC
PC4	stSG401/ A001W15	D20227		121	L: TCACTGGCATCAACAAAAGG R: TAAGGTTAGCAACCTTTCTGGC	RHMC
	SGC33824	T96957	514.40	137	L: CAAATTGAATGTACCTAAGCATTTG R: GAACTCGTAAGAAAATCTAGATTGC	RHMC
	WI-18254	H67393	514.50	150	L: TATACGGATCATGTATTTGTGTGA R: AAATGTCTAGGAACGGAAAACA	RHMC
	WI-17786	H44425	515.60	128	L: AAGAGCCCAGGTTGTTAGCA R: AGGTGAAGGTGTGAATTGTAAATG	RHMC
	WI-18408	D80021	520.20	150	L: GCTAGTCTATGAGTCCCTCCAG R: AGAAGACAGCCACATCTTTTAAA	RHMC
	stSG10289	H04293		130	L: GTCTAACAAGCAATAAAACAACA R: ATTCTTCCTCATATTCAATGACT	RHMC

	stSG3600	T67153		164	L: ATGTGTATAATATGTGTTCCTTC R: GGCTGAATTTCAGATTATT	RHMC
	stSG464	T32808		229	L: GGACATTCACACTCAATTGGG R: TGGCATCAAGAGAGCACAAG	RHMC
	stSG4807	R61547		124	L: GGTAGAAAATGGTGGAGGCA R: AGGGAATAGAATTAAATTGGGG	RHMC
D7S2785	WI-8693	T50974		106	L: AAAACTGTGTTGGAATAAGAAAACG R: AGTACCATACCTTACAGAGCTGGG	RHMC
D7S2787	WI-8726	T52325		124	L: TATATCCATGGACAGGCTTGG R: GAGACCATGATTTATATTGGATTGC	RHMC
D7S2756/ *CAVEOLIN -1*	SHGC-10385/ D29206	G11600	494.10	156	L: TTTAAAACAGACACTGGCATGG R: AGTTTACAGATTGAATCACAAAGCC	RHMC
	SHGC-31795	G26992		126	L: GTGCATTTTTAATCCAAAATAGAAA R: ACCTCGTGGGTGTTGAGG	RHMC
D7S2776/ *CAPZa2*	WI-7882	U03851		324	L: GATGCTACAGGAATTTCAAGCC R: TTATATTCTCAATGCTTTCAAAGCC	RHMC
	sWSS3428/ 7B07A03	AA077041		105	L: CAGACACTGTATTACAGG R: ATGGTAATGAGTTGGTGG	NHGRI
	WI-18209	H63719	515.20	128	L: CCTAAAGTCCACTTAAGCCAGG R: CCATAACCCAGGCTTACCAA	RHMC
D7S2097E	Cda0zb11/ SHGC-5660	Z39076	496.30	101	L: AAACTGGAATGAGAAGTCTG R: GATTTTCTGGTGGTGATG	RHMC
D7S2769/ *WNT2*	WI-7597	X07876	496.70	328	L: CAAGTGGTTTTACAGCTACCACC R: CACCCAATAGTTACAGAATATTGCC	RHMC
CFTR	CFEX1			933	L: GGAGTTCACTCACCTAAA R: ACACGCCCTCCTCTTTCGTG	Zielenski *et al.*, 1991
CFTR	CFEX3			309	L: CTTGGGTTAATCTCCTTGGA R: ATTCACCAGATTTCGTAGTC	Zielenski *et al.*, 1991

CFTR	CFEX4			438	L: TCACATATGGTATGACCCTC R: TTGTACCAGCTCACTACCTA	Zielenski *et al.*, 1991
CFTR	CFEX6b			417	L: TGGAATGAGTCTGTAACAGCG R: GAGGTGGAAGTCTACCATGA	Zielenski *et al.*, 1991
CFTR	CFEX10			491	L: GCAGAGTACCTGAAACAGGA R: CATTCACAGTAGCTTACCCA	Zielenski *et al.*, 1991
CFTR	CFEX12			426	L: GTGAATCGATGTGGTGACCA R: CTGGTTTAAGCATGAGGCGGT	Zielenski *et al.*, 1991
CFTR	CFEX20			473	L: GGTCAGGATTGAAAGTGTGCA R: CTATGAGAAAACTGCACTGGA	Zielenski *et al.*, 1991
D7S2742/ *CFTR*	D7S2742	M28668	497.10	265	L: AGCAGCATAAATGTTGACATGG R: TCTGGCTTGCAAAACACAAG	RHMC
D7S2114E	SHGC-8708	Z39427		132	L: GGAAGTCCAGAAATCATACTGC R: CCATTCTAGTCATTCCCCATC	RHMC
	A005W09/ Cda19h02	G20547		154	L: GCAGAATATACCTGTTGAAT R: TAGCCTTGGATTATTTCA	RHMC
D7S2757	SHGC-10294	G11397	499.20	237	L: AATTTTACAGCTTTAGGGCAAGG R: TCCTTTGCATCAATAAATCGC	RHMC
D7S2093E	A004D07/ SHGC-5654	Z41622	508.10	109	L: TAGGTTATATATCCATTCTTGC R: ACTAGTTACCTAAACAAGAATCC	RHMC
	A004D12	Z40586		214	L: TGTACAGAGTTTAATTTTACAGC R: TATAATGTACAACCAAGAATCTC	RHMC
PTPRZ1		M93426		130	L: GGGACTCACATCTGAGCATTG R: TAAATTTGGCGGCAGAAT	Krueger and Saito, 1992

Primer		Source
alu IV	CAGAATTCGCGACAGAGCGAGACTCCGTCTC	Cotter *et al.*, 1990
alu V	GTGAGCCACCGCGCCCGGCC	Cotter *et al.*, 1990
BK33	CTGGGATTACAGGCGTGAGCC	Nelson *et al.*,1989
BK34	CCACTGCACTCCAGCCTGGG	Nelson *et al.*,1989
TC-65	AAGTCGCGGCCGCTTGCAGTGAGCCGAGAT	Nelson *et al.*,1989
517	CGACCTCGAGATCTYRGCTCACTGCAA	Nelson *et al.*,1989

Table A3. Oligonucleotide primers used for inter*Alu* PCR

Primer	
Sequencing from pCR2.1 or pBluescript	
M13	Forward: TTGTAAAACGACGGCCAGTG Reverse: GGAAACAGCTATGACCATG
T7	GTAATACGACTCACTATAGGGC
T3	AATTAACCCTCACTAAAGGG
KS	TCGAGGTCGACGGTATC
SK	CGCTCTAGAACTAGTGGATC
RT-PCR from pSPL3	
SA2	ATCTCAGTGGTATTTGTGAGC
SA4	CACCTGAGGAGTGAATTGGTCG
SD2	GTGAACTGCACTGTGACAAGC
SD6	TCTGAGTCACCTGGACAACC

Table A4. Sequence of oligonucleotide primers used during sequencing and exon-amplification.