Involvement of p53 and RB-1 in the immortalisation of human cells

Noel Whitaker

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The Children's Medical Research Institute

University of Sydney

Summary

Normal diploid mammalian cells undergo a finite number of population doublings in culture before they undergo senescence [Hayflick & Moorhead, 1961]. In contrast, tumours often contain "immortalised" cells that exhibit an apparently unlimited *in vitro* and *in vivo* proliferative potential. Fusion of normal and immortalised cells usually results in hybrids with limited proliferative potential [Bunn & Tarrant, 1980; Muggleton-Harris & DeSimone, 1980] indicating that immortalisation is probably due to loss of normal gene function. Similarly, fusion of different immortalised human cell lines with each other often results in mortal hybrids, indicating that the cell lines have become immortalised via different genetic events. Such studies have identified at least four complementation groups for immortalisation, referred to as groups A, B, C and D (Pereira-Smith & Smith, 1988).

Some of the genes involved in the control of proliferative potential appear to have been identified. Studies of the transforming proteins of DNA tumour viruses have indicated the involvement of the p53 gene and the retinoblastoma susceptibility (RB-1) gene in senescence [reviewed in Shay *et al.*, 1991; Bryan & Reddel, 1994]. DNA tumour viruses that are able to increase the proliferative potential of human cells encode proteins that bind to, and inactivate the function of, the protein products (p53 and $p110^{RB}$, respectively) of these genes. Examples of such proteins include the simian virus 40 (SV40) large T antigen, the adenovirus E1A and E1B proteins, and the E6 and E7 proteins of the oncogenic human papillomaviruses: SV40 large T antigen binds both p53 and $p110^{RB}$, E1A and E7 bind to $p110^{RB}$, and E1B and E6 bind to p53. Likewise, down-regulation of p53 and RB-1 expression by antisense methods resulted in extension of the lifespan of normal human fibroblasts [Hara *et al.*, 1991]. Although functional inactivation of the p53 and RB-1 genes by DNA tumour virus genes or by antisense oligomer treatment results in an increased proliferative potential this is usually not associated with immortalisation, therefore other genetic changes must be required.

Other genes that might be involved in control of proliferative potential include the $p16^{INK4}$ gene [Serrano *et al.*, 1993] which has recently been shown to be mutated in many immortalised cell lines [Kamb *et al.*, 1994; Spruck *et al.*, 1994; Okamoto *et al.*, 1994]. In 29 cell lines from tumours of the lung, oesophagus, liver, pancreas and colon there was an inverse correlation between the presence of $p16^{INK4}$ protein and the presence of the protein product ($p110^{RB}$) of the RB-1 gene [Okamoto *et al.*, 1994]. A possible explanation of this finding is that the growth suppressive properties of $p110^{RB}$ are inactivated by its phosphorylation, a process which is inhibited by $p16^{INK4}$ binding to cdk4 [Serrano *et al.*, 1993]. Thus, loss of $p16^{INK4}$ expression may be the functional equivalent of loss or mutation of RB-1. Phosphorylation of $p110^{RB}$

is catalysed by the complex of cdk4 with cyclin D1 or D2. In addition, RB-1–induced growth arrest has been shown to be reversed by the overexpression of cyclins A, E [Hinds *et al.*, 1992], D2 and mutant, but not wild type, D1 [Ewen *et al.*, 1993; Dowdy *et al.*, 1993]. It is thus conceivable that the normal role of RB-1 in senescence could be abrogated by loss of $p16^{INK4}$ expression or over-expression of these cyclins.

The involvement of p53 and $p110^{RB}$ in the immortalisation of human cells was examined in this study utilising the well characterised model of SV40-induced immortalisation of human cells. In this model, the inactivation of both p53 and $p110^{RB}$ by the TAg has long been known to be important. SV40-induced immortalisation of human cells occurs in two phases. Initially the affected cells are transformed to form multilayered foci of proliferating cells, associated with an extension of the normal *in vitro* lifespan. Eventually this population of cells ceases proliferation and enters culture crisis. In some of these populations, a minority of cells recommence proliferation in the second phase of SV40-induced immortalisation, i.e., escape from crisis.

The requirement for inactivation of $p110^{RB}$ and p53 in SV40-induced immortalisation was examined by comparing the focus forming ability and extension of lifespan induced by the introduction of a wild type TAg expression plasmid with that induced by expression plasmids encoding either non-$p110^{RB}$- or non-p53-binding mutants of TAg. I also considered the possibility that mutation of RB-1 or p53 genes might give the cells an advantage that exceeds that conferred by binding of their protein products to TAg, and might contribute to escape from crisis. Potential involvement of these tumour suppressor genes in escape from crisis was determined by examining their status in SV40-immortalised cell lines.

In the second part of this study I have examined whether any of the cellular genes discussed above, with known or potential involvement in immortalisation or control of proliferative capacity, correspond to the previously described immortalisation complementation groups. I determined whether cell lines from each of the four complementation groups have mutations in p53 or RB-1, lack $p16^{INK4}$ expression, exhibit over-expression of the cyclin or MDM2 genes.

An intact $p110^{RB}$ binding region proved to be essential for both focus formation and extension of lifespan. Further, TAg-induced focus formation was reduced by co-transfection with an $p110^{RB}$ expression plasmid, indicating that binding to $p110^{RB}$ was necessary for this process. Binding of TAg to p53, in contrast, appeared to be unnecessary although it did significantly increase the efficiency of focus formation.

In the cell lines known to contain DNA tumor virus proteins (SV40 large T, HPV-16 E6 and E7, or adenovirus-5 E1A and E1B), there was no evidence of abnormalities of the p53, RB-1 or $p16^{INK4}$ proteins (Tables 4.1 and 4.2). Mutations of these genes presumably confer no selective advantage in the presence of viral proteins that bind to p53 and $p110^{RB}$. In contrast, in cell lines without these viral proteins, either wild type $p110^{RB}$ was absent (J82) or $p16^{INK4}$ expression was undetectable (all other cell lines). This novel finding in immortal cell lines indicates that mutational inactivation of RB-1, and loss of $p16^{INK4}$ expression, are functionally equivalent and potentially involved in the immortalisation of human cells.

p53 mutations, while not universal in non-virally immortalised cell lines, were common and found in each immortalisation complementation group (Table 4.2). In cell lines without p53 mutations it is not known whether alteration in some other gene results in inactivation of p53 function or otherwise substitutes for p53 mutations.

It has been known for several years that loss of functional p53 or $p110^{RB}$ is insufficient for immortalisation of human cells since cells expressing DNA tumor virus proteins that bind and inactivate p53 and $p110^{RB}$ have an increased proliferative potential but usually do not become immortalised without a period of crisis. Furthermore, since I found that there was no correlation between immortalisation complementation group and abnormalities of p53 (or MDM2), RB-1 (or $p16^{INK4}$), it is clear that other genes must also be involved in immortalisation.

Maintenance of telomere length by expression of telomerase may contribute to the immortal phenotype. The gene(s) encoding telomerase have not yet been identified, but telomerase activity, not detectable in normal human somatic cells, has been detected in most immortal human cell lines examined to date (Morin, 1989; Counter *et al.*, 1992; 1994; Klingelhutz *et al.*, 1994; Kim *et al.*, 1994). The combination of p53 and $p16^{INK4}$ mutations together with the presence of telomerase activity, however, is also insufficient for immortalisation. This is illustrated by the cell lines T98G and 143BTK-, which are both shown to contain telomerase activity [Whitaker *et al.*, 1995], mutant p53, and no detectable $p16^{INK4}$ expression, yet are in different complementation groups for immortalisation. This indicates that they contain further mutations in different genes responsible for their immortal phenotype. It is possible, however, that one or more of these genes are involved in repression of telomerase activity in normal somatic cells.

STATEMENT OF ORIGINALITY

The contents of this thesis have not been presented for the award of a degree at this or any other university. The research presented in this thesis is the work of the author, except where specifically indicated.

Noel Whitaker

PUBLICATIONS

The results presented in this thesis represent part of a long term study into the immortalisation of human cells, initiated in 1992 with somatic cell hybridisation analysis of immortalisation [Whitaker *et al.*, 1992]. The work presented has contributed to the following publications.

1. Maclean, K., Rogan, E.M., Whitaker, N.J., Chang, A.C.-M., Rowe, P.B., Dalla-Pozza, L., Symonds, G. and Reddel, R.R. *In vitro* transformation of Li-Fraumeni syndrome fibroblasts by SV40 large T antigen mutants. **Oncogene**, 9: 719-725, 1994.

2. De Silva, R., Whitaker, N.J., Rogan, E.M. and Reddel, R.R. Human papillomavirus type 16 E6 and E7 genes, like SV40 early region genes, are insufficient for immortalization of human mesothelial and bronchial epithelial cells. **Exp. Cell Res.**, 213: 418-427, 1994.

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Book Chapter

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6. Reddel, R., De Silva, R., Duncan, E., Maclean, K., Moy, E., Rogan, E., Warneford, S., Whitaker, N. and Zahra, D. SV40 early regions genes and the analysis of human cell immortalization. **Symposium on Aging, Immortalization and Neoplastic Transformation of Human Cells**, Okayama, Japan, 1994.

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ABBREVIATIONS

3'UTR	3'-untranslated region
4NQO	4-nitroquinoline 1-oxide
AA	amino acid
ATP	adenosine 5'-triphosphate
bp	base pairs
BSA	bovine serum albumin
CAK	cyclin activating kinase
CAP	Chloramphenicol
cdk	cyclin dependent kinase
cDNA	complementary DNA
ced	*Caenorhabditis elegans* development genes
CKII	casein kinase II
cyc	cyclin gene
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
dGTP	deoxyguanosine 5'-triphosphate
DME	Dulbecco's Modified Eagle's medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol-bis (b-aminoethyl ether) N,N,N',N'-tetraacetic acid
FAP	familial adenomatous polyposis
FBS	foetal bovine serum
G0	Gap 0 (resting phase) in the cell cycle
G1	Gap 1 (growth phase 1) in the cell cycle
Ha-MSV	Harvey murine sarcoma virus
HBS	HEPES buffered saline
HEPES	N-2-hydroxyethyl-piperazine-N'-2-ethane sulphonic acid
HNPCC	hereditary non-polyposis colon cancer
HPV	Human Papillomavirus
ICE	interleukin-1β converting enzyme
IGF-1	Insulin-like growth factor-1
kb	kilobases
kD	kilodalton
Ki-MSV	Kirstin murine sarcoma virus
LB	Luria-Bertani
LHC	Laboratory of Human Carcinogenesis medium
LN	Lesch Nyhan Syndrome
LTR	long terminal repeat
MMTV	mouse mammary tumour virus
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MOPS	3-[N-morpholino] propanesulphonic acid
mRNA	messenger RNA

NF	neurofibromatosis
NHDF	normal human diploid fibroblast
ori	origin of replication
p	passage
$p110^{RB}$	protein product of the RB-1 gene
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDL	population doubling
PET	PVP-EGTA-trypsin
PVP	polyvinylpyrrolidone
RB	retinoblastoma gene family
RB-1	retinoblastoma gene
REF	rat embryo fibroblasts
RNA	ribonucleic acid
RNAase	ribonuclease
RPMI	Roswell Park Memorial Institute medium
S	DNA synthesis phase of the cell cycle
SAG	senescence associated gene
SCLC	small cell lung carcinoma
SD	standard deviation
SDS	sodium dodecyl sulphate
ssDNA	single stranded DNA
SSC	sodium chloride-trisodium citrate buffer
SV40	Simian Virus 40
TAg	SV40 large transforming protein
tAg	SV40 small transforming protein
TE	tris-EDTA
Tris	tris (hydroxymethyl) aminomethane
TS	thymidylate synthetase
v/v	volume per volume
w/v	weight per volume
WT	Wilms' tumour
wt	wild type

Chapter 1

General Introduction

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Chapter 1

General Introduction

Whereas normal somatic cells have a finite proliferative potential, many cancer cells are able to divide an apparently unlimited number of times, i.e. they have become immortalised. The evidence that immortalisation is an important aspect of cancer cell biology is summarised below (section 1.1). Evidence that immortalisation results from inactivation of the function of putative growth suppressor genes that are active in normal cells is discussed in section 1.2.

For reasons that are described in section 1.3, much of what is known about the mechanisms of immortalisation has been obtained from studies of simian virus 40 (SV40)-immortalised human cells. SV40, like other DNA tumour viruses that can induce immortalisation, encodes an oncoprotein that binds and inactivates the protein products (p53 and $p110^{RB}$, respectively) of the p53 and retinoblastoma genes. Studies of the normal function of p53 and $p110^{RB}$ are reviewed in section 1.4, and the reasons for studying the role of these proteins/genes in immortalisation are described.

The studies described in this thesis were designed to investigate molecular mechanisms of cellular immortalisation, especially the roles of p53 and $p110^{RB}$ in this process. The specific aims of these studies are described briefly in section 1.5, with a more

detailed description of the aims being given in the introductions to chapters 3 and 4.

1.0 Senescence of somatic cells

Techniques are now well established for growing many types of human primary cells, i.e., cells isolated from tissue, and resulting secondary cells or cell strains. When provided with a suitable environment these cell populations grow exponentially, as illustrated in the growth curve in Figure 1.1. Eventually, however, the proliferation rate decreases and finally ceases, as indicated by the levelling of the growth curve in Figure 1.1. August Weismann [1891] first speculated that the somatic cells of higher animals would be found to have a limited proliferative potential. At that time, however, no experimental evidence was available to support this theory. In 1961 Hayflick and Moorhead [1961] reported that normal mammalian somatic cells have only a limited proliferative potential *in vitro*. The eventual loss of proliferative potential is termed senescence and cells are defined as senescent when they no longer respond to mitogens and when the population does not double within a set time period (two to three weeks, depending on the laboratory) [Smith & Pereira-Smith, 1990]. This is in contrast to quiescence, a non-proliferative state from which cells can be induced to resume proliferation.

Hayflick [1965] first proposed that the finite lifespan of unaltered diploid cell strains *in vitro* may be an expression of ageing at the cellular level and therefore an innate characteristic of the cells. This is supported by experiments *in vivo* where normal mouse

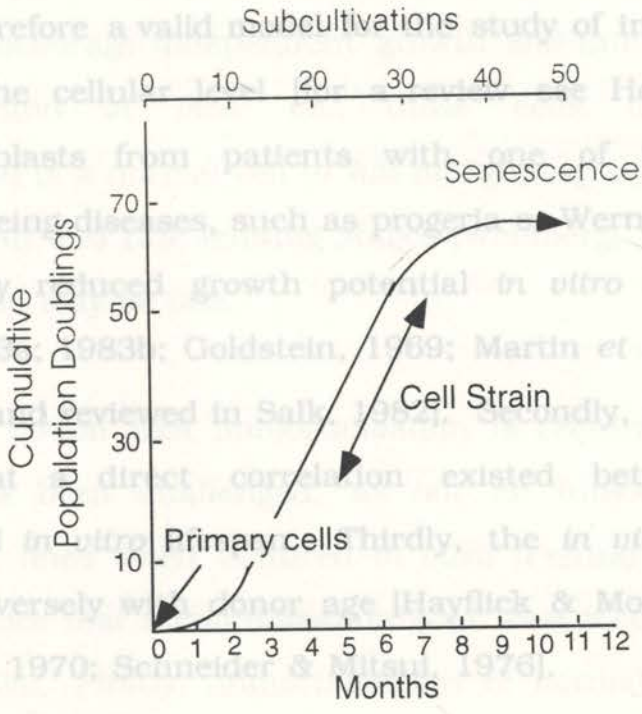


Figure 1.1. Stages of growth of human fibroblast cell strains in culture

The primary culture (referred to as phase I by Hayflick and Moorhead [1961]) terminates with the passaging of the first confluent sheet into a new culture vessel. These cells are a cell strain (phase II) and are characterised by exponential growth necessitating many subcultivations. Cell strains characteristically senesce (phase III), and are usually lost after a finite period of time; this is indicated by the levelling off of the growth rate at the top of the curve. After Hayflick and Moorhead [1961].

tissue can only be transplanted a finite number of times, in contrast to mouse tumours which can be transplanted indefinitely. There are other grounds for believing that *in vitro* senescence is related to the ageing of the organism and that the cell culture system is therefore a valid model for the study of intrinsic ageing changes at the cellular level [for a review see Holliday, 1988]. Firstly, fibroblasts from patients with one of the inherited premature ageing diseases, such as progeria or Werner's syndrome, have a greatly reduced growth potential *in vitro* [Thompson & Holliday, 1983a; 1983b; Goldstein, 1969; Martin *et al.*, 1970; Salk *et al.*, 1981; and reviewed in Salk, 1982]. Secondly, Röhme [1981] observed that a direct correlation existed between species longevity and *in vitro* lifespan. Thirdly, the *in vitro* lifespan is correlated inversely with donor age [Hayflick & Moorhead, 1961; Martin *et al.*, 1970; Schneider & Mitsui, 1976].

Senescence has been most extensively studied in fibroblasts and is characterised by a reduced number of cells at confluency, detachment of cells or failure to reattach after trypsinisation, variable cell size with the appearance of giant polyploid or multinucleate cells, excessive granularity and formation of autofluorescent compounds and secondary lysosomes [reviewed in Holliday, 1988]. These cells remain viable for long periods of time but eventually there are no cycling cells left, and population growth ceases. As cells in this population die they are not replaced and the population size decreases.

1.1 Relevance of immortalisation to cancer

In contrast to normal somatic cells, cancer cell populations often have altered or transformed growth properties *in vitro* and *in vivo*. These altered growth properties include ability to form tumours in nude mice, anchorage independent growth and unlimited lifespan in culture and *in vivo*, i.e., these cells are immortal. Transformation of a normal cell to full malignancy occurs in five or six independent and rate limiting stages [Weinberg, 1989] of which immortalisation may be one.

Although the notion that immortalisation is required for tumour formation has been challenged, as not all tumours will form immortal cell lines when cultured *in vitro* [Freund *et al.*, 1992], there is evidence that immortalisation is at least a common feature of tumour cells. Firstly, immortalisation of normal human cells, either spontaneously or induced by carcinogenic agents, is a rare event [McCormick & Maher, 1988; Namba *et al.*, 1988]. This is in contrast to the relative ease with which continuous cell lines can be obtained *in vitro* from human tumours, provided favourable growth conditions can be achieved [Klein, 1959; Pontén, 1976; Fogh *et al.*, 1977; Klein, 1979; Rheinwald & Beckett, 1981]. Secondly, telomerase activity, thought to be associated with immortality [Counter *et al.*, 1994a; Kim *et al.*, 1994], has been demonstrated recently in a wide variety of tumour types [Counter *et al.*, 1994b; Nilsson *et al.*, 1994; Kim *et al.*, 1994]. Finally, the correlation between oncogenicity of various Human Papillomavirus (HPV) strains *in vivo* and their ability to immortalise human cells in culture [Schlegel *et al.*, 1988; Pecoraro *et al.*, 1989; Woodworth *et*

al., 1989], suggests that immortalisation is an important aspect of at least HPV-induced oncogenesis.

Although it is not clear if immortalisation is necessary for tumourigenesis, the acquisition of the immortal phenotype has clearly been shown to be a prerequisite for neoplastic transformation in many experimental systems [Marshall, 1991]. Several investigators have found that in murine and human cell culture systems, immortal cell lines can be neoplastically transformed using various agents, while their mortal precursor counterparts cannot (see Table 1.1). Namba *et al.* [1988] suggested that the immortalisation process is a pivotal or rate-limiting step in the carcinogenesis of human cells. By occurring as an early event in the development of tumours, immortality may provide an enlarging population of transformation-competent cells within otherwise normal tissue. This is supported by the observation of Lee *et al.* [1989] that immortal rodent liver epithelial cells are possible precursor populations for the generation of hepatocellular carcinoma.

1.2 Analysis of immortalisation

Somatic cell hybridisation analysis of immortalisation

Somatic cell hybridisation studies have yielded some useful information about the molecular genetic events which result in immortalisation. Littlefield [1973] fused senescent normal human cells with each other and with young cells and found that he was unable to obtain hybrids that could grow to any significant extent, and concluded that ageing is dominant in such hybrids. His

Table 1.1. Examples of mammalian cell culture systems that require immortalisation for neoplastic transformation.

Transformation incompetent cell type	Transformation competent cell type	Transforming agent	Reference
SHD (primary Syrian hamster dermal fibroblasts)	'Immortalised' SHD cells	EJ bladder carcinoma c-Ha-*ras*1 oncogene	Newbold & Overell, 1983
HEK (primary human foreskin epithelial cells)	RHEK-1 (HEK cells immortalised with Ad12-SV40 hybrid virus)	Ki-MSV[a], activated human oncogenes (*ras*, *erb*B, *fms*, *fes*, *src*), X-irradiation or chemical carcinogens (MNNG[b], 4NQO[c]).	Rhim *et al.*, 1990
NHBE (normal bronchial epithelial cell strain)	BEAS-2B (NHBE cells immortalised with Ad12-SV40 hybrid virus)	Ki-MSV	Reddel *et al.*, 1988b
KMS-6 (cell strain from whole human embryo)	KMST-6 (^{60}Co γ-irradiated KMS-6 cells)	Ha-MSV[d]	Namba *et al.*, 1988
FS-2 (human neonatal fibroblasts)	FSSV cells (SV40 immortalised FS-2 cells)	Ki-MSV	O'Brien *et al.*, 1986

[a]Ki-MSV: Kirsten murine sarcoma virus
[b]MNNG: *N*-methyl-*N'*-nitrosoguanidine
[c]4NQO: 4-nitroquinoline oxide
[d]Ha-MSV: Harvey murine sarcoma virus

conclusion has since been confirmed by others [reviewed in Smith & Pereira-Smith, 1990].

In early studies [reviewed in Pereira-Smith & Smith, 1981], it was noted that hybrids of normal and SV40-immortalised human cells often had an apparently unlimited proliferative potential; it was therefore concluded that the phenotype of immortality is dominant. The interpretation of this result was that the senescent cells did not express some of the genes necessary for DNA synthesis and cell division and that these were turned on again in a dominant fashion in the immortal cells. This view was held until Bunn and Tarrant [1980] reported that some hybrids obtained from the fusion of HeLa cells with normal human diploid fibroblasts yielded hybrids that had limited division potential. If these non-doubling hybrid populations were maintained in culture, foci of dividing cells appeared at a frequency of about one to two per 10^5 cells. These hybrids had regained the immortal phenotype and could grow without limit. Many examples of mortal hybrids from the hybridisation of tumour-derived and SV40-immortalised cell lines to normal cells now exist (Table 1.2) lending support to the notion that senescence is dominant over immortality.

Four complementation groups for immortalisation

The implication of immortality being recessive is that immortal cells have lost the function of a growth regulatory gene(s) responsible for senescence. If two different immortal cell lines have lost the same gene or gene pathway, that is, they were immortalised by the same mechanism, then hybrids between these two cell lines should also be immortal. If two immortal cell lines

Table 1.2. Studies showing that fusion of immortalised human cell lines to normal human cells results in senescent hybrids.

Cell Line	Hybridised to Cell Strain[a]	Reference
HEB7A (CAP[b] resistant and tk[-c] derived HeLa)	GM377 (fibroblasts from LN[d] donor - formerly GM29 and referred to as GM1662 by Pereira-Smith & Smith [1981; 1983])	Bunn & Tarrant, 1980
CL-1 (SV40-transformed WI-38)	WI-38 (normal diploid fibroblasts)	Muggleton-Harris & DeSimone, 1980
VA13 (SV40-transformed lung fibroblast)	GM1662 (diploid skin fibroblasts from LN donor)	Pereira-Smith & Smith, 1981
VA13	CSC-301 (human foetal lung cells)	Pereira-Smith & Smith, 1981
GM 639 (SV40-transformed skin fibroblasts)	GM1662	Pereira-Smith & Smith, 1981
GM639	CSC-301	Pereira-Smith & Smith, 1981
HT1080 (fibrosarcoma)	GM1662	Pereira-Smith & Smith, 1983
T98G (glioblastoma)	GM1662	Pereira-Smith & Smith, 1983.
143BTk- (Ki-MSV - transformed osteosarcoma)	GM1662	Pereira-Smith & Smith, 1983.
GM847 (SV40-immortalised skin fibroblast)	CSC-303 (diploid fibroblasts)	Pereira-Smith & Smith, 1983
HeLa (cervical carcinoma)	Normal fibroblasts	Bosch *et al.*, 1990
GM847	T lymphocytes	Pereira-Smith *et al.*, 1990

[a] In all examples listed, hybridisations between human cell lines and normal human cell strains resulted in senescent hybrids.
[b] chloramphenicol
[c] thymidine kinase deficient
[d] Lesch Nyhan Syndrome

were immortalised by different mechanisms, then hybrids between these two cells should have a normal complement of growth regulatory genes, and thus should exhibit the senescent phenotype. Pereira-Smith and Smith [1983] fused different immortal cell lines to determine whether there was complementation resulting in senescent hybrids. When SV40-immortalised cell lines were fused to other SV40-immortalised cell lines there was no complementation and the hybrids were all immortal, but when SV40 immortalised cells were fused to cell lines immortalised by different mechanisms the hybrids were mostly mortal. This study was extended [Pereira-Smith & Smith, 1988] by fusing 21 different human immortal cell lines to one another in order to determine the number of complementation groups. All 21 lines were assigned to one of only four complementation groups, called groups A, B, C and D (Table 1.3) and this has now been extended to include additional cell lines [e.g. Ning *et al.*, 1991a; Whitaker *et al.*, 1992].

The initial results indicated that all of the SV40-immortalised cell lines, except for one SV40-transformed xeroderma pigmentosum line which was assigned to complementation group B, were in complementation group A (Table 1.3). This suggested that SV40-induced immortalisation usually occurred via the same genetic mechanism. However, it has subsequently been found that the hybrids formed by cells from two SV40-immortalised cell lines had a limited lifespan and that four additional SV40-immortalised cell lines were in complementation groups other than A [Whitaker *et al.*, 1992; Duncan *et al.*, 1993]. This indicates that the mechanism of SV40-induced immortalisation is not always the same.

Table 1.3 Cell lines assigned to complementation groups for indefinite proliferation potential

Cell line	Description	Group Assigned[a]
GM639	SV40-transformed skin fibroblasts	A
GM847	SV40-transformed skin fibroblasts ([b]HGPRT$^-$, Lesch Nyhan)	A
VA13	SV40-transformed lung fibroblasts	A
wtB	SV40-transformed keratinocytes	A
A268IV	SV40-transformed amnion	A
SVHF39	Origin-defective SV40-transformed bone fibroblasts	A
CW12 XP	SV40-transformed XP skin fibroblasts	A
HT1080	Fibrosarcoma (N-*ras*$^+$)	A
EJ (T24)	Bladder carcinoma (H-*ras*$^+$)	A
GM2096SV9	Origin-defective SV40-transformed xeroderma pigmentosum skin fibroblasts	B
T98G	Glioblastoma	B
HeLa	Cervical carcinoma	B
J82	Bladder carcinoma	B
CMV-Mj-HEL-1	Cytomegalovirus-transformed lung fibroblasts	C
143BTk-	TE85 osteosarcoma secondarily transformed by Kirsten murine sarcoma virus (Ki-*ras*$^+$)	C
293	Adenovirus-transformed embryonic kidney	not A, B or C
WI38-Ct1	Co-irradiated lung fibroblasts	D
SUSM-1	4NQO-transformed liver fibroblasts	D
A549	Lung carcinoma	not A, B or C
A2182	Lung carcinoma (Ki-*ras*$^+$)	D
A1698	Bladder carcinoma (Ki-*ras*$^+$)	D
BET-1A	SV40-immortalised bronchial epithelial cells	D[c]
KMST-6	γ-irradiation-transformed fibroblasts	D[d]
HepG2	Hepatoma cell line	D[e]
Jurkat	Cutaneous T cell lymphoma	D[f]
Hut-78	Acute T cell leukaemia	D[f]
Molt-4	Acute lymphoblastic leukaemia	D[f]
Daudi	Burkitt's lymphoma (EBV$^+$)	D[f]
Raji	Burkitt's lymphoma (EBV$^+$)	D[f]
MC/CAR	Plasmacytoma (EBV$^+$)	D[f]
U266	Myeloma	D[f]

[a] From Pereira-Smith & Smith, 1988, unless otherwise indicated

[b] HGPRT-: Hypoxanthine-Guanine Phosphoribosyl Transferase deficient

[c] Whitaker *et al.*, 1992; [d] Ogata *et al.*, 1993: [e] Ogata *et al.*, 1995: [f] Goletz *et al.*, 1995

The assignment of cells to four complementation groups implies that there are a limited number of mechanisms by which cells can become immortal. Thus, immortalisation must result from mutations or damage in specific genes rather than in random locations. Furthermore, the results from these experiments showed that complementation group was independent of cell type, embryonal layer of origin and tumour type. Thus, it is likely that only a small group of genes control senescence in all cells. Identifying and characterising the mechanisms of action of these genes may be valuable in understanding the cell cycle, ageing and development.

Microcell mediated chromosome transfer studies

Putative senescence genes have, in some cases, been localised at the chromosome level. Fusion of a Syrian hamster cell line (10W-2) to a human foetal lung fibroblast cell strain resulted in senescent hybrids [Sugawara *et al.*, 1990]. The loss of human chromosome 1 correlated with the appearance of proliferating segregants indicating that the loss of both copies of chromosome 1 was essential for continued proliferation of these hybrids. This was confirmed by showing that the introduction of a single copy of human chromosome 1 into the hamster cells by microcell fusion caused typical signs of cellular senescence, while the transfer of chromosome 11 had no effect on the growth of the cells. The frequency of senescence was greater if there was selective pressure for retention of human chromosome 1, by G418 selection of the neomycin resistance gene integrated in the introduced chromosome. Human chromosome 1 was shown to induce senescence in three human cell lines that had been assigned to

complementation group C [Hensler *et al.*, 1994], while inducing no change in the proliferative potential of cell lines representative of the other three groups. A derivative of chromosome 1, missing most of the q arm, was unable to induce senescence in any of the immortal cell lines. This can be interpreted to mean that chromosome 1q contains the senescence gene that is altered in cell lines in group C.

Fusion of a mouse A9 donor cell line containing Chinese hamster chromosome X to an immortal nickel-transformed Chinese hamster cell line resulted in senescence in 100% of colonies [Klein *et al.*, 1990]. However, senescence was seen in only 50% of colonies when Chinese hamster X chromosomes were transferred from a later passage A9 cell. Interestingly, the full senescence-inducing activity was restored upon demethylation of DNA by treatment with 5-azacytidine, suggesting that escape from senescence can occur by epigenetic mechanisms.

SV40-immortalised cell lines contain many chromosomal aberrations. An analysis of these aberrations indicated that chromosome 6q is frequently involved. Thus it was thought that chromosome 6q had a role in immortalisation of these cells and this was confirmed by induction of senescence upon microcell mediated transfer of chromosome 6q into two such fibroblast cell lines [Sandhu *et al.*, 1994]. One of these cell lines, SV/HF39, belongs to complementation group A, indicating that the gene corresponding to group A may lie on chromosome 6q.

Chromosome 4 was introduced into cell lines representative of each of the four complementation groups [Ning *et al.*, 1991b]. This resulted in senescence of three cell lines from group B but not of cell lines representative of the other three complementation groups, indicating that the gene whose disruption is associated with the immortalisation of group B cell lines, may lie on chromosome 4.

Introduction of chromosome 7, but not chromosomes 11 or 1, into two immortalised cell lines containing abnormalities on chromosome 7q, SUSM-1 and KMST-6, resulted in senescence [Ogata *et al.*, 1993]. Chromosome 7 was also shown to induce senescence in another cell line assigned to group D, HepG2 [Ogata *et al.*, 1995]. It is possible, therefore, that a gene responsible for loss of senescence in cell lines of complementation group D is located on chromosome 7.

Molecular analysis of senescence

Many genes have been isolated whose expression changes with senescence (Table 1.4). Some of these genes, such as statin [Wang *et al.*, 1985] and more recently $p21^{CIP1}$ (originally isolated as *sdi1*) [Noda *et al.*, 1994], are also associated with quiescence. Yet others appear to change in a way specific to the onset of, or escape from, senescence. The senescence-associated gene (SAG), for example, is expressed at a threefold higher level in senescent fibroblasts [Wistrom & Villeponteau, 1992] and the expression of p53 increases as cells approach senescence [Kulju & Lehman, 1995]. A 60 kD form of terminin, an alternative to the normal 90 kD form, is expressed as cells senesce [Wang & Tomaszewcki, 1991].

Table 1.4 Examples of altered protein content of senescent cells

Protein	State in senescent cells	Reference
c-fos (AP-1)	down regulated	Seshadri & Campisi, 1990; Riabowol *et al.*, 1992; Campisi, 1992
p34cdc2	down regulated	Richter *et al.*, 1991; Stein *et al.*, 1991
cycA, cycB	down regulated	Stein *et al.*, 1991
cdk2	down regulated	Stein *et al.*, 1991
statin	upregulated	Wang *et al.*, 1985
$p21^{CIP1}$	upregulated	Noda *et al.*, 1994
SAG	upregulated	Wistrom & Villeponteau, 1992
terminin	alternatively spliced	Wang & Tomaszewcki, 1991
IGF-1	expression inhibited	Farber *et al.*, 1993
$p110^{RB}$	hypo-phosphorylated	Stein *et al.*, 1990; Futreal & Barrett, 1991
p53	upregulated	Kulju & Lehman, 1995

Mortalin's subcellular localisation in mouse cells changes from cytosolic in mortal cells to perinuclear in immortal cells [Wadhwa *et al.*, 1993]. Subcellular localisation of mortalin correlates with immortalisation complementation group in human cells [Wadhwa *et al.*, 1995].

An important feature of senescent fibroblasts is that, unlike quiescent fibroblasts, they cannot be induced to enter S phase of the cell cycle [reviewed in Goldstein, 1990]. This suggests that mechanisms involved in the regulation of progression through the cell cycle are also involved in senescence. In contrast to quiescent cells, the expression of Insulin-like growth factor-1 (IGF-1) [Farber *et al.*, 1993] *c-fos* [Seshadri & Campisi, 1990; Campisi, 1992] and the cell cycle associated genes, *cdc2, cycA* and *cycB* [Richter *et al.*, 1991; Stein *et al.*, 1991], is not induced in senescent normal human diploid fibroblasts (NHDF) in response to mitogen stimulation. Finally, although both quiescent and senescent NHDF contained hypophosphorylated $p110^{RB}$, after serum stimulation only the quiescent NHDF cells phosphorylated $p110^{RB}$ and entered S-phase [Stein *et al.*, 1990; Futreal & Barrett, 1991].

Telomerase

A problem all immortal cells face is replication of the ends of the chromosomes, the telomeres. The telomeres contain many copies of short repetitive nucleotide sequences; in human cells the repeat is TTAGGG. Approximately 50 base pairs at each end of the chromosome fail to be replicated during each cell cycle [Harley *et al.*, 1990]. The telomeres probably function to protect the ends of the chromosomes and loss of the telomeric sequences in terminal

phase fibroblasts is thought to contribute to the dramatic increase in chromosomal abnormalities. The observed loss of telomeric DNA in somatic cells presumably leads to the eventual loss of essential sequences and may explain the loss of proliferative ability [Harley *et al.*, 1990]. A yeast model exists with the *est-1* mutation which causes gradual loss of telomeric DNA and eventual decrease in the cell population mimicking senescence in higher eukaryotic cells [Harley *et al.*, 1990].

Normal mammalian embryonic stem cells and germ cells appear to be able to proliferate indefinitely [Matsui *et al.*, 1992; Resnick *et al.*, 1992]. If telomere shortening is associated with senescence, then presumably these cells have a terminal polymerase activity to maintain telomere length. This conclusion is supported by the observation that in adults the telomeres of sperm are longer than those of other tissues [Allshire *et al.*, 1989; Cross *et al.*, 1989; Hastie *et al.*, 1990; de Lange *et al.*, 1990] and that their length is maintained throughout adult life [Harley *et al.*, 1992]. A ribonucleotide enzyme, telomerase, is responsible for the maintenance of telomere length in germ cells. The RNA component of this enzyme acts as the template for the addition of telomere repeat sequences onto the replicating chromosome. Bone marrow and peripheral blood lymphocytes have been shown to have a low level of telomerase activity, possibly due to the presence of haemopoietic stem cells [Counter *et al.*, 1995].

Telomerase was first identified in *Tetrahymena* [Greider & Blackburn, 1985] and has been isolated since from other unicellular organisms, as well as from immortalised human cells

[Morin, 1989; Counter *et al.*, 1992]. Telomerase activity has recently been shown to be a common feature of immortal cell lines [Counter *et al.*, 1992; Counter *et al.*, 1994a; Kim *et al.*, 1994].

1.3 SV40 model for immortalisation

1.3.1 Spontaneous immortalisation

The frequency of spontaneous immortalisation is between 10^{-5} to 10^{-6} for murine cells [McCormick & Campisi, 1991] and very rare in normal human cells [McCormick & Maher, 1988; Namba *et al.*, 1988; Boukamp *et al.*, 1988]. Shay *et al.* [1991] explain the difference between human and rodent rates of immortalisation by suggesting that at least two rate limiting stages for the immortalisation of human cells are required, while in murine cells, only one of these steps is rate limiting. It is because of this lack of background of spontaneous immortalisation that the majority of studies in the field of cellular ageing have utilised human cells and why a model system, such as SV40-induced immortalisation, is useful.

1.3.2 SV40-induced immortalisation

Although DNA tumour viruses, such as SV40, readily immortalise rodent cells [Jat & Sharp, 1986; Linder & Marshall, 1990] and often induce tumours in rodents [Lewis & Martin, 1979; van Dyke *et al.*, 1987; Choi *et al.*, 1988; Efrat & Hanahan, 1989] and tumourigenesis in rodent cells [Theile *et al.*, 1976], they only sometimes immortalise human cells [Chang, 1986; Reddel *et al.*, 1988b] and rarely induce tumourigenesis.

The SV40 virus is a member of the Papovavirus family and its genome is a double-stranded circular DNA containing 5243 bp. The genome is divided into two functionally distinct regions of approximately equal size named "early" and "late" according to the order in which they are transcribed during the infection cycle. The early region encodes three proteins referred to as large T antigen (TAg), small t antigen (tAg) and 17kT. The late region encodes the viral coat proteins. Between the coding sequences of these two regions is a small region that contains the origin of DNA replication (*ori*) and the early and late promoter/enhancers. Most immortalisation experiments have been done with the whole viral genome, either by infection with the wild type virus or by transfection of a plasmid containing the whole SV40 genome [reviewed in Chang, 1986]. In many recent experiments, only early region-containing subgenomic fragments of SV40 have been transferred into cells by transfection or infection with recombinant viruses.

SV40 genes are insufficient to immortalise cells

Although SV40 and variants of SV40 have occasionally been reported to establish (i.e., immortalise) a few types of human cells in a single step [Miranda *et al.*, 1988; Shearer & Taylor-Papadimitriou, 1981], SV40 genes usually induce immortalisation via at least two steps [Chang, 1986; Neufeld *et al.*, 1987; Bartek *et al.*, 1991; Reddel *et al.*, 1995]. When SV40-early region genes are transferred into normal human cells, foci of morphologically altered cells form. Cell populations derived from these foci have an extended *in vitro* lifespan, indicated by the thicker black line in Figure 1.2. This extended proliferative potential is finite, however,

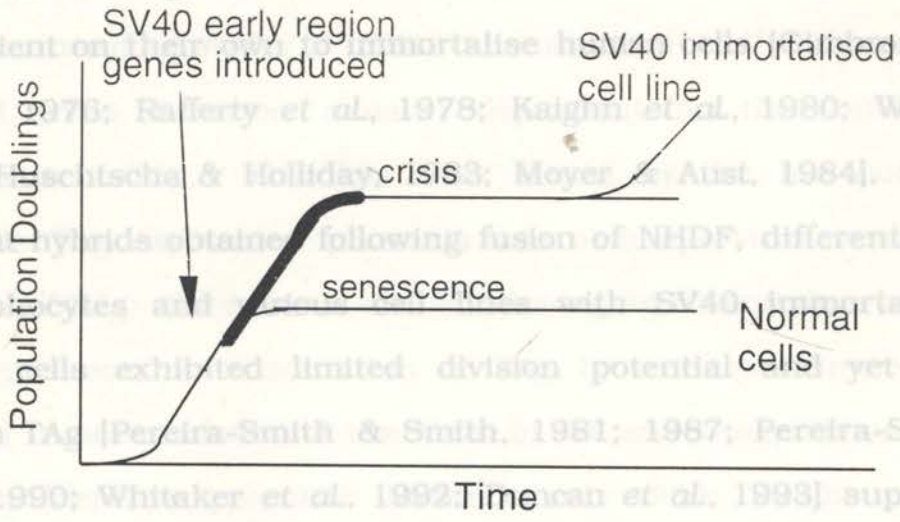


Figure 1.2 Model: Two stages in the immortalisation of normal human cells with the SV40 large tumour antigen (TAg)

The introduction of SV40 TAg increases the *in vitro* lifespan of normal human cells, as indicated by the thicker line (an increase of ~10-20 population doublings is common in fibroblasts). Eventually, however, this population of cells ceases to grow in a phase called "crisis". In some cultures, rare colonies of cells begin to proliferate and these colonies give rise to immortal cell lines.

and the cultures eventually reach a state of "crisis" when the cells undergo characteristic morphological changes and cease to proliferate [Chang, 1986]. This indicates that SV40 genes are insufficient on their own to immortalise human cells [Gimbrone & Fareed, 1976; Rafferty *et al.*, 1978; Kaighn *et al.*, 1980; Walen, 1981; Huschtscha & Holliday, 1983; Moyer & Aust, 1984]. The fact that hybrids obtained following fusion of NHDF, differentiated T lymphocytes and various cell lines with SV40 immortalised human cells exhibited limited division potential and yet still express TAg [Pereira-Smith & Smith, 1981; 1987; Pereira-Smith *et al.*, 1990; Whitaker *et al.*, 1992; Duncan *et al.*, 1993] supports the notion that SV40-genes are insufficient to immortalise cells.

Occasionally a subpopulation of proliferating cells arises that has escaped from crisis; these cells are usually able to be established as an immortalised cell line. This indicates that the SV40-early region genes do not directly immortalise cells, and that additional change is required. The frequency of escape from crisis ranges from 10^{-9} [E. Duncan, unpublished] to 10^{-5} [Shay *et al.*, 1993], and is clearly greater than that of spontaneous immortalisation. The frequency of this second immortalising step suggests that it is due to mutation, probably resulting in the loss of a normal function as indicated by the hybridisation results discussed above (Section 1.2). This change may be facilitated by the karyotypic derangements caused by SV40 [Sack & Obie, 1981; Huschtscha & Holliday, 1983; Meisner *et al.*, 1988; Ray *et al*, 1990], especially the N-terminal 147 amino acids of TAg [Woods *et al.*, 1994]. The combination of this genetic destabilisation and the 10 to 30 population doubling (PDL) extension in lifespan typically induced

by the SV40-early region presumably increases the probability that an immortalising mutation will occur.

The continued growth of SV40-immortalised cells that have escaped crisis, however, remains dependent on the continued expression of TAg. This has been shown by the use both of inducible TAg expression plasmids and of temperature sensitive mutants of TAg: the removal of the inducing agent and thus loss of TAg expression, or inactivation of TAg by a temperature shift, resulted in accumulation of the cells in G1 and reacquisition of a finite proliferative potential [Radna *et al.*, 1989; Wright *et al.*, 1989; Shay *et al.*, 1991; Resnick-Silverman *et al.*, 1991].

1.3.3 The early region genes have many effects

The three proteins encoded by the early region genes of SV40 virus are shown schematically in Figure 1.3. Some of the functions of TAg and tAg are discussed below. No functions, however, have yet been assigned to the newly discovered 17kT [Zerrahn *et al.*, 1993]. The SV40 expression plasmids used in the studies described in this thesis encode all of the early region proteins.

Small t antigen

tAg associates with PP2A, thereby inhibiting the ability of PP2A to dephosphorylate TAg [Scheidtmann *et al.*, 1991a; 1991b; Yang *et al.*, 1991]. This implies that tAg would allow a larger quantity of phosphorylated replication-inactive TAg to accumulate prior to the onset of PP2A-catalysed dephosphorylation. Accumulation of higher amounts of active TAg maybe significant for immortalisation

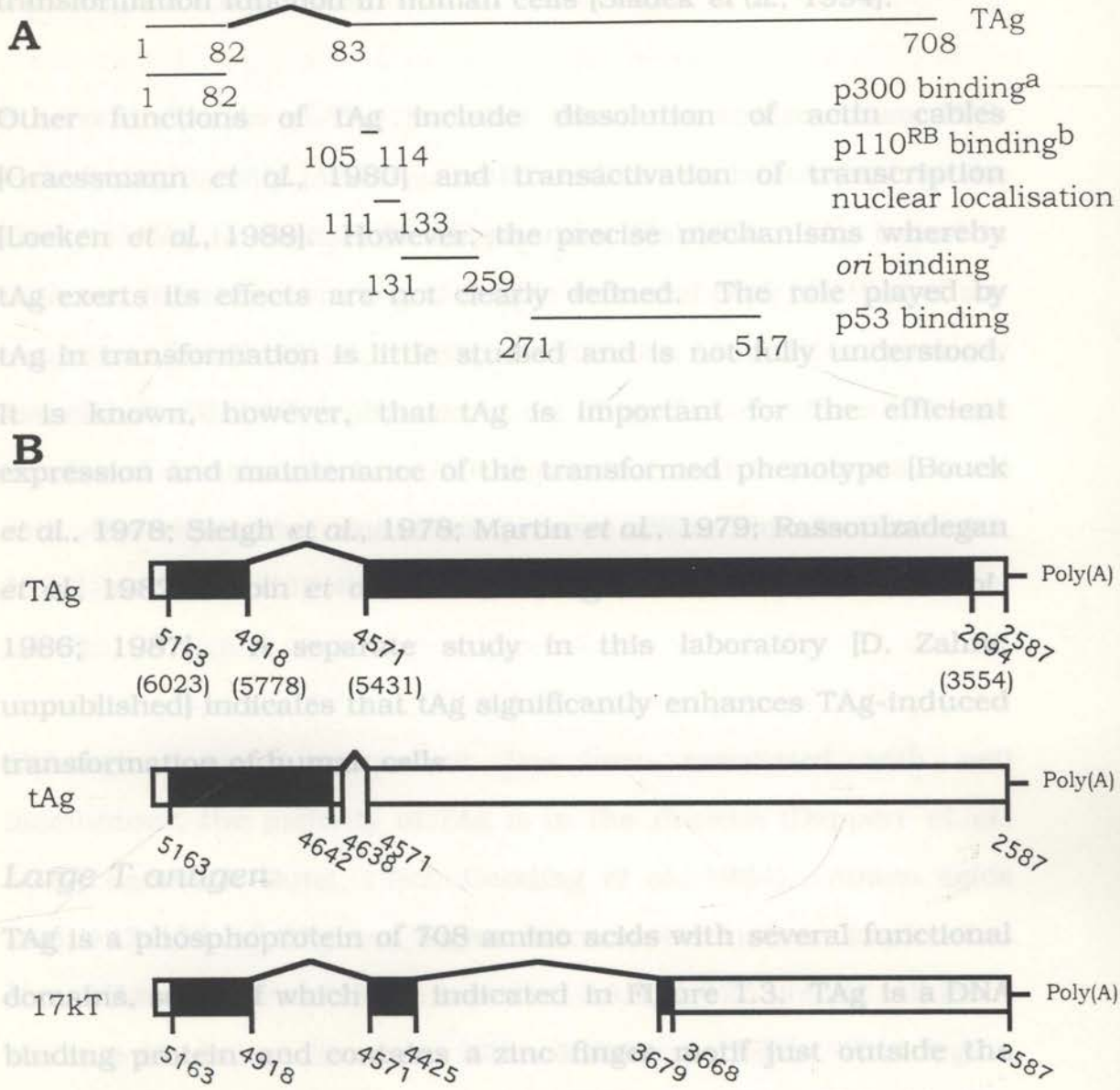


Figure 1.3. SV40 early region genes

A. Functional regions of large T antigen (see text). Numbering refers to amino acids

[a]Based on homology to p300 binding region of E1A [Yaciuk *et al.*, 1991]

[b]Region also binds p107 and p130

B. Schematic overview of the SV40 early region and the alternatively spliced mRNAs encoding large T antigen (TAg), small T antigen (tAg), and 17kT. Boxes represent spliced mRNAs; filled boxes represent translated regions. Numbering refers to nucleotides of the SV40 genome (nucleotide numbering for plasmid pRSV-T in parentheses).
Modified from Bryan and Reddel [1994].

studies as there is evidence that the amount of TAg affects its transformation function in human cells [Sladek *et al.*, 1994].

Other functions of tAg include dissolution of actin cables [Graessmann *et al.*, 1980] and transactivation of transcription [Loeken *et al.*, 1988]. However, the precise mechanisms whereby tAg exerts its effects are not clearly defined. The role played by tAg in transformation is little studied and is not fully understood. It is known, however, that tAg is important for the efficient expression and maintenance of the transformed phenotype [Bouck *et al.*, 1978; Sleigh *et al.*, 1978; Martin *et al.*, 1979; Rassoulzadegan *et al.*, 1982; Rubin *et al.*, 1982; Chang *et al.*, 1984; Bikel *et al.*, 1986; 1987]. A separate study in this laboratory [D. Zahra, unpublished] indicates that tAg significantly enhances TAg-induced transformation of human cells.

Large T antigen

TAg is a phosphoprotein of 708 amino acids with several functional domains, some of which are indicated in Figure 1.3. TAg is a DNA binding protein and contains a zinc finger motif just outside the DNA binding domain, a motif frequently found in DNA binding proteins [Klug & Rhodes, 1987]. TAg binds to two sites between the early and late regions of the SV40 genome. By binding to these sites, TAg auto-regulates its own expression, probably by repression of transcription [Rio & Tjian, 1983] and initiates viral DNA replication [Dean *et al.*, 1987; Wold *et al.*, 1987; Stahl *et al.*, 1988]. Newly synthesised phosphorylated TAg does not induce viral DNA replication. As the concentration of phosphorylated TAg

increases it is dephosphorylated by PP2A and this form is active in inducing DNA replication.

A second domain of TAg includes the active centre of an ATP-hydrolysing activity (ATPase), which is likely to be involved in the ability of TAg to unwind double-stranded (ds)-DNA. TAg bound to DNA can initiate unwinding of double stranded DNA (dsDNA) of any sequence [Stahl *et al.*, 1988], a function which is normally associated with DNA replication at *ori*. TAg also contains a helicase functional domain which is ATP-dependent and is also associated with the replication initiation process [Wun-Kim & Simmons, 1990], and a binding site for DNA polymerase α [Gannon & Lane, 1987].

Although a small component has been associated with cell membranes, the majority of TAg is in the nucleus [Deppert *et al.*, 1980; Santos & Butel, 1982; Gooding *et al.*, 1984]. Amino acids (AA) 127-133 of TAg act as an autonomous nuclear localisation signal [Lanford *et al.*, 1986], however, AA 111-125 greatly influence the rate of nuclear transport [Rihs & Peters, 1989]. This region includes the casein kinase II (CKII) phosphorylation sites $Ser^{111/112}$ [Jans & Jans, 1994] as well as a cdc2 site at Thr^{124} [Jans *et al.*, 1991].

TAg mutants without a nuclear localisation signal are able to transform established mouse or rat cell lines to tumourigenicity, but fail to immortalise primary cells [Lanford *et al.*, 1985], suggesting that nuclear TAg is required for immortalisation but not for transformation to tumourigenicity. One explanation for how

TAg can still be active while being excluded from the nucleus is indicated by the observation that p53 is retained in the cytoplasm via its complexing with cytoplasmic TAg [Montenarh *et al.*, 1987]. Nuclear localisation has been shown to be essential for the growth and transformation suppressor effects of p53 [Shaulsky *et al.*, 1991; Gannon & Lane, 1991; Ginsberg *et al.*, 1991].

TAg inactivates the protein products of the p53 and RB-1 tumour suppressor genes

Several other DNA tumour viruses (polyoma virus, papilloma viruses and adenoviruses) produce functionally similar proteins to SV40 [Vousden & Jat, 1989] that associate with many of the same cellular proteins, including the products of the RB-1 and p53 tumour suppressor genes (Table 1.5). These cellular genes have been shown to be involved in cell cycle regulation [Shay *et al.*, 1991; DeCaprio *et al.*, 1989]. Although the role of these interactions is not yet fully understood, it is likely that they play a critical role in growth regulation since diverse viruses appear to extend the proliferative potential by targetting the same two proteins [reviewed in Cantley *et al.*, 1991]. For SV40, at least, there is good evidence that this dysregulation of the cell cycle and extension of *in vitro* lifespan requires intact p53 protein and $p110^{RB}$ binding regions of TAg [Shay *et al.*, 1991; Resnick-Silverman *et al.*, 1991; Levine, 1990; Maclean *et al.*, 1994]. Chen & Paucha [1990], however, found the $p110^{RB}$ protein binding region dispensable for this function in primary mouse embryo fibroblasts.

Table 1.5 Examples of interactions between DNA tumour virus proteins and cellular proteins

Cellular Protein[a]	Viral Protein	Reference
$p110^{RB}$	SV40 large T	DeCaprio *et al.*, 1988
	Polyoma large T	Dyson *et al.*, 1989a;b
	HPV E7	Münger *et al.*, 1989
	Adenovirus E1A	Whyte *et al.*, 1988
p107/p120	SV40 large T	Ewen *et al.*, 1989
	HPV E7	Davies *et al.*, 1993
	Adenovirus E1A	Whyte *et al.*, 1989
		Dyson *et al.*, 1989a
p130/RB-2	Adenovirus E1A	Harlow *et al.*, 1986
	SV40 large T	Ludlow & Skuse, 1995
	HPV E7	
p300	Adenovirus E1A	Harlow *et al.*, 1986
	SV40 large T[b]	Yaciuk *et al.*, 1991
p53 protein	SV40 large T	Linzer & Levine, 1979
	Adenovirus E1B	Lane & Crawford, 1979
	HPV-16/18 E6	Sarnow *et al.*, 1982;
	EBV EBNA5	Werness *et al.*, 1990
	Lymphotrophic papovavirus large T	Szekely *et al.*, 1993
		Symonds *et al.*, 1991
cycA	Adenovirus E1A	Giordano *et al.*, 1989
	HPV E7	Pines & Hunter, 1990
	SV40 large T	Tommasino *et al.*, 1993
		Adamczewski *et al.*, 1993
DNA polymerase α	SV40 large T	Donrieter *et al.*, 1990
cycE	Adenovirus E1A	Faha *et al.*, 1993
cdk2	Adenovirus E1A	Tsai *et al.*,, 1991
	SV40 large T	Adamczewski *et al.*, 1993
PP2A	Polyoma middle T	Pallas *et al.*, 1988
	Polyoma small t	Walter *et al.*, 1988
	SV40 small t	Walter *et al.*, 1989
		Pallas *et al.*, 1990

[a]Cellular proteins that interact with viral proteins associated with transformation and/or immortalisation of cells.

[b]Suggested by structural homology to p300 binding region of E1A.

The minimum region of TAg that binds $p110^{RB}$, AA 105-114, has been shown to be sufficient, as a synthetic peptide, for $p110^{RB}$ binding *in vitro* [DeCaprio *et al.*, 1989]. Although the presence of TAg does not affect the cyclical nature or the gross timing of the phosphorylation of $p110^{RB}$ [Ludlow *et al.*, 1990], TAg has been shown to bind only the active, hypophosphorylated form of $p110^{RB}$ [Ludlow *et al.*, 1989].

The region of TAg necessary for p53 binding is less well defined, although an intact region AA 271-517 is required [Mole *et al.*, 1987; Schmeig & Symmons, 1988]. The length of this region suggests that the binding site is formed by folding of TAg. Deletions and some point mutations in this region disrupt this binding, presumably due to alteration of the secondary structure of TAg which disrupts the p53 binding site. The N-terminus of TAg has been reported to be sufficient for transforming mouse cells, indicating that binding to p53 is not required for transformation of these cells. A study of human cells, however, showed that disruption of the p53-binding region severely reduced focus formation and possibly abrogated extension of lifespan associated with wild type TAg [Maclean *et al.*, 1994].

From the discussions above, it appears that the function of SV40 early region genes in the immortalisation of human cells is two-fold: firstly, it exerts cell cycle arrest override activity, thus stimulating cells to proliferate beyond their normal lifespan and provide a larger transformed population; and secondly, it induces karyotypic instability and thus provides an environment for the occurrence of mutations that allow cells to escape senescence.

The binding of TAg to p53 and $p110^{RB}$ appears to be integral to the role of SV40 in the immortalisation of human cells.

1.4 The p53 and RB-1 tumour suppressor genes

Tumour suppressor genes normally function to negatively regulate cell proliferation and thereby maintain the nonmalignant phenotype. Mutations of tumour suppressor genes are usually recessive, i.e. it is the loss of function of tumour suppressor genes which results in tumourigenesis. This is supported by the fact that tumourigenicity can be suppressed in hybrids between normal and malignant cells, and reversion to a malignant phenotype is associated with chromosomal loss. Many tumour suppressor genes are associated with heritable cancer syndromes, such as NF1 in Neurofibromatosis type 1 (von Recklinghausen's disease), WT1 in Wilms' tumour, APC in familial adenomatous polyposis (FAP), MSH2, MLH1 and related genes in hereditary non-polyposis colon cancer (HNPCC), BRCA1 and BRCA2 in familial breast cancer, p53 in Li-Fraumeni syndrome and RB-1 in hereditary retinoblastoma. Reintroduction of tumour suppressor genes into cells that lack the endogenous wild-type gene has validated the tumour suppression properties of some of these genes. Both RB and p53 have been tested in this manner and found to suppress the growth rate and/or tumourigenic potential of transformed cells [Huang *et al.*, 1988; Baker *et al.*, 1990; Diller *et al.*, 1990; Mercer *et al.*, 1990a; 1990b].

1.4.1 The p53 gene

A lot of information has been published concerning p53 but much of what is known about its normal role was summarised in its description as the "guardian of the genome" [Lane, 1992]. If DNA is damaged, p53 accumulates and switches off replication to allow extra time for its repair. If the repair fails, p53 may trigger cell suicide by apoptosis [Yonish *et al.*, 1991]. Tumour cells in which p53 is inactivated by mutation, or by binding to host or viral proteins, cannot carry out this arrest. They are therefore genetically less stable and will accumulate mutations and chromosomal rearrangements at an increased rate, leading to rapid selection of increasingly malignant clones.

The p53 protein (53 kD in mouse and 55 kD in human cells) is a highly conserved nuclear phosphoprotein. A cross-species comparison of p53 proteins shows five highly conserved regions within the AA 13-19, 117-142, 171-181, 234-258 and 270-286 [Soussi *et al.*, 1990]. These five evolutionarily conserved domains within the coding regions are regarded as essential to the function of p53 [Pavletich *et al.*, 1993].

The p53 gene has been found to be mutated in at least some cases of almost every type of human cancer so far examined. Functional loss of p53 occurs most commonly via loss of one allele and point mutation, usually missense, of the second allele giving rise to an altered protein [Baker 1989; Nigro *et al.*, 1989; Vogelstein, 1990; Takahashi *et al.*, 1989; Iggo *et al.*, 1990; James *et al.*, 1989; McKay *et al.*, 1988; Prosser *et al.*, 1990; Kelman *et al.*, 1989]. This suggests that the wild-type (wt) tumour suppressor p53 is often

dominant over the mutant p53 (but see the comments below about dominant mutants).

A review of p53 mutations in various human tumours by Levine *et al.* [1991] indicated a pattern to the point mutations of the p53 gene. Firstly, most are missense mutations, giving rise to an altered protein for which there would seem to be positive selection. Secondly, the mutations are not randomly scattered. The majority are clustered between AA 130 and 290 and most of these are localised in four of the conserved regions of the protein identified by Soussi *et al.* [1990] (AA 117-142, 171-181, 234-258 and 270-286). Finally there are at least three mutation hot spots affecting AA 175, 248 and 273. This non-random pattern of point mutations of p53 supports the notion that at least some forms of mutant p53 may give cells a growth advantage over and above the effect of loss of normal p53 function, that is, some mutant p53 genes may act as oncogenes [Gannon *et al.*, 1990]. Interestingly, many functional mutations of p53 appear to produce a common conformational effect [Gannon *et al.*, 1990], i.e., different mutant p53 proteins are recognised by the same p53 conformation specific antibodies.

The p53 mRNA level is very low in growth arrested cells but is induced upon serum stimulation [Reich & Levine, 1984]. In continuously proliferating cells the mRNA levels do not vary significantly throughout the cell cycle [Khochbin *et al.*, 1988]. In the presence of DNA damaging agents such as irradiation and chemotoxins, the p53 level is increased, primarily by the post-transcriptional stabilisation of the protein. The mRNA contains a

long 3' untranslated region (3'-UTR) that is possibly also involved in the stabilisation of the mRNA molecule [Harlow *et al.*, 1985]. The trigger for the accumulation of p53 appears to be single stranded (ss)-DNA and DNA breaks, to which the molecule has been shown to bind [Steinmeyer & Deppert, 1988; Bakalkin, *et al.*, 1994; 1995; Jayaraman & Prives, 1995].

Accumulation of p53 has a number of effects, largely geared towards inhibiting cell cycling and facilitating DNA repair. An important factor in this is the transcriptional activity of p53, which is able to induce the expression of genes which contain p53 recognition sequences in the promoter. Genes reported to be upregulated by wild-type p53 include murine double minute 2 (MDM2) [Momand *et al.*, 1992; Oliner *et al.*, 1992; Barak & Oren, 1992], kappa immunoglobulin [Aloni-Grinstein *et al.*, 1993], the DNA-repair associated gene GADD45 [Kastan *et al.*, 1992], RB-1 [Shiio *et al.*, 1992] and the cyclin-dependent kinase inhibitor $p21^{CIP1}$ [Harper *et al.*, 1993; El Deiry *et al.*, 1993; 1994; Dulic *et al.*, 1994]. p53 also down-regulates other genes whose promoters do not contain p53 recognition sequences. Cellular genes down-regulated by p53 include proliferating cell nuclear antigen (PCNA), histone 3 [Mercer *et al.*, 1990a; 1990b], c-fos, c-jun, [reviewed in Donehower & Bradley, 1993], B-*myb*, and DNA polymerase-α [Lin *et al.*, 1992].

As mentioned, the transactivation is dependent on the presence of specific sequences in the promoter regions of the p53-regulated genes. This indicates the importance of the specific dsDNA binding function of p53 [Kern *et al.*, 1991] for its role in

transcriptional regulation and, significantly, functional mutants of p53 do not bind these specific DNA sequences. The region of p53 that binds to recognition sequences is AA 91-309 [Bargonetti *et al.*, 1993], and this is commonly mutated in human cancers, often resulting in abrogation of DNA-binding. DNA-binding has also been shown to be dependent upon the ability of p53 to oligomerise. The C-terminus contains the dimerisation (AA 334-356) and tetramerisation (AA 363-386) domains [Sturzbecher *et al.*, 1992; Taurina and Jenkins, 1993]. p53 is predominantly found *in vivo* as tetramers, which are able to bind DNA [Friedman *et al.*, 1993].

Oligomerisation of the mutant p53 with the wt p53 has been demonstrated *in vivo* [Eliyahu *et al.*, 1988; Rovinski & Benchimol, 1988], and could explain how some p53 mutants may be able to inactivate or inhibit the functions of wild-type p53. The existence of dominant-negative p53 mutants is supported by the observation that transgenic mice carrying a mutant murine p53 allele (along with two endogenous wild-type copies) have offspring with a much higher risk of developing cancer [Lavigueur *et al.*, 1989] while the introduction of mutant p53 does not accelerate tumour development in p53 nullizygotes [Harvey *et al.*, 1995].

Both wild type and mutant p53 bind to a nuclear phosphoprotein, MDM2, which conceals the N-terminal transactivating domain of p53 and has been shown to abrogate the p53 induced G1 block in the cell cycle [Khatib *et al.*, 1993; Reifenberger *et al.*, 1993; 1994]. The fact that p53 function is inhibited by MDM2 suggests that amplification/overexpression of MDM2 may constitute an

alternative pathway to mutational inactivation of p53 [Oliner *et al.*, 1992]. Indeed, many subsequent studies have documented the amplification and/or overexpression of MDM2 in a subset of tumours not containing p53 mutation [Ladanyi *et al.*, 1993; Reifenberger *et al.*, 1993; Forus *et al.*, 1993; Leach *et al.*, 1993; Habuchi *et al.*, 1994; Lianes *et al.*, 1994]. It now appears that overexpression of MDM2 does not necessarily correlate with amplification [Lianes *et al.*, 1994; Flørenes *et al.*, 1994] and some tumour cells both overexpress MDM2 and contain mutant p53 [Flørenes *et al.*, 1994]. It should also be noted that MDM2 maps to chromosome 12q14.3-15 [Heighway *et al.*, 1994] and the region amplified in tumour cells is frequently quite large and includes numerous flanking genes. Genes located centromeric to MDM2 that are variably coamplified with it include SAS [Jankowski *et al.*, 1994], cdk4 [Khatib *et al.*, 1993], CHOP [Aman *et al.*, 1992] and GLI [Kinzler *et al.*, 1987]. Each of these genes can be plausibly related to the regulation of the cell cycle.

The net function of p53 appears to be in maintenance of the genome rather than normal cell cycle progression control. Strand breaks appear to be a cue for the induction of p53 [Nelson & Kastan, 1994], suggesting that p53 acts as a cell cycle checkpoint control protein, temporarily halting progression through the cell division cycle until any DNA damage that has previously occurred has been repaired [Tominaga *et al.*, 1992]. Loss of this normal function may result in the accumulation of damage in many other genes, including other tumour suppressor genes [Milner, 1991]. The p53 protein may therefore play a key role in maintaining genetic stability.

1.4.2 The RB-1 gene

Like p53, RB-1 is highly conserved. The encoded protein, $p110^{RB}$, is present in all normal mammalian cells (110 kDa in human and 105 kDa in murine cells [Lee *et al.*, 1987; Bernards *et al.*, 1989]). Loss of RB-1 function is also associated with a wide variety of tumours such as osteosarcoma [Friend *et al*, 1986; Fung *et al.*, 1987], soft tissue sarcomas [Abramsom *et al.*, 1984], breast carcinoma [Lee *et al.*, 1988], bladder carcinoma [Horowitz *et al.*, 1990; Logothetis *et al.*, 1992], small cell lung carcinoma (SCLC) [Horowitz *et al.*, 1990] and non-SCLC [Reissmann *et al.*, 1993].

The $p110^{RB}$ protein has been identified as a nuclear phosphoprotein [Lee *et al.*, 1987] which has a key role in controlling the cell division cycle [Mihara *et al.*, 1989]. It has also been shown to have a critical role in normal mouse neurogenesis and haematopoiesis [Lee *et al.*, 1992; Jacks *et al.*, 1992; Clark *et al.*, 1992]. By subverting $p110^{RB}$, the DNA tumour viruses appear to induce continuous cell cycling.

RB-1 is normally expressed throughout the cell cycle. Consistent with its ubiquitous expression, the RB-1 promoter has many characteristics similar to those of housekeeping gene promoters [Hong *et al.*, 1989]. Regulation of $p110^{RB}$ appears primarily to be by its cyclical phosphorylation/dephosphorylation. The phosphorylation of $p110^{RB}$ fully accounts for its heterogeneity on SDS-PAGE, where it ranges from 110-114 kD [Takahashi *et al.*, 1991]. Only the hyperphosphorylated form of $p110^{RB}$ appears to be active in inhibiting cellular proliferation.

Association of $p110^{RB}$ with transcriptional factors Sp1 (via $p110^{RB}$ binding to an inhibitor SP1-I) [Chen *et al.*, 1994], ATF-2 [Kim *et al.*, 1992], MyoD and myogenin [Gu *et al.*, 1993a] is not well understood but is proposed to cause transcriptional activation. On the other hand, $p110^{RB}$ binding to other transcriptional factors E2F (also known as DRTF1) [Bagchi *et al.*, 1991; Chellappan *et al.*, 1991; Hiebert *et al.*, 1992; Zamanian & La Thangue, 1992; Helin & Harlow, 1993], Elf-1 [Wang *et al.*, 1993], and TBF, via the binding of $p110^{RB}$ to PU.1 [Hagemeier *et al.*, 1993], c-myc and N-myc [Rustgi *et al.*, 1991], has been proposed to result in repression of transcription. The interaction between E2F and $p110^{RB}$ is the best studied and appears critical for the ability of $p110^{RB}$ to regulate cell growth [Nevins, 1992].

E2F consists of a group of proteins, including E2F-1 and DP-1, that bind to DNA as a heterodimeric transcription factor [Girling *et al.*, 1993]. E2F binds exclusively to hypophosphorylated $p110^{RB}$ via an ~18 residue region in its C-terminal transactivating unit [Helin *et al.*, 1992]. While bound by hypophosphorylated $p110^{RB}$, E2F retains DNA binding activity and functions as a sequence-specific transcriptional repressor [Dalton, 1992; Hamel *et al.*, 1992; Hiebert *et al.*, 1992; Weintraub *et al.*, 1992; Zamanian & La Thangue, 1992; Adnane *et al.*, 1995]. Phosphorylation of $p110^{RB}$, or the presence of viral proteins such as TAg, disrupts the E2F/$p110^{RB}$ complex generating "free" transcriptionally active E2F [Nevins, 1992].

E2F binding sites have been identified in the promoter region of a variety of cellular genes involved in the control of cell proliferation

and associated with the S-phase of the cell cycle. In particular, E2F mediates the activation late in G1 phase of the B-myb [Lam & Watson, 1993], c-myc [Oswald *et al.*, 1994], cycA [Henglain *et al.*, 1994], DHFR [Blake & Azizkhan, 1989; Means *et al.*, 1990; 1992; Slansky *et al.*, 1993], cdc2 [Dalton, 1992], RB-1 [Hamel *et al.*, 1992] and thymidylate synthetase (TS) [Johnson, 1994] genes.

The region of RB-1 conserved in each of the RB family members, the RB pocket, and much of the adjacent C-terminus must be intact for stable binding to E2F [Qin *et al.*, 1992; Qian *et al.*, 1992; Hiebert, 1993; Huang *et al.*, 1992] and is sufficient for inducing G1-phase arrest in RB-/- cells [Qin *et al.*, 1992; Goodrich *et al.*, 1991]. To date, all spontaneously occurring RB-1 mutations which do not grossly compromise $p110^{RB}$ stability map to this E2F/TAg/E1A binding, or pocket, region [Bookstein *et al.*, 1990; Horowitz *et al.*, 1989; 1990; Kaye *et al.*, 1990; Shew *et al.*, 1990a; 1990b; Hu *et al.* 1990; Kaelin *et al.*, 1991]. The critical pocket region of $p110^{RB}$ is conserved in at least two other proteins within the RB family, p107 [Whyte *et al.*, 1989] (also referred to as p120 due to the variation in apparent size of the protein on SDS-PAGE) and p130 [Mayol *et al.*, 1993; Yeung *et al.*, 1993; Li *et al.*, 1993].

1.4.3 p53 and RB in the cell cycle

This is certainly not a comprehensive description but serves to link the functions of p53 and $p110^{RB}$. Although p53 and $p110^{RB}$ have different biological functions their known biochemical functions appear to be united in the cell cycle. Specifically they act at three checkpoints in the cell cycle, the restriction or R-point,

the G1/S and the G2/M checkpoints, each of which appear to be disrupted in immortalised cell lines.

Restriction-point

Like yeast cells, mammalian cells are sensitive to mitogenic stimuli until they reach a restriction point late in G1, after which they can complete their division if supplied with factors that support their viability [Pardee, 1989]. This restriction point, or R-point, in mammals is the equivalent of START in yeast. The R-point seems to be under the control of an accumulation of unstable proteins whose properties match those of cyclins [Pardee, 1989]. The D-type cyclins (cycD) are the most likely proteins involved as microinjection of antibodies against the cycD into normal fibroblasts during G1 phase prevents their entry into S phase, but injection near the G1/S transition does not [Baldin *et al.*, 1993; Quelle *et al.*, 1993] (see Figure 1.4).

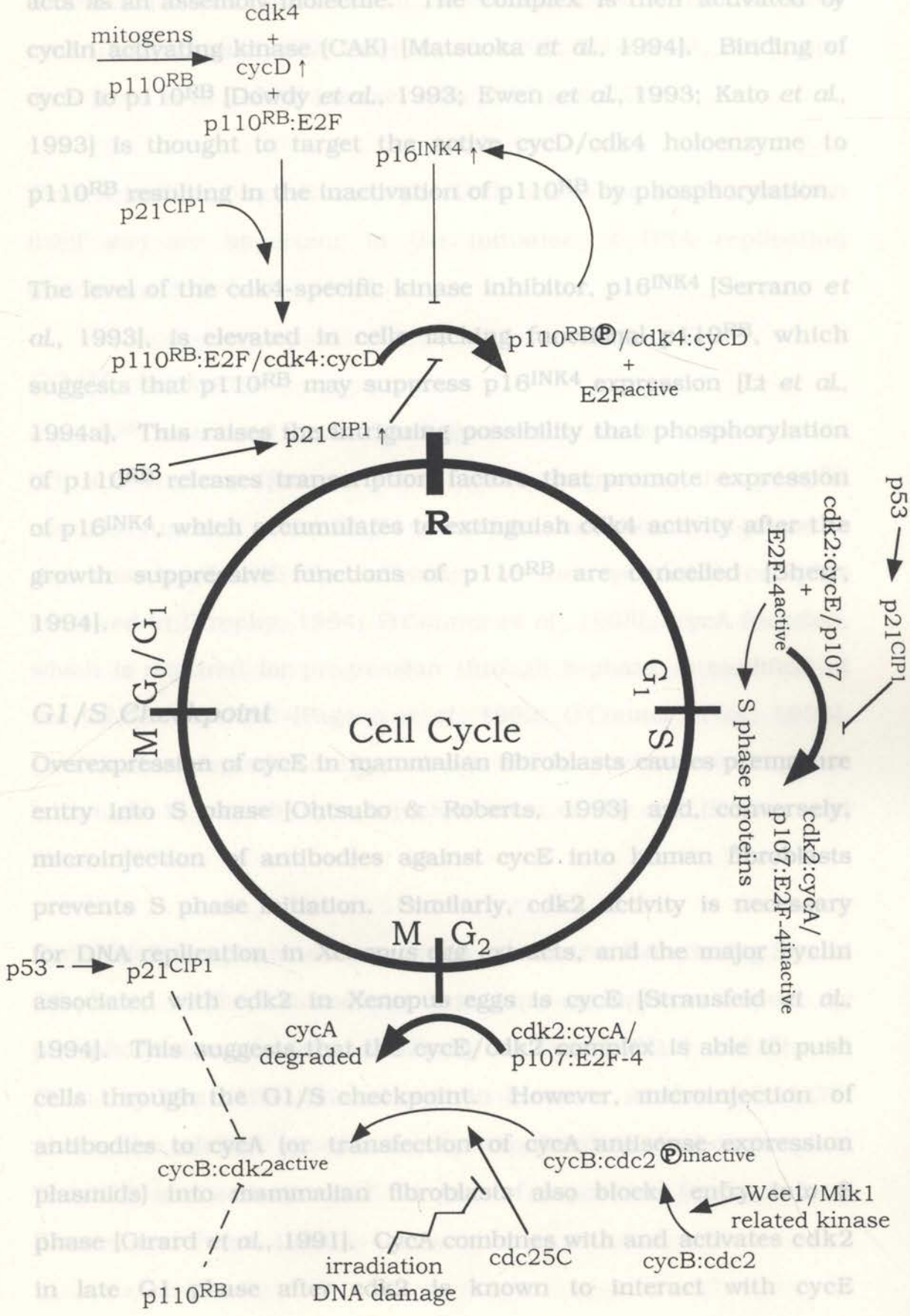
The release of "free" active EF2 from $p110^{RB}$, by phosphorylation of $p110^{RB}$ by the cycD/cdk4 holoenzyme [Xiong *et al.*, 1992; Kato *et al.*, 1993; Bates *et al.*, 1994; Meyerson & Harlow, 1994], appears the most likely candidate mechanism for the R-point. Induction of D-type cyclin expression is dependent on mitogen stimulation, and cycD is quickly degraded in the absence of mitogens [Matsushime *et al.*, 1991; Won *et al.*, 1992]. In contrast, cdk4 is present throughout the cell cycle but the cycD/cdk4 kinase activity is not present until mid G1 [Matsushime *et al.*, 1992; Geng and Weinberg, 1993]. The fact that $p21^{CIP1}$ expression is stimulated by mitogens [Michieli *et al.*, 1994; Macleod *et al.*, 1995] and that it binds the cycD/cdk4 complex [Xiong, 1992] suggests that $p21^{CIP1}$

Figure 1.4 Interaction of p53 and $p110^{RB}$ at specific control points in the cell cycle.

Thicker curved arrows indicate a traverse of the relevant checkpoint: R-point, G1/S or G2/M

$\longrightarrow$ indicates this process is induced

$\dashv$ indicates this process is inhibited


mitogens
cdk4
+
cycD ↑
+
p110RB:E2F
p110RB
p16INK4 ↑
p21CIP1
p110RB:E2F/cdk4:cycD
p110RB Ⓟ/cdk4:cycD
+
E2Factive
p21CIP1 ↑
p53
R
Cell Cycle
M
G0/G1
G1
S
M
G2
p53
p21CIP1
cycA
degraded
cdk2:cycA/
p107:E2F-4
cycB:cdk2active
p110RB
irradiation
DNA damage
cdc25C
cycB:cdc2 Ⓟinactive
Wee1/Mik1
related kinase
cycB:cdc2
p53
p21CIP1
cdk2:cycE/p107
+
E2F-4active
S phase proteins
cdk2:cycA/
p107:E2F-4inactive

acts as an assembly molecule. The complex is then activated by cyclin activating kinase (CAK) [Matsuoka *et al.*, 1994]. Binding of cycD to $p110^{RB}$ [Dowdy *et al.*, 1993; Ewen *et al.*, 1993; Kato *et al.*, 1993] is thought to target the active cycD/cdk4 holoenzyme to $p110^{RB}$ resulting in the inactivation of $p110^{RB}$ by phosphorylation.

The level of the cdk4-specific kinase inhibitor, $p16^{INK4}$ [Serrano *et al.*, 1993], is elevated in cells lacking functional $p110^{RB}$, which suggests that $p110^{RB}$ may suppress $p16^{INK4}$ expression [Li *et al.*, 1994a]. This raises the intriguing possibility that phosphorylation of $p110^{RB}$ releases transcription factors that promote expression of $p16^{INK4}$, which accumulates to extinguish cdk4 activity after the growth suppressive functions of $p110^{RB}$ are cancelled [Sherr, 1994].

G1/S Checkpoint

Overexpression of cycE in mammalian fibroblasts causes premature entry into S phase [Ohtsubo & Roberts, 1993] and, conversely, microinjection of antibodies against cycE into human fibroblasts prevents S phase initiation. Similarly, cdk2 activity is necessary for DNA replication in *Xenopus* egg extracts, and the major cyclin associated with cdk2 in Xenopus eggs is cycE [Strausfeld *et al.*, 1994]. This suggests that the cycE/cdk2 complex is able to push cells through the G1/S checkpoint. However, microinjection of antibodies to cycA (or transfection of cycA antisense expression plasmids) into mammalian fibroblasts also blocks entry into S phase [Girard *et al.*, 1991]. CycA combines with and activates cdk2 in late G1 phase after cdk2 is known to interact with cycE [reviewed in Sherr, 1993; Heichman & Roberts, 1994], and this is

concomitant with the onset of measurable DNA synthesis. Both cycA [Devoto *et al.*, 1992; Ewen *et al.*, 1992; Faha *et al.*, 1992] and cycE [Lees *et al.*, 1992] interact with p107 and E2F.

Thus it is thought cycE and cycA both act at the G1/S transition itself and are important in the initiation of DNA replication [Heichman & Roberts, 1994].

G2/M checkpoint

A $p110^{RB}$ associated kinase has been recently shown to be active in G2- and M-phase [Sterner *et al.*, 1995], suggesting that $p110^{RB}$ may also function at the G2/M transition. Initiation of M-phase is also associated with the activation of the cycB/cdc2 complex [reviewed in Dunphy, 1994; O'Connor *et al.*, 1993]. cycA function, which is required for progression through S-phase, is modified at the G2/M transition [Pagano *et al.*, 1992; O'Connor *et al.*, 1993]. CycA is then degraded during mitosis and its presence in M-phase is thought to contribute to transformation of the cell [Hunter and Pines, 1994].

CycB/cdc2 complexes accumulate in an inactive form during S- and G2-phase. The kinase is kept in an inactive state by phosphorylation of cdc2, specifically on Tyr-15 (Y15) and Thr-14 (T14), by Wee1/Mik1-related protein kinases. At the end of G2-phase, the cdc25C phosphatase is stimulated to dephosphorylate T14/Y15 and activate cdc2 as part of a positive feedback loop [Hunter and Pines, 1994].

1.4.4 p53 and RB in apoptosis

Apoptosis has proven to be a popular field for study particularly as a number of the genes involved in the process have been cloned. This discussion, however, is primarily restricted to the role of p53 and $p110^{RB}$ in apoptosis. Apoptosis [Kerr *et al.*, 1972], or programmed cell death, is defined by morphologic changes resulting in cell loss and is relevant to a wide spectrum of biology. The process is best characterised in *Caenorhabditis elegans* where *ced* genes mediate the death of specific cells during development. Two of the corresponding genes have been identified in mammalian cells: ced9 suppresses apoptosis and is homologous to bcl-2 [Hengartner & Horvitz, 1994] and ced3 is homologous to a mammalian protease interleukin-1β converting enzyme (ICE) [Miura *et al.*, 1993]. Apoptosis also appears to be a cellular response to situations in which normal control is lost. These situations may include irreparable DNA damage and uncontrolled proliferation or cell cycling in response to the presence of sub-lethal toxic agents, irradiation or viral infection.

Apoptosis in response to DNA damage

DNA damage, as a result of irradiation, chemical or physical insult induces the expression of p53 and the resultant p53-dependent G1 block. This G1 block appears to be activated by the long term induction of $p21^{CIP1}$ [Di Leonardo *et al.*, 1994] but also appears to be dependent on the presence of $p110^{RB}$ [Slebos *et al.*, 1994; Almason *et al.*, 1995]. $p21^{CIP1}$, which at low mitogen stimulated levels acts as an assembly factor for cycD/cdk4 in G1, is also a kinase inhibitor. $p21^{CIP1}$ binds to and inhibits a wide variety of cyc/cdk complexes including cycD/cdk4, cycE/cdk2 and

cycA/cdk2 [Gu *et al.*, 1993b; Harper *et al.*, 1993; Xiong *et al.*, 1993; El-Diery *et al.*, 1994; Zhang *et al.*, 1994]. This suggests that the inactivation of cycD/cdk4 by $p21^{CIP1}$ accounts for the p53 induced G1 block [Diller *et al.*, 1990; Kastan *et al.*, 1992; Hartwell, 1992; Lane, 1992], although $p21^{CIP1}$ can also inhibit cdk2 at the G1/S check point.

Little is known about the DNA damage response mechanisms in G2, but p53 [Aloni-Grinstein *et al.*, 1995] and overexpression of $p110^{RB}$ after the G1/S boundary [Karantza *et al.*, 1993] have been shown to elicit a G2 block. This is consistent with the observation that degradation of the G2 checkpoint response to DNA damage is an event that precedes SV40-immortalisation [Kaufmann *et al.*, 1995]. In normal cells, DNA damage prevents dephosphorylation of the amino acids, T14/Y15, resulting in arrest in the G2 phase. The arrest mechanism is unknown in mammalian cells, but in *Xenopus* egg extracts unreplicated DNA activates a Wee1/Mik1 kinase to block entry into M phase [Smythe & Newport, 1992]. In contrast, many tumour cell lines activate cycB/cyc2 regardless of the state of the DNA and enter M-phase with damaged DNA [O'Connor *et al.*, 1992; 1993], suggesting that there is a defect in the regulation of T14/Y15 phosphorylation.

Presumably the p53-activation of the checkpoints allows DNA repair, directed by $p21^{CIP1}$. In normal cells, $p21^{CIP1}$ is found in a quaternary complex that includes not only a cyclin and cdk but also PCNA [Xiong, 1992], which functions in both DNA replication and repair as a subunit of DNA polymerase δ. $p21^{CIP1}$ can directly inhibit processive PCNA-dependent DNA replication in the absence

of cyclin-cdk complexes [Flores-Rozas *et al.*, 1994; Waga *et al.*, 1994] without interfering with PCNA-dependent nucleotide excision repair [Li *et al.*, 1994b]. The G2/M checkpoint has an important function in protection against chromosome breakage [Zambetti-Bosseler & Scott, 1981] and may also be important in protecting against genetic destabilisation.

If DNA damage cannot be repaired, presumably p53 mediated apoptosis is initiated. What the precise signal is for this decision is not clear. Presumably, in this context, it occurs when the DNA cannot be repaired within a certain period of time.

Apoptosis in response to inappropriate cell cycling

Induction of cellular proliferation in the presence of p53 and the absence of $p110^{RB}$ results in apoptosis [Morgenbesser *et al.*, 1994; Almason *et al.*, 1995; Almason *et al.*, *PNAS* In Press]. Alternatively, entry into S-phase induced in quiescent fibroblasts by induction of E2F-1 results in apoptosis, which is suppressed by wild type $p110^{RB}$ and transdominant mutant p53 [Qin *et al.*, 1994; Almason *et al.*, 1995]. This suggests that entry into S-phase when wt p53 is present and wt $p110^{RB}$ is absent results in apoptosis, but when there is no wt p53, or when wt p53 and wt $p110^{RB}$ are both absent, S-phase entry does not result in apoptosis.

DNA tumour viruses circumvent these controls by inactivating both tumour suppressor proteins, since inactivation of $p110^{RB}$ alone results in apoptosis. Introduction of E1A or E7 into primary cells and transgenic mice can induce apoptosis [Debbas & White, 1993; Lowe *et al.*, 1993; White *et al.*, 1991; Clark *et al.*, 1993; Lowe *et al.*,

1993; White *et al.*, 1994], or enhance normal regulated apoptosis in the lens of the developing eye [Howes *et al.*, 1994; Pan & Griep, 1994].

In respect to the viral transforming proteins there is a correlation between stimulation of cell growth and ability to induce apoptosis. This activity is dependent on the capacity to disrupt the interaction between RB family members and E2F [Kowalik *et al.*, 1995]. This correlation in virally infected cells perhaps reflects the normal cellular situation in which the ability of E2F-1 to stimulate growth correlates with ability to stimulate apoptosis, suggesting that cell growth is induced by E2F and apoptosis is counteracted by a p53 antagonist [Kowalik *et al.*, 1995].

1.5 Aims of this project

The involvement of p53 and $p110^{RB}$ in immortalisation of human cells was examined in a SV40 model system of immortalisation as well as in existing cell lines which had been immortalised via a number of different mechanisms. In order to determine if inactivation of either, or both, of these genes is necessary for SV40-induced immortalisation, the process was examined by inactivating either $p110^{RB}$ or p53 separately. Specifically, the ability of mutant TAg plasmids, which encode proteins that bind to $p110^{RB}$ or p53 only, to induce focus formation and lifespan extension was compared with that of a wild type TAg plasmid. To further test if inactivation of $p110^{RB}$ is necessary for focus formation and extension of lifespan, an exogenous source of RB-1

was introduced with TAg. The intention was to see if RB-1 expression was able to inhibit these two early effects of TAg.

As discussed in Section 1.3.2, the escape of SV40-transformed cells from crisis, or immortalisation, is probably due to mutational inactivation of a cellular gene. It was considered that mutation of p53 and $p110^{RB}$ might give the cells a selective growth advantage beyond that provided by inactivation of these cellular proteins by binding to TAg. Thus, the status of $p110^{RB}$ and p53 was examined in post crisis, SV40-immortalised, cell lines to determine if mutation of these genes is necessary for escape from crisis.

The involvement of inactivation of $p110^{RB}$ and p53 was also examined in cell lines, including tumour derived cell lines, which had been immortalised via a number of different mechanisms. These cell lines had previously been assigned to complementation groups for immortalisation and the status of $p110^{RB}$ or p53 was examined in cell lines in each of these complementation groups. The aim of this part of the project was to determine if inactivation of $p110^{RB}$ or p53 may account for the immortalisation event in any of these complementation groups. To determine whether inactivation of $p110^{RB}$ and p53 is important in non-virally-induced immortalisation, the status of the RB-1 and p53 genes was examined in non-viral cell lines that had previously been assigned to immortalisation complementation groups.

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Materials and Methods

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Chapter 2

Materials and Methods

2.1 Tissue Culture

2.1.1 Materials

I Solutions

HEPES buffered saline (HBS) was prepared by dissolving HEPES (4.76 g), NaCl (7.07 g), KCl (0.20 g), glucose (1.70 g), and Na_2HPO_4 (1.022 g) in 900 ml of distilled water. 0.05% w/v phenol red solution (0.25 ml) was added, the pH adjusted to 7.5, and the final volume adjusted to 1 litre.

2 X transfection-HBS was prepared by adding 10 ml of each of the following 20 X stock solutions to 100 ml of double 0.2 µm (Nalgene) filtered water. The solution was pH adjusted to 7.60 and stored at 4°C.

20 X stock solutions:

Na_2HPO_4: $7H_2O$ 1.88 g in 500 ml H_2O

Dextrose: 10.8 g in 500 ml H_2O

NaCl: 80.8 g in 500 ml H_2O

KCl: 3.72 g in 500 ml H_2O

HEPES: 47.6 g in 500 ml H_2O

Collagen/fibronectin coating solution was prepared essentially as described in Lechner and LaVeck [1985]. Bovine fibronectin (2 mg; Sigma) was dissolved in LHC-Basal medium (2 ml; Biofluids, Inc.) by heating to 37°C for 1 hour. To this was added 20 ml 1 mg/ml BSA

(Boehringer Mannheim), Vitrogen 100 (2 ml) (Collagen Corp.), and 200 ml LHC-Basal medium.

PET trypsin solution (PET) was prepared by mixing 10% PVP/HBS (50 ml), 0.2% Ethyleneglycol-bis(b-aminoethyl ether) N,N,N',N'-tetraacetic acid (EGTA)/HBS (50 ml) and 1% trypsin XII-S (Sigma)/HBS with HBS (390 ml).

II Media

Roswell Park Memorial Institute (RPMI)-1640 (GIBCO/BRL), **Dulbecco's Modified Eagles (DME)** (GIBCO/BRL), **DME without methionine** (GIBCO/BRL), **Leibovitz 15 (L15)** (Sigma), **Laboratory of Human Carcinogenesis-Basal Medium (LHC-BM)** (Biofluids Inc.) and **LHC-BM without methionine** (Biofluids Inc.) were obtained as powders and prepared according to the manufacturers' instructions.

LHC-8 growth medium, and LHC-8 without methonine (for ^{35}S-Methionine labelling), were prepared from LHC-BM, or LHC-BM without methionine essentially as described in Lechner and LaVeck [1985]. Each of the ingredients from Table 2.1 was added to LHC-BM (500 ml) as a stock solution. The medium was then 0.2 µm filter sterilised and stored at 4°C in the dark.

LHC-MM was prepared from LHC-BM essentially as described in Lechner and LaVeck [1985]. Growth additives (Table 2.2) were added to LHC-BM (500 ml) as stock solutions, the medium was 0.2 µm filter sterilised and stored at 4°C in the dark.

Table 2.1 Preparation of LHC-8 medium

Ingredient	Concentration in medium
LHC-BM	N/A
EGF (Sigma)	0.83 nM
Gentamicin (Sigma)	50 µg/ml
Hydrocortisone (Sigma)	0.2 µM
Insulin (Sigma)	0.87 µM
Phosphoethanolamine (Sigma)	0.5 µM
Ethanolamine (Sigma)	0.5 µM
Trace Elements	
$MnSO_4$ (Sigma)	1 nM
$(NH_4)_6Mo_7O_{24}.4H_2O$ (Sigma)	1 nM
$NiCl_2.6H_2O$ (Sigma)	0.5 nM
$NaSeO_3$ (Sigma)	30 nM
$Na_2SiO_3.9H_2O$ (Sigma)	0.5 µM
$SnCl_2$ (Sigma)	0.5 nM
NH_4VO_3 (Sigma)	5 nM
Transferrin (Sigma)	0.13 µM
Triiodothyronine (Sigma)	10 nM
Bovine Pituitary Extract (Clonetics)	0.35 µg/ml

Table 2.2 Preparation of LHC-MM medium

Ingredient		Concentration in medium
LHC-BM		N/A
$CaCl_2$		1.92 mM
EGF (Sigma)		0.83 nM
Gentamicin (Sigma)		50 µg/ml
Hydrocortisone (Sigma)		0.2 µM
Insulin (Sigma)		0.87 µM
Na_2SO_4		26 µM
Trace Elements		
	$MnSO_4$ (Sigma)	1 nM
	$(NH_4)_6Mo_7O_{24}.4H_2O$ (Sigma)	1 nM
	$NiCl_2.6H_2O$ (Sigma)	0.5 nM
	$NaSeO_3$ (Sigma)	30 nM
	$Na_2SiO_3.9H_2O$ (Sigma)	0.5 µM
	$SnCl_2$ (Sigma)	0.5 nM
	NH_4VO_3 (Sigma)	5 nM
Transferrin (Sigma)		0.13 µM
FBS		3%

2 X antibiotic freezing medium was prepared by adding foetal bovine serum (FBS) (40 ml), 50 mg/ml gentamicin sulphate (0.4 ml), 1 M HEPES (4 ml; pH7.6) to L15 medium (156 ml). This was mixed, 0.2 µm filter sterilised and stored at -20°C.

2 X DMSO freezing medium was prepared by adding 10% PVP/HBS (40 ml), DMSO (30 ml), 1 M HEPES (4 ml) to L15 medium (126 ml). This was mixed, 0.2 µm filter sterilised and stored at -20°C.

III Cell lines and cell strains

Fibroblast cell strains

NHDF cell strain, **HFF5**, was a gift from R. Boehmer and grown in RPMI 1640 (GIBCO/BRL) + 10% FBS (Cytosystems). NHDF cell strains **MRC-5**, **WI-38** and **IMR-90** were obtained from ATCC and grown in DME (GIBCO/BRL) + 10% FBS.

GM01879A and **GM01880B** are constitutionally RB-1 +/- fibroblasts isolated from the proband and affected mother respectively, and were obtained from Coriell Cell Repository and grown in DME + 20% FBS. **GM03313** are from the non-affected father from the same family, and were also obtained from Coriell and grown in DME + 20% FBS.

Bronchial epithelial (BE) cell lines

BET-1A and **BET-2A** are immortalised clonally derived non-tumourigenic human bronchial epithelial cell lines [Reddel *et al.*, 1995], established after transfecting NHBE cells with the plasmid pRSV-T encoding the early region of SV40. We have previously

assigned BET-1A to Immortalisation Complementation Group D [Whitaker *et al.*, 1992]. As the growth of these cells is inhibited by serum they were grown in LHC-8 medium in tissue culture flasks coated with a collagen/fibronectin matrix.

BEAS-2B is an immortalised clonally derived human bronchial epithelial cell line [Reddel *et al.*, 1988b], transformed by an adenovirus 12-SV40 hybrid virus. BEAS-2B cells express SV40 genes but no adenovirus genes. The culture conditions were identical to those of BET-1A.

BEAS-2B/R1 cells are a serum resistant subclone of BEAS-2B cells [Ke *et al.*, 1988]. The cells do not require a collagen/fibronectin coating matrix and grow in DME + 10% FBS.

BEAS-2B/S6 cells are a serum sensitive subclone of BEAS-2B cells which undergo terminal differentiation in the presence of FBS [Ke *et al.*, 1988]. These cells require culture conditions identical to those of parental BEAS-2B cells.

BES-1A1 and **BES-1A1.6** are immortalised clonally-derived non-tumourigenic human bronchial epithelial cell lines [Reddel *et al.*, 1988b], transformed by infection with the SV40 virus. These cell lines were grown in identical conditions to those of BET-1A.

HB56B/5T is an immortalised clonally-derived human bronchial epithelial cell line, from cells with an extraordinarily long *in vitro* lifespan which were transformed with the plasmid pRSV-T [Reddel

et al., 1991]. The culture conditions were identical to those of BET-1A.

Control cell lines

MePV-23I is an immortalised clonally-derived human mesothelial cell line, transformed with the plasmid p1321 expressing HPV-16 E6 and E7 [DeSilva *et al.*, 1994]. These cells have apparently lost the E6 and E7 genes during crisis and have been previously shown to contain normal $p110^{RB}$ [DeSilva *et al.*, 1994]. This cell line was grown in LHC-MM medium (Table 2.2) in tissue culture flasks coated with a collagen/fibronectin matrix.

Saos-2 is a cell line derived from a primary osteogenic sarcoma and was obtained from ATCC. The cells express no p53 protein and only a non-phosphorylated, cytoplasmic and truncated (~95 kDa) $p110^{RB}$ due to deletion of RB-1 exons 21-27 [Xu *et al.*, 1989]. This cell line was grown in RPMI 1640 + 10% FBS.

C-33A is a cervical carcinoma cell line obtained from ATCC. The cells express only a slightly truncated (due to a small in-frame deletion at the 5' end of exon 20) $p110^{RB}$ that is not hyperphosphorylated [Wrede *et al.*, 1991; Scheffner *et al.*, 1991]. The cells were grown in DME + 10% FBS.

NCI-H209 is the only suspension culture used in this study. It is a small cell lung carcinoma (SCLC)-derived cell line obtained from ATCC. The cells express only hyperphosphorylated $p110^{RB}$ which does not bind TAg due to a point mutation within RB-1 exon 21

(a non-conservative C to F substitution at codon 706) [Kaye *et al.*, 1990]. The cells were grown in RPMI 1640 + 10% FBS.

WM1175 is a melanoma derived cell line which was a gift from G. Mann. The cells contain a homozygous deletion of $p16^{INK4}$. These cells were grown in DME + 10% FBS.

Complementation group cell lines

15 cell lines from known complementation groups (Table 1.5) were used in this study, including BET-1A already described. **WI-38 VA13/2RA**, **T24**, **HT1080**, **T98G**, **J82** and **293** were obtained from ATCC. **GM847**, **HeLaCOT**, **143BTK-**, and **A1698** were a gift from O. Pereira-Smith. Importantly, the clone of A1698 used in this study was exclusively the $A1698^{DM}$ supplied by O. Pereira-Smith. **CMV-Mj-HEL-1** was a gift from F. Rapp and **SUSM-1** was a gift from M. Namba. **A2182** was a gift from T. Lehman. Each of these cell lines was grown in DME + 10% FBS and were as described by Pereira-Smith and Smith [1988].

2.1.2 General tissue culture techniques

NCI-H209 cells were grown as a suspension culture, and all other cells utilised in this project were grown as monolayer cultures. All cells were grown in disposable sterile plastic cell culture vessels (Corning) in a water-jacketed incubator with an atmosphere of 3.5% CO_2 in air at 36.5°C. Manipulation of cells was carried out in a class II Bio-Hazard Hood.

I Passaging cells

In order to harvest adherent cells and plate them out at a lower cell density their attachment to the surface of the culture vessel must be disrupted. In this method [Lechner and LaVeck, 1985] the cells were washed in PBS without Ca^{++}/Mg^{++} (GIBCO/BRL) and trypsinised using PET solution (2 ml/75cm^2) for epithelial and mesothelial cells, or Multicel™ trypsin (0.05% w/v)/EDTA (0.02% w/v) solution (Cytosystems) for all other cells. Cells were harvested in PBS (Cytosystems) containing 10% FBS and centrifuged for 5 minutes at 200 g before resuspension in fresh growth medium. Bronchial epithelial cells are particularly sensitive to trypsin-induced toxicity, so prior to resuspension of these cells in their growth medium the cell pellet was resuspended in 3% soybean trypsin inhibitor/HBS (Sigma) (0.15 ml) and incubated at room temperature for 3 minutes. Under most circumstances, cells were seeded at a density such that they became subconfluent and ready for passaging at weekly intervals.

II Cryopreservation of cells

Between 2-4x10^6 cells were frozen in 2 X antibiotic solution (0.5 ml) and 2 X DMSO (0.5 ml) in a 1 ml cryogenic freezing vial (Corning). The cells were frozen overnight in a -80°C freezer and then stored in a -195°C liquid nitrogen cryostorage unit.

III Cloning

Isolation of colonies of cells which had taken up the transfected DNA was achieved by G418 selection or focus formation.

Selection of G418 resistant colonies

In this procedure, a plasmid containing the gene encoding resistance to the antibiotic neomycin (pRcCMV) was cotransfected into the cells along with the gene of interest. G418 (Geneticin™; Sigma), an analogue of neomycin is toxic to mammalian cells. Only those cells rendered G418 resistant through their expression of the neomycin resistance (neo^R) gene were able to proliferate in the presence of G418, giving rise to colonies of cells which could be selected with cloning cylinders (see below).

A cytotoxicity assay was performed on each of the cell strains to determine the optimal dose of G418 required to kill all cells within 7-10 days. To do this, cells (3×10^5) were transferred to 60 mm dishes and incubated overnight in appropriate growth medium. The following day the medium was removed and replaced with fresh medium containing G418. Medium containing the appropriate concentration of G418 was replaced every third or fourth day. The optimal lethal concentration of G418 varied from 100 µg/ml for HFF5 cells to 200 µg/ml for MRC-5 cells. G418 selections were carried out in two or more 100 mm dishes (Corning) per cell type at each G418 concentration with 1×10^6 cells per dish.

Isolation of colonies with cloning cylinders

In the majority of cases of SV40 early region induced transformation, it was possible to see foci of rapidly proliferating transformed cells on a background of untransformed and non-proliferating cells. It was therefore possible to isolate these foci, and also G418 resistant colonies, using sterile glass cloning

cylinders (0.8 cm internal diameter, Bellco Glass Inc.). These were placed around the colonies of interest, with autoclaved vacuum grease (DOW Corning) to attach the cloning cylinders to the dish. Cells within the cloning cylinder were harvested by trypsinisation with Multicel™ trypsin (0.2 ml) and collected in PBS + 10% FBS (0.8 ml). The cells were then transferred to 6 well tissue culture plates (Corning). When confluent in a 6 well dish, the cells were transferred to a T25 tissue culture flask and from there to a T75 flask for subsequent passaging in 100 mm dishes.

2.1.3 Transfections

I Plasmids

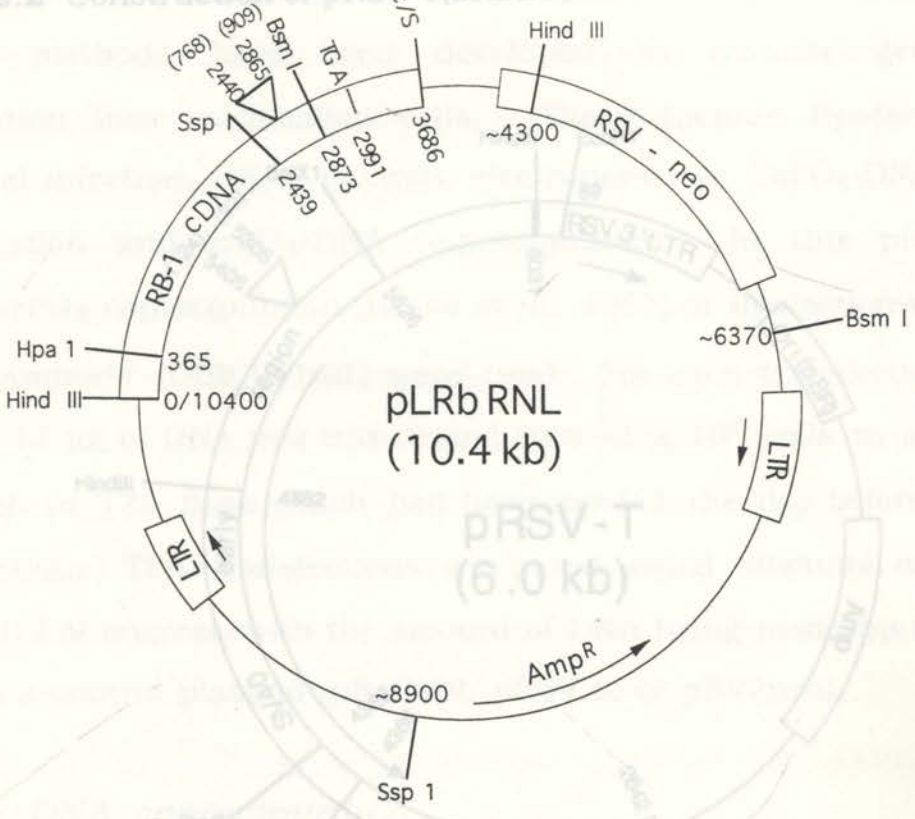
Plasmids used in this study (Table 2.3) were introduced into *E. coli* DH5α cells by electroporation with a Gene Pulser™ (Bio-Rad) as described [Dower *et al.*, 1988]. Plasmid-containing bacteria were grown in Luria-Bertani (LB) medium containing 50 μg/ml ampicillin and the plasmid DNA was extracted and purified using Qiagen maxi kit columns (Qiagen Inc.) according to the manufacturer's instructions.

Construction of pLRb(Δ768-909)RNL (Figure 2.1) involved replacing the HpaI/BsmI fragment in pLRbRNL with the corresponding fragment from RBΔ768-909. This deletion has been shown to disrupt the binding to the viral transforming proteins SV40 TAg and adenovirus E1A in the RBΔ768-909 plasmid [Hu *et al.*, 1990]. Construction of pRSV-T(ΔRBBS) (Figure 2.2) involved replacing the BstXI/BamHI fragment in pRSV-T with the corresponding fragment

Table 2.3 Plasmids used in this study

Plasmid	Features/ Reference	Source
pRSV-T	Expresses wt SV40 early region genes, driven by Rous sarcoma virus (RSV) long terminal repeat (LTR) promoter/enhancer [Sakamoto *et al.*, 1993]	B. Howard
pRSV-T(ΔRBBS)	pRSV-T with the PvuII/BstX1 fragment replaced by the equivalent region from T50-L11 [DeCaprio *et al.*, 1988] resulting in deletion of codons 92-124 which encompass the $p110^{RB}$-binding site of TAg [Maclean *et al.*, 1994]	N. Whitaker
pRSV-T(K1)	pRSV-T containing a point mutation resulting in a Glu › Lys change at codon 107 in TAg: this mutation abrogates binding to $p110^{RB}$ [Kalderon & Smith, 1984; DeCaprio *et al.*, 1989; Sakamoto *et al.*, 1993]	B. Howard
pbssv402DE	Consists of the SV40 genome with a point mutation resulting in an Asp › Glu change at codon 402 in TAg, cloned into Bluescript vector; this mutation abrogates p53-binding [Lin & Simmons, 1991]	D. Simmons
pRSV-T(402DE)	pRSV-T with the BstX1/BamHI fragment replaced by the equivalent region from pbssv402DE [McLean *et al.*, 1994]	E. Rogan
pLRbRNL	Human RB-1 cDNA in pLux virus containing RSVneo [Huang *et al.*, 1988]	W.-H. Lee
pLRb(Δ768-909)RNL	pLRbRNL with the HpaI/BsmI fragment replaced by the equivalent region from RBΔ768-909 which contains a deletion of codons 768-909 shown to disrupt binding to TAg and E1A [Hu *et al.*, 1990]	N. Whitaker
pRcCMV	Eukaryotic expression vector containing the CMV promoter and enhancer and bovine growth hormone polyadenylation signal. Also expresses neomycin resistance gene from the SV40 promoter	InVitrogen
pRSV.3	A eukaryotic expression vector containing the RSV 3' LTR [Jacobson *et al.*, 1989]	E. Long
pSV2neo	Expresses the neomycin resistance gene from the SV40 promoter [Southern & Berg, 1982].	R. Reddel

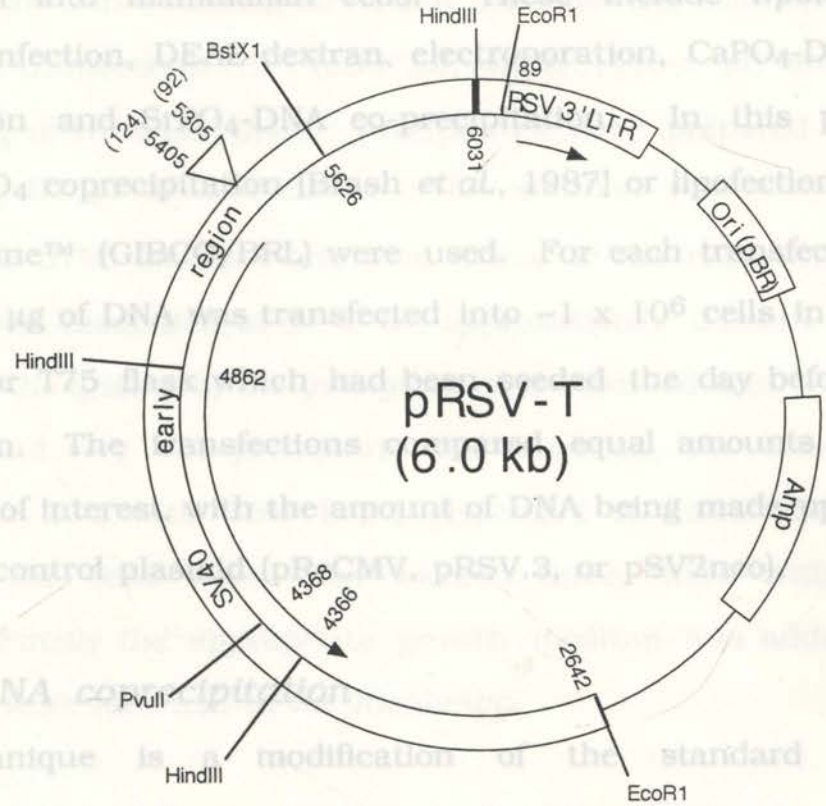
Figure 2.1 Construction of pLRb(Δ768-909)



pLRbRNL was linearised by HpaI digestion (site at NA 365). This linear molecule was then partially digested with BsmI (site at NA 2450 and ~5950) to release the 2.5 kb fragment containing the region to be deleted. The 8.0 kb fragment was isolated and was ligated with the HpaI/BsmI 2.0 kb fragment from RBΔ768-909.

Construction was confirmed with the following digestions: HindIII/SspI digestion of pLRbRNL resulted in 4.6 kb, 2.4 kb, 1.9 kb and 1.5 kb fragments while this digestion of pLRb(Δ768-909)RNL resulted in 4.6 kb, 4.0 kb and 1.5 kb fragments; BsmI/HpaI digestion of pLRbRNL resulted in 4.4 kb, 3.5 kb and 2.5 kb fragments while this digestion of pLRb(Δ768-909)RNL resulted in 4.4 kb, 3.5 kb and 2.0 kb fragments.

Figure 2.2 Construction of pRSV-T(ΔRBBS)



The 1.3 kb BstXI/PvuII fragment of pRSV-T was replaced with the corresponding BstXI/PvuII fragment from T50-L11 which has codons 92-124 deleted. Construction was confirmed by sequencing across the deletion.

from T50-L11 [DeCaprio *et al.*, 1988] which contains a deletion of codons 92-124, including the $p110^{RB}$ binding region of TAg.

II Methods of gene transfer

Several methods have been developed to transfer genetic information into mammalian cells. These include lipofection, retroviral infection, DEAE dextran, electroporation, $CaPO_4$-DNA co-precipitation and $SrPO_4$-DNA co-precipitation. In this project either $SrPO_4$ coprecipitation [Brash *et al.*, 1987] or lipofection using lipofectamine™ (GIBCO/BRL) were used. For each transfection, a total of 12 µg of DNA was transfected into ~1 x 10^6 cells in a 100 mm dish or T75 flask which had been seeded the day before the transfection. The transfections compared equal amounts of the plasmid/s of interest, with the amount of DNA being made up to 12 µg with a control plasmid (pRcCMV, pRSV.3, or pSV2neo).

$SrPO_4$ -DNA coprecipitation

This technique is a modification of the standard $CaPO_4$ coprecipitation method developed for fibroblasts, and was developed to circumvent the terminal differentiation that calcium phosphate induces in NHBE cells. The protocol used for experiments described in this thesis was a slightly modified version of that detailed in the addendum to the original description of the method [Brash *et al.*, 1987].

LHC-8 medium was equilibrated at 3.5% CO_2, 36.5°C overnight. The following day the medium was removed from the cells and the dishes were washed three times with HBS. 10 ml of pre-equilibrated LHC-8 was then added to the dishes. The dishes were

returned to the CO_2 incubator and allowed to equilibrate for at least 1 hour.

All water used for transfections was double filtered through Nalgene disposable filtration units (0.2 µm). Polypropylene tubes were warmed to 37°C in a heating block and the following solutions were added: 2 X transfection-HBS (500 µl), water (440-x µl) and x µl DNA (12 µg of DNA per dish). Precipitates were prepared by the addition of 62 µl of 2 M $SrCl_2$. The solution was mixed and incubated at 37°C for 30 s and then added to the cells dropwise. The dish was incubated at 37°C for approximately 2 hours. After two hours, the quality of the precipitate was observed and, when an abundant and very fine precipitate was observed, the cells were shocked for 30 s with 15% (w/v) glycerol/1 X transfection-HBS solution. The cells were then washed three times with basal medium. Finally the appropriate growth medium was added and the dishes were returned to the incubator.

Lipofection

Transfections using Lipofectamine™ (Gibco/BRL) were carried out according to the manufacturer's instructions. For each transfection, 12 µg of DNA, 970 µl of RPMI 1640 and 30 µl of Lipofectamine™ were mixed and incubated at room temperature for 30-60 minutes. 7 ml of RPMI 1640 was then added to each tube (a total of ~8 ml per transfection). The plates of cells were then washed three times with RPMI 1640 and finally the 8 ml of the RPMI 1640/Lipofectamine™ /DNA mix was added. The cells were then incubated at 37°C for two hours after which the

RPMI/Lipofectamine/DNA mix was removed and the cells were re-fed with appropriate growth media.

2.1.4 Focus formation assay/crystal violet staining

A feature of SV40-transformation of human cells is focus formation, which is the appearance of proliferating cells with a transformed morphology. Importantly these cells appear to be able to continue to proliferate when in contact with other cells, an environment which inhibits the growth of normal cells. Thus the transformed cells in these foci pile up and are macroscopically visible against a background of normal and non-proliferating cells in a monolayer.

To increase the contrast between the foci of transformed cells and the normal monolayer cells, the cells were fixed and stained with a formalin/crystal violet solution. The cells were washed 2 X with PBS (10 ml) and then stained for 30 minutes with the 2% crystal violet/10% formalin/PBS solution (5 ml/100 mm dish). The solution was removed and the cells were washed with excess tap water. The plates of cells were allowed to dry, and the foci were counted.

2.1.5 Lifespan determination

Another feature of SV40-transformation is an extension of the normal *in vitro* lifespan beyond the point at which the normal cell population would cease proliferation, i.e. senescence (Figure 1.2). The point at which the population of transformed cells ceases proliferation is termed crisis to distinguish it from senescence in

the untransformed cell population. To determine the lifespan of a population the cells were passaged (Section 2.1.2) until the cells ceased proliferating. The number of population doublings was determined at each passage and this was plotted against time (e.g. Figure 3.9).

2.2 Protein analyses

2.2.1 Primary antibodies

PAb108, the monoclonal antibody against the N-terminus of both SV40 TAg and tAg was isolated from the medium conditioned by the hybridoma PAb108 obtained from ATCC. Likewise, **PAb122**, a monoclonal antibody against both mutant and wt conformational p53, was isolated from medium conditioned by the hybridoma PAb122 obtained from ATCC. Both antibodies were used at 1 mg per immunoprecipitation and 1 mg/ml for western blotting. Conformation specific antibodies against p53 were also used and included **PAb1620** (Ab5; Oncogene Sciences), wt conformation only (Milner *et al.*, 1987); and **PAb240** (a gift from D. Lane), mutant conformation only (Gannon *et al.*, 1990).

NCL-RB, a mouse monoclonal antibody against human $p110^{RB}$, was obtained from Novocastra Laboratories Ltd. The antibody was used according to the manufacturer's instructions, 25 µl per immunoprecipitation.

For immunoprecipitation and western detection a rabbit polyclonal **anti-$p16^{INK4}$ antibody** (PharMingen) was used according to the

manufacturer's instructions, 3 µl per immunoprecipitation or 3 µl/ml for western blotting.

2.2.2 Western blot analysis

Protein from each cell line was extracted using RIPA lysis buffer (10 mM Tris-HCl pH8.0, 150 mM NaCl, 1 mM EDTA, 1% NP40, 0.1% SDS, 0.1 mg/ml phenylmethylsulphonylfluoride (PMSF; Sigma)). Protein was quantitated using the BioRad protein quantitation kit and 30 µg of protein from each lysate was separated on an SDS-polyacrylamide (4-20% gradient) gel at 19V/cm for 90 minutes. The protein separated in the gels was electrotransferred onto Immobilon™ PVDF membrane (Millipore) at 30 V for 2 hours. The membrane was then blocked overnight at 4°C with blocking solution (2% BSA in TBS (NaCl 29.25 g/l, Tris-HCl 24.25 g/l; pH7.4)). The following day the membranes were incubated with the appropriate primary antibody for 2 hours at room temperature in blocking solution. The membranes were then washed three times with TTBS (Tween20 0.5 ml/l, NaCl 29.25 g/l, Tris-HCl 24.25g/l; pH7.4) and then incubated for one hour with the alkaline phosphatase-linked goat anti-rabbit antibody (Bio-Rad) in blocking solution. After a further three washes with TTBS, the antibody was then detected by colour reaction using the chromogenic substrate for the enzyme alkaline phosphatase, BCIP/NBT (GIBCO-BRL).

2.2.3 Immunoprecipitation analysis

The RB-1, $p16^{INK4}$ and p53 status of the cell lines was determined by immunoprecipitation of the protein essentially as previously

described (De Silva *et al.*, 1994; Whitaker *et al.*, 1995). Cells at ~70% confluence in a T75 flask were washed twice with PBS for ^{35}S-AA (^{35}S-amino acid), or HBS for $^{32}PO_4$, labelling. The cells were then methionine or phosphate starved for 90 minutes with three changes of LHC-8 without methionine or DME without phosphate. The cells were labelled with 100 µCi ^{35}S-methionine/^{35}S-cysteine (Express Protein Labeling Mix, Dupont) in 2 ml of LHC-8 without methionine, or 2 mCi of $^{32}PO_4$ free acid (Dupont) in DME without phosphate. The labelled protein was extracted with a lysis buffer. For co-immunoprecipitation of p110^{RB} and SV40 large T antigen the cells were lysed with Low Stringency Lysis Buffer (50 mM Tris-HCl pH8.0, 120 mM NaCl, 5 mM EDTA, 0.5% NP40, 0.3 mg/ml PMSF), otherwise the cells were lysed with RIPA lysis buffer. The efficiency of the labelling was determined by TCA precipitation: 5 µl of the labelled sample was mixed with 100 µl of 1% BSA/water and precipitated with ice cold 10% trichloroacetic acid (TCA). The precipitate was dried onto glass filters (Millipore) and the glass fibre filters were placed into scintillation fluid for counting in a scintillation counter. Equal counts of cell lysate were immunoprecipitated with protein-A/agarose (GIBCO-BRL) and with a primary antibody against the protein of interest. The protein-A/agarose beads were washed five times with the relevant lysis buffer and resuspended in 2 X loading buffer (4.6% SDS, 20% glycerol, 0.125 M Tris-HCl). The samples were separated on SDS-polyacrylamide (4-20% gradient) for p16^{INK4}, and SDS-polyacrylamide-8% for all other immunoprecipitations, at 19V/cm for 90 min. Gels were fixed in 7% acetic acid/25% methanol in water, dried on a gel dryer and exposed to Kodak XAR film.

2.2.4 Immunostaining

For each cell type to be stained, approximately 5000 cells in growth medium were placed into each well of an 8 well glass slide (Nunc Inc.) which had been previously coated with collagen/fibronectin solution (Section 2.1.1). Following overnight incubation at 37°C, the growth medium was removed and the cells were washed with PBS. Following the washes in PBS, the cells were fixed for 5 min in 100% ice-cold methanol. The cells were then rehydrated in PBS and incubated at 37°C in a humidified chamber with PAb108. The cells were washed with PBS and incubated at 37°C in a humidified chamber with the fluorescein (DTAF)-goat-α-mouse secondary antibody (Jackson Immunoresearch Laboratories Inc). The cells were rinsed with PBS, and a coverslip was mounted with a few drops of DABCO anti-fading mounting solution (20 mM Tris-HCl, pH8.0; 90% glycerol containing 2.3% of the DABCO anti-fade, 1,4 diazabicyclo-(2.2.2) octane [Johnson *et al.*, 1982]). The cells were then examined using fluorescein excitation with a fluorescence microscope.

2.3 Nucleic acids

2.3.1 Probes

Each of the probes used for Southern and Northern analysis is shown in Table 2.4.

Labelling the probe

Radioactively labelled probes were generated via the random priming method [Feinberg and Vogelstein, 1983]. The DNA probe fragments, 100 ng, were labelled with α-^{32}P-dCTP using the

Table 2.4 Southern and Northern probes

Probe	Description	Reference/Source
MDM2	900 bp Xho I fragment of MDMC14-2, (NA -312 to ~NA +600)	Oliner *et al.*, 1992
	or	
	Oligo probe A201[1] - 5' coding sequence, labelling primed with oligo A203[2]	
$p16^{INK4}$	Eco RI/Xho I fragment of pcDNA3/p16	J. Noble, CMRI
RB-1	3.3 kb Stu I/Hind III fragment of pLRbRNL	[Huang *et al.* 1988]
neo	1.0 kb Smal fragment of pMOL-NEO	D. Sauer, CMRI

[1]A201 5'-G CAG GCA AAT GTG CAA TAC CAA CAT GTC TGT ACC TAC TGA TGG TGC TGT AAC CAC CTC ACA GAT TCC AGC TTC GCA ACA AGA GAC CCT GGT TAG ACC AAA GCC ATT GCT TTT GAA GTT ATT AAA GTC TGT TGG TGC-3'

[2]A203 5'-GCA CCA ACA GAC TTT AAT AAC-3'

GIGAprime-labelling kit according to the manufacturer's protocol (Bresa). For labelling the oligonucleotide probe for MDM2, the decanucleotide solution from the GIGAprime-labelling kit was replaced with specific oligonucleotide primers as shown in Table 2.4. Unincorporated α-^{32}P-dCTP was separated from the probe using a Centricon 30 microconcentrator (Amicon). The specific activity of the probe was quantitated on a scintillation counter. The specific activity of the probe was calculated as disintegrations per min (dpm)/µg DNA. The probes were hybridised to the membranes at 2 x 10^6 counts per min (cpm) of probe per ml of hybridisation solution.

2.3.2 Isolating DNA and RNA

I Isolating DNA

This method made use of the DNA extraction kit developed by Stratagene®. Cells from one confluent T150 flask (Corning, USA) were harvested as described in Section 2.1.2 and homogenised in solution 2 (50 mM Tris-HCl pH8.0, 20 mM EDTA pH8.0, 2% SDS). Pronase (225 mg/ml) was added to a final concentration of 100 µg/ml and the samples were incubated for 1 hour at 60°C. Solution 3 (saturated NaCl; 4 ml) was added to the mixture which was incubated on ice for 5 min. Each sample was then centrifuged for 15 min at 2000 g at 4°C. The supernatant was retrieved and to this RNase (10 mg/ml) was added to a final concentration of 20 µg/ml. Samples were incubated at 37°C for 15 minutes. DNA was precipitated, spooled on flame sealed Pasteur pipettes and resuspended in TE.

II Isolating DNA from crystal violet stained foci

The plates of stained cells were rehydrated with PBS (5 ml) for 30 minutes. The focus or area of cells was isolated with a cloning cylinder and vacuum grease as described in Section 2.1.2. The isolated cells were then lysed with Trizol (0.2 ml; Life Technologies Inc.) and collected in a 1 ml eppendorf tube (Eppendorf). After 2 minutes $CHCl_3$ was added (0.1 ml) and mixed vigorously. The aqueous phase, containing the RNA, was then resolved and removed after centrifuging at 12,000 g. To the remaining interface, 100% ethanol (0.1 ml) was added to precipitate the DNA. After centrifuging at 2,000 g the phenol/ethanol supernatant was removed and the pellet washed 2 X with 0.1 M sodium citrate/10% ethanol (0.2 ml). The pellet was suspended in 75% ethanol (0.5 ml) for 20 minutes and then centrifuged at 2,000 g. The pellet was air dried and dissolved in 8 mM NaOH (40 µl).

III Isolating RNA

RNA was isolated using Trizol according to the manufacturer's instructions. Cells from two confluent T150 flasks were harvested as described in Section 2.1.2 and washed with PBS. The cells were lysed with Trizol (1 ml). After 2 minutes $CHCl_3$ was added (0.2 ml) and mixed vigorously. The aqueous phase, containing the RNA, was then resolved by centrifugation at 12,000 g and removed. The RNA was precipitated with iso-propanol (0.5 ml), and the resultant pellet was washed with 75% ethanol and then dissolved in H_2O.

IV Poly-A$^+$ RNA isolation

Poly-A$^+$ RNA was isolated from total RNA using the PolyATract® mRNA Isolation System III (Promega) according to the

manufacturer's instructions. 100 µg of total RNA was denatured at 65°C and annealed to Biotinylated-Oligo(dT) Probe. The annealed RNA/Biotinylated(dT) Probe was mixed with Streptavidin-Paramagnetic Particles in 0.5 X SSC. The complexed paramagnetic beads were captured using a magnetic stand and the complexed beads washed four times with 0.1 X SSC. The mRNA was eluted in H_2O and the paramagnetic beads and complexed Biotinylated-Oligo(dT) Probe were separated from the mRNA by capturing with the magnetic stand.

2.3.3 Southern analysis

I Digesting and separating DNA

Each sample of genomic DNA (10 µg) was digested overnight with appropriate enzyme (10 X excess) under the manufacturer's recommended conditions. 5 µl of 10 X loading buffer (0.1% Bromophenol Blue, 50% glycerol, 500 mM EDTA) was added to each sample and the digested DNA fragments were separated on a 0.8% w/v agarose gel in 1 X TBE buffer (0.09 M Tris-borate, 0.002 M EDTA). Electrophoresis was performed in a MAX Submarine Agarose Gel Unit (Hoefer Scientific Instruments) in 1 X TBE buffer with a potential difference of 2 V/cm applied across the gel for 16-18 hours. Bacteriophage λ DNA fragments generated by digestion with *Hin*dIII (New England Biolabs) were used as size markers.

II Transfer

After electrophoresis the gel was rinsed in 1 X TBE and then soaked for 45 mins-1 hour in denaturing solution (0.5 M NaOH,

1.5 M NaCl). The gel was rinsed in 1 X TBE and then soaked twice for 30-60 minutes in neutralising solution (0.5 M Tris-HCl, 1.5 M NaCl; pH7.2). The genomic DNA was transferred overnight by capillary action to Hybond-N membrane (Amersham) with 20 X SSC (3 M NaCl, 0.4 M trisodium citrate). The membrane was then air dried for at least 30 minutes and the DNA fixed to the membrane by UV baking for 5 minutes.

III Prehybrisation and hybridisation

The membrane was incubated with 10 ml prehybridisation solution of 6 X SSC (20 X SSC (7.5 ml); 1 X SSC is 0.15 M NaCl, 0.02 M trisodium citrate), 5 X Denhardt's solution (50 X Denhardt's solution (2.5 ml): Ficoll (5 g), polyvinylpyrrolidone (5 g), and bovine serum albumin (5 g) were dissolved in distilled water and the final volume adjusted to 500 ml)), 0.5% SDS (10% SDS (1.25 ml)), heat denatured herring sperm DNA (10 mg/ml; 50 µl), and distilled water to a final volume of 25 ml, in a Hybaid bottle (Hybaid). Prehybridisation of the membrane was performed for a minimum of 4 hours at 65°C in a Hybaid rotisserie oven.

Probes were labelled by the random priming method as described above, and hybridised to the Southern blots at 2 x 10^6 counts per min (cpm) of probe/ml of prehybridisation solution. The membrane was incubated at 65°C for 12-16 hours. The membrane was washed twice with 2x SSC/0.1% SDS (100 ml) for 10 minutes at room temperature, once with 1x SSC/0.1% SDS (100 ml) for 30 minutes at 65°C and then twice with 0.1x SSC/0.1% SDS at 65°C for 10 minutes. The membrane was exposed to Kodak XAR film (Kodak) with an intensifying screen at -70°C for up to 4 weeks.

2.3.4 Northern analysis

I Separating RNA

This method is described in Sambrook *et al.* [1989]. To the RNA sample (10 µg) made up to 12 µl with H_2O the following was added: 5 µl 10x MOPS buffer (41.86 g/l MOPS, 4.1 g/l NaAc, 10 mM EDTA; pH7.0), 8 µl 37% formaldehyde, 25 µl deionised formamide. The samples were then heat denatured at 65°C for 5 minutes and chilled on ice. Loading buffer (5 µl) was added to each sample and the RNA was separated by electrophoresis in a 1% agarose gel containing 2.2 M formaldehyde. Electrophoresis was performed in a 1 X MOPS/6.2% formaldehyde buffer with a potential difference of 2-4 V/cm applied across the gel for approximately 16-18 hours.

II Transfer

Following electrophoresis the RNA was transferred to Hybond-N transfer membrane by capillary action. The membrane was then air dried for at least 30 minutes and the RNA was fixed to the membrane by UV baking for 5 minutes

III Prehybridisation and hybridisation

The northern transfer was incubated with 10 ml prehybridisation solution (4 X SSC, 5 X Denhardt's solution (see Section 2.3.3), 10% Dextran, 50 mM NaH_2PO_4; pH7.4), in a Hybaid bottle (Hybaid, UK). Prehybridisation of the membrane was performed for a minimum of 4 hours at 60°C in a Hybaid rotisserie oven.

The membranes were incubated at 65°C for 12-16 hour in 10 ml hybridisation solution (prehybridisation solution without Dextran) containing 2 x 10^6 cpm/ml labelled probe. The membrane was

washed three times with 0.5 X SSC/0.1% SDS (100 ml) for 30 minutes at 50°C. The membrane was exposed to Kodak XAR film with an intensifying screen at -70°C for up to 4 weeks.

2.3.5 PCR

PCR amplification of RB-1 intron 14

The primers to check for integration of either pLRbRNL or pLRb(Δ768-909)RNL in normal cells were chosen either side of intron 14 (402 bp). Thus, there is a positive internal control for PCR conditions in each reaction, generating a larger fragment from the endogenous RB-1 gene and a smaller (without the intron) fragment from the plasmid. The fragment generated by the RB-1 cDNA copy present in the two plasmids is 81 bp while the fragment generated by the genomic RB-1 is 483 bp. The two primers used were:

Forward primer (A214): 5'-CGA TAC AAA CTT GGA GTT CGC-3'

Reverse primer (A215): 5'-TTG AAT GGA TAA TCG TTC TTC TTC-3'

The PCR reaction conditions for each reaction were: 4 µl 10 X Taq buffer (Boehringer Mannheim), 1 µl 10 mM dNTPs, 5 µl each 10 pmol/µl primers A214 and A215, 0.2 µl Taq polymerase (50U/µl), 100 ng template DNA, made up to 40 µl with H_2O. The PCR reaction mix was denatured at 94°C for 5 minutes then subjected to 30 cycles of 90°C for 40 s, 50°C for 40 s and 72°C 90 s. The PCR reaction was completed with a single extension step at 72°C for 10 minutes. 10 µl of the reaction mix was run on a 2% agarose gel stained with ethidium bromide to visualise the resultant DNA fragments.

Nested PCR of TAg

Due to the small amount of DNA expected from the crystal violet stained cells a nested PCR strategy was employed to detect the presence of the SV40 early region. The first reaction produces a 558 bp fragment using the following primers:

Reaction 1 (NA 4236-4792 of pRSV-T)

Forward primer (D57): 5'-TC CCC TCC AGT GCC CTT-3'

Reverse primer (D49): 5'-CA GTA CAG TTT TGA AAT-3'

The second, nested, reaction produces a 407 bp fragment using the following primers:

Reaction 2 (NA 4348-4756 of pRSV-T)

Forward primer (A20): 5'-AC ATA ATT CAA GCA AAG CAG-3'

Reverse primer (A21): 5'-AA AGA ACA GCC CAG CCA CTA-3'

The reaction conditions for Reaction 1 were as described for PCR of RB-1 intron 14. 2 µl of the reaction mix from Reaction 1 was used as the template DNA for Reaction 2, otherwise the conditions were as for PCR of RB-1 intron 14. For Reaction 1: the PCR reaction mix was denatured at 95°C for 5 minutes then subjected to 30 cycles of 92°C for 60 s, 47°C for 120 s and 72°C 120 s, and the reaction was completed with a single extension step at 72°C for 10 minutes. For Reaction 2: the PCR reaction mix was denatured at 95°C for 5 minutes then subjected to 30 cycles of 92°C for 60 s, 58°C for 120 s and 72°C 120 s, and the reaction was completed with a single extension step at 72°C for 10 minutes. 10 µl of the reaction mix from Reaction 2 was electrophoresed on a 2% agarose gel stained with ethidium bromide to visualise the resultant DNA fragments.

Chapter 3

Involvement of p110^{RB} and p53 in SV40-induced immortalisation

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Chapter 3

Involvement of p110^{RB} and p53 in SV40-induced immortalisation

3.1 Introduction

Introduction of SV40-early region genes has proven the most reliable way of immortalising a large variety of cell types and was used as a model system in this study. SV40-induced immortalisation of human cells is known to proceed via two main phases (Figure 1.2). In the first phase, cells containing SV40 early region genes, encoding large (TAg) and small (tAg) tumour antigens, continue doubling beyond the point at which normal cells cease doubling, *i.e.*, they temporarily escape from senescence. This property of the SV40 early region is insufficient for immortalisation since the great majority of cells expressing the early region genes eventually enter culture crisis. Some, but not all, clones of cells expressing the T antigens are able to become immortalised. The genetic events resulting in escape from crisis are unknown, but loss of function of normal genes is indicated by somatic cell hybridisation experiments (Section 1.2).

Role of p110^{RB} and p53 binding in focus formation and lifespan extension

Focus formation is an early effect of introducing SV40 early region genes, by transfection or infection, into adherent cells. As well as having a transformed appearance, the cells expressing the T antigens are not contact inhibited for proliferation. This loss of contact inhibition allows piling up of the transformed cells, forming

contact inhibition allows piling up of the transformed cells, forming a macroscopically visible and multilayered focus. This is in contrast to the normal background cells, which form a monolayer.

The cells in these foci have an extended *in vitro* lifespan, the population proliferating 10 to 30 PDL beyond that of normal cells. The early transformation and extended lifespan exhibited by these cells is always associated with the expression of TAg [Chang, 1986]. For mouse cells this focus forming ability and extension of lifespan has been localised to the N-terminal 121 AAs of TAg, which include the $p110^{RB}$ binding region, with a second and independent transforming region located within ~AA 370-630, which includes the region necessary for p53 binding [Srinivasan *et al.*, 1989].

The fact that both p53 and $p110^{RB}$ are bound by the transforming proteins of a number of DNA tumour viruses (see Table 1.5), including TAg, indicates the importance of these genes in the control of cellular proliferation. This is supported by the known role of p53 in G1/S block [Diller *et al.*, 1990; Kastan *et al.*, 1992; Hartwell, 1992; Lane, 1992] and G2/M block [Aloni-Grinstein *et al*, 1995] and the role of $p110^{RB}$ in controlling the cell division cycle [Mihara *et al.*, 1989].

The role of $p110^{RB}$ and p53 binding in focus formation and lifespan extension was examined using two strategies. The first strategy involved disrupting the $p110^{RB}$ region or the p53 binding region of TAg and comparing focus formation and lifespan extension induced in NHDF cells by these mutants with wt TAg. Focus formation of the non-$p110^{RB}$-binding mutants of TAg in NHDF was also compared

with that induced in fibroblasts which contained just one allele of the RB-1 gene. In a previous study we had shown that non-p53-binding mutants of TAg induced foci in LFS fibroblasts, which contain just one copy of wt p53, at a significantly higher frequency than fibroblasts containing both copies of the p53 gene [Maclean *et al.*, 1994]. The second strategy involved co-transfecting a $p110^{RB}$ expression plasmid with the wt TAg expression plasmid to see if the focus formation and lifespan extension induced by wt TAg was decreased by the exogenous source of $p110^{RB}$.

Role of $p110^{RB}$ and p53 binding in immortalisation

Cells which have an extended lifespan and still express the T antigens, however, reach a period of "crisis". During crisis the cells undergo characteristic morphological changes and cease to proliferate in culture, indicating that SV40 genes are insufficient to immortalise human cells. In some cultures, rare proliferating colonies appear at a frequency of 10^{-9} to 10^{-5} (Section 1.3). The low frequency suggests that a mutational event allows these cells to escape crisis to form an immortal cell line.

In this study I considered the possibility that mutation of the p53, and/or RB-1 genes, might give the cells an advantage that exceeds that conferred by binding of their protein products to TAg, and might contribute to escape from crisis. Specifically, I examined the status of RB-1 in nine independently derived SV40-immortalised cell lines. p53 status was assessed by immuno-precipitation studies, sequence analysis (in one case) and sequence data obtained in other laboratories.

3.2 Focus formation

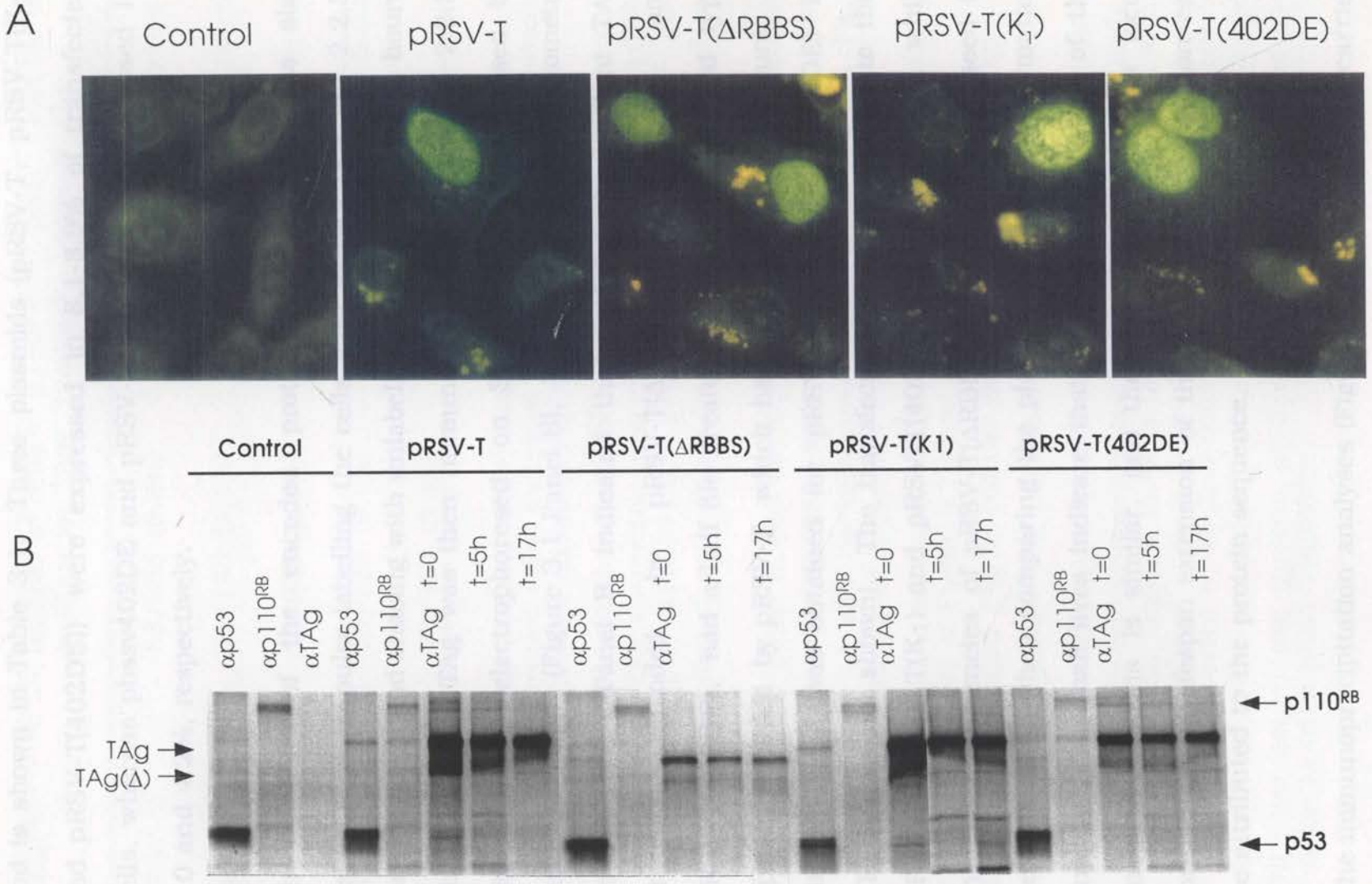
The SV40 early region expression plasmids to be used in this study (Table 2.3) were tested for their efficiency of transfection in a transient assay using HT1080 cells (Section 2.1.1). These cells were chosen because they have both wt $p110^{RB}$ and p53 (see Sections 4.2 and 4.4.1). The eukaryotic expression plasmid, pRSV-T, expresses wt SV40 early region genes, driven by Rous sarcoma virus (RSV) long terminal repeat (LTR) promoter/enhancer [Sakamoto *et al.*, 1993]. pRSV-T(ΔRBBS) was constructed by replacing the PvuII/BstX1 fragment in pRSV-T with the equivalent region from T50-L11 [DeCaprio *et al.*, 1988], resulting in deletion of codons 92-124 which encompass the $p110^{RB}$-binding site of TAg (Figure 2.2). pRSV-T(K_1) differs from the pRSV-T plasmid by a point mutation resulting in a Glu › Lys change at codon 107 in TAg: this mutation abrogates binding to $p110^{RB}$ [Kalderon & Smith, 1984; DeCaprio *et al.*, 1989; Sakamoto *et al.*, 1993]. pbssv402DE consists of the SV40 genome with a point mutation resulting in an Asp › Glu change at codon 402 in TAg, cloned into Bluescript vector; this mutation abrogates p53-binding [Lin & Simmons, 1991]. pRSV-T(402DE) is pRSV-T with the relevant BstX1/BamHI fragment replaced by the equivalent region from pbssv402DE [Maclean *et al.*, 1994].

Forty-eight hours after transfection, using lipofectamine, of the HT1080 cells with 10 μg of the relevant plasmid, the cells were methanol fixed and immunostained with a monoclonal antibody against TAg (Figure 3.1 Panel A). The primary antibody used, PAb108, binds the amino-terminus of TAg and thus recognises the proteins encoded by each of these plasmids. The efficiency of

Figure 3.1 Analysis of TAg expressed transiently from wild type and mutant TAg expression plasmids

A. Photomicrographs of TAg immunostained transfected HT1080 cells 60 hours post transfection. HT1080, non-transfected negative control; pRSV-T, HT1080 cells transiently transfected with wild type TAg expressing plasmid pRSV-T; pRSV-T(Δ), HT1080 cells transiently transfected with $p110^{RB}$ binding region deleted TAg expressing plasmid pRSV-T(ΔRBBS); pRSV-T(K1), HT1080 cells transiently transfected with TAg expressing plasmid pRSV-T(K1) containing a point mutation which disrupts $p110^{RB}$ binding; 402DE, HT1080 cells transiently transfected with TAg expressing plasmid pRSV-T(402DE) containing a point mutation which disrupts p53 binding.

B. Immunoprecipitation of cells from panel A. Transiently transfected cells were pulse labelled with ^{35}S-AA (section 2.2.3), and lysed with a low stringency buffer to maintain any complexes between cellular proteins, p53 and $p110^{RB}$, and TAg. The cells were lysed at 0 , 5 and 17 hours after labelling. The lysates were then immunoprecipitated with: αp53, PAb122 conformational independent antibody against p53; $\alpha p110^{RB}$, NCL-RB monoclonal antibody against $p110^{RB}$; αTAg, PAb108 monoclonal antibody against the N-terminus of TAg. The arrows indicate the positions of TAg protein, TAg protein containing the deletion of the $p110^{RB}$-binding region, $p110^{RB}$ and p53.


A
Control
pRSV-T
pRSV-T(ΔRBBS)
pRSV-T(K1)
pRSV-T(402DE)
B
Control
pRSV-T
pRSV-T(ΔRBBS)
pRSV-T(K1)
pRSV-T(402DE)
αp53
αp110RB
αTAg
αTAg t=0
t=5h
t=17h
TAg
TAg(Δ)
p110RB
p53

transfection for each plasmid, as indicated by the number of cells positive for TAg expression, was determined from duplicate wells and is shown in Table 3.1. Three plasmids (pRSV-T, pRSV-T(K_1) and pRSV-T(402DE)) were expressed in 8.1-8.9% of transfected cells, whereas pbssv402DE and pRSV-T(ΔRBBS) were expressed in 3.0 and 4.2%, respectively.

The stability of the encoded proteins (Table 3.1) was also determined by pulse labelling the cells with ^{35}S-AA (Section 2.2.3) for two hours and chasing with unlabelled AAs for 0, 5 and 17 hours. The expressed TAg was then immunoprecipitated (Section 2.2.3) with PAb108, electrophoresed on SDS-PAGE and visualised by autoradiography (Figure 3.1 Panel B). The results from densitometry on Figure 3.1 Panel B, indicated that each of the mutant TAg proteins, encoded by pRSV-T(ΔRBBS), pRSV-T(K1) and pRSV-T(402DE), had a half life greater than that of the wild type protein encoded by pRSV-T, which had a half life of ~13 hours. A similar result was obtained for pRSV-T and pRSV-T(402DE) in NHDF (data not shown). The transfection efficiencies indicate that pRSV-T, pRSV-T(K_1) and pRSV-T(402DE) are comparable, while the lower efficiencies of pRSV-T(ΔRBBS) and pbssv402DE need to be considered when comparing the plasmids in the focus formation analysis. The half lives indicate that the stability of each of the encoded proteins is similar, and therefore, differences in focus formation and lifespan extension of the cells after transfection can be attributed to the protein sequence.

The immunoprecipitation analyses (Figure 3.1 Panel B) were carried out on subpopulations of transfected cells that were immunostained

Table 3.1 Transient transfection of the TAg expressing plasmids and analysis of encoded proteins in HT1080 cells

Plasmid	number of cells positive for TAg[a]	$p110^{RB}$ binding[b]	p53 binding[c]	half life of the protein[d]
pRSV-T	55/666 (8.3%)	+[e]	+	13 hours
pRSV-T(ΔRBBS)	31/733 (4.2%)	-[f]	+	>17 hours
pRSV-T(K1)	62/695 (8.9%)	-	+	>17 hours
pRSV-T(402DE)	59/725 (8.1%)	+	reduced[g]	>17 hours
pbssv402DE	20/665 (3.0%)	ND[h]	ND	ND

[a] number of TAg positive cells/total number of cells counted (percentage positive cells) indicated by immunostaining (Section 2.2.4). Examples are shown in Figure 3.1 panel A

[b] from Figure 3.1 panel B, $p110^{RB}$ binding is indicated by the presence of TAg co-immunoprecipitated with the α-$p110^{RB}$ antibody and also the presence of $p110^{RB}$ co-immunoprecipitated with the α-TAg antibody (as described in Section 2.2.3)

[c] from Figure 3.1 panel B, p53 binding is indicated by the presence of TAg co-immunoprecipitated with the α-p53 antibody and also the presence of p53 co-immunoprecipitated with the α-TAg antibody (as described in Section 2.2.3)

[d] from densitometry estimates of amount of protein present in a lighter exposure of Figure 3.1 panel B

[e] binding of encoded TAg to cellular protein of interest detected

[f] no binding of encoded TAg to cellular protein of interest detected

[g] p53 protein co-immunoprecipitated with the α-TAg antibody, and TAg protein co-immunoprecipitated with the α–p53 antibody, are present but appears to be a lesser amount compared with that seen in the wild type TAg. This mutantion in TAg has previously reported to abolish detectable binding to p53 [Lin & Simmons, 1991]

[h] ND: not determined (protein produced from pbssv402DE is the same as from pRSV-T(402DE)

for TAg expression (Figure 3.1 Panel A). The differences in the amount of TAg present, indicated by the intensity of the immunoprecipitated band, appears to correspond with the percentage of positive cells in each of the populations, although the immunoprecipitations were not quantitated by densitometry.

The lysis of the cells for immunoprecipitation was done using a low stringency lysis buffer which maintained the complex between TAg and $p110^{RB}$ as well as TAg and p53. The presence of a p53 band co-immunoprecipitated by the α-TAg antibody, and the presence of a TAg band co-immunoprecipitated by the α-p53 antibody indicates TAg binding to p53. Similarly, the presence of a $p110^{RB}$ band co-immunoprecipitated by the α-TAg antibody, and the presence of a TAg band co-immunoprecipitated by the α-$p110^{RB}$ antibody indicates TAg binding to $p110^{RB}$. Binding of $p110^{RB}$ and p53 to TAg can be seen clearly in the HT1080 cells transfected with pRSV-T. p53 binding to TAg can also be clearly seen in the cells transfected with pRSV-T(K_1). Weak binding is suggested by faint bands in HT1080 cells transfected with pRSV-T(ΔRBBS) or pRSV-T(402DE). The amount of TAg protein immunoprecipitated by *PAb108 in the HT1080 + pRSV-T(ΔRBBS) cells is less than that* present in the other lanes, presumably partly due to the lower transfection efficiency. The binding of p53 to the pRSV-T(402DE) encoded protein appears to be less compared to binding between pRSV-T wt encoded TAg and p53. This indicates that, while pRSV-T(402DE) has a reduced level of binding to p53, some binding is present. The pRSV-T(402DE) encoded TAg is also shown to bind $p110^{RB}$ while the non-$p110^{RB}$-binding mutant TAgs encoded by

pRSV-T(ΔRBBS) or pRSV-T(K_1) do not appear to bind $p110^{RB}$ (Figure 3.1 Panel B).

3.2.1 $p110^{RB}$ binding is necessary for focus formation

I Focus formation in NHDF with non-$p110^{RB}$-binding mutant TAg plasmids

NHDF cell strains, MRC-5 and HFF5 (Section 2.1.1), were co-transfected with a TAg expression plasmid and a control plasmid, or control plasmid alone, using either the $SrPO_4$ precipitation or lipofection transfection technique (Section 2.1.3). Following transfection the cells were maintained in culture without passaging. In some of the plates, after approximately three weeks, foci of transformed and proliferating cells appeared on the background of normal confluent, non-proliferating cells. The plates of transfected cells were then fixed and stained with a formalin/crystal violet solution (Section 2.1.4) to better reveal the foci (Figure 3.2).

As shown in Table 3.2, co-transfection of pRSV-T and a control plasmid (pRcCMV, pRSV.3 or pSV2neo) resulted in an average of 44 foci/10 μg DNA/10^6 NHDF cells transfected. The number of foci obtained was similar in each of the NHDF cell strains: an average of 51 foci in HFF5 cells, 39 foci in MRC-5 cells and 36 foci in GM03313 cells. As expected, foci were never induced in plates of cells transfected with the control plasmids alone, or in the water controls. Table 3.3 shows that the transfection efficiency was unaffected by the choice of co-transfection plasmid (i.e., pRSV.3, pSV2neo or pRcCMV).

Figure 3.2 Focus formation in NHDF

NHDF cell strains, HFF5 and MRC-5, were stably co-transfected with the SV40-early region plasmids and the control plasmid pRcCMV, or with the control plasmid alone, as indicated. The cells were allowed to grow without passaging. In some of the dishes, foci of transformed cells appeared and these were better revealed by fixing and staining with a formaldehyde/crystal violet solution. The plates of cells were then photographed as shown.

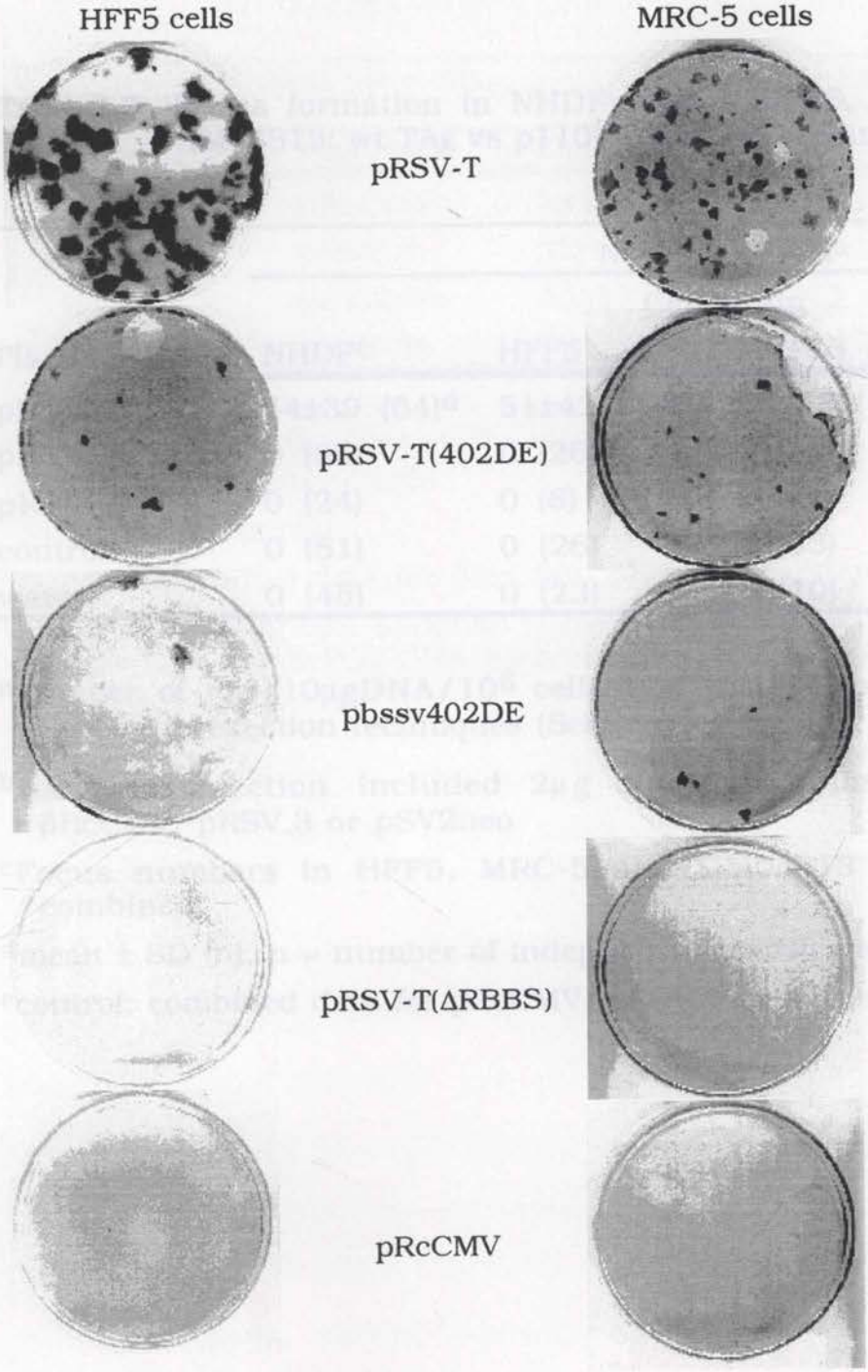

HFF5 cells
MRC-5 cells
pRSV-T
pRSV-T(402DE)
pbssv402DE
pRSV-T(ΔRBBS)
pRcCMV

Table 3.2 Focus formation in NHDF strains HFF5, MRC-5 and GM03313: wt TAg vs $p110^{RB}$-binding mutants

	Number of foci[a]			
	Cell strain			
Plasmid[b]	NHDF[c]	HFF5	MRC-5	GM03313
pRSV-T	44±39 (64)[d]	51±42 (33)	39±43 (16)	36±25 (15)
pRSV-T(ΔRBBS)	0 (55)	0 (26)	0 (13)	0 (16)
pRSV-T(K1)	0 (24)	0 (6)	0 (6)	0 (12)
control[e]	0 (51)	0 (26)	0 (13)	0 (12)
water	0 (45)	0 (23)	0 (10)	0 (12)

[a]number of foci/10μgDNA/10^6 cells ± SD using Lipofection and $SrPO_4$ transfection techniques (Section 2.1.3)

[b]Each transfection included 2μg of control plasmid, either pRcCMV, pRSV.3 or pSV2neo

[c]Focus numbers in HFF5, MRC-5 and GM03313 cell strains combined

[d]mean ± SD (n), n = number of independent transfections

[e]control: combined data for pRcCMV, pRSV.3 or pSV2neo

Table 3.3 TAg-induced focus formation in NHDF strains HFF5, MRC-5 and GM03313, using either Lipofectamine or $SrPO_4$-DNA co-precipitation transfection techniques

Co-transfection plasmids		Number of foci Transfection Method	
Plasmid 1	Plasmid 2	Lipofectamine	$SrPO_4$
pRSV-T	pRcCMV	73±40 (21)[a]	22±21 (27)
pRSV-T	pRSV.3	ND[b]	24±17 (11)
pRSV-T	pSV.2neo	90±27 (5)	ND
pRSV-T	control[c]	76±38 (26)	23±20 (38)[d]

[a]number of foci/10μgDNA/10^6 cells, mean ± SD (n), n = number of independent transfections

[b]ND: not determined

[c]control: combined data for pRcCMV, pRSV.3 or pSV2neo

[d]mean number of foci obtained using lipofection was significantly greater than for $SrPO_4$-DNA co-precipitation (P=0.0002)

The lipofection method resulted in higher efficiency of transfection than the $SrPO_4$-DNA co-precipitation (Table 3.3; P = 0.0002). Transfection using $SrPO_4$ resulted in approximately one third the number of foci obtained by lipofection and increased the variation in the number of foci in each experiment. The explanation for this might be that the $SrPO_4$ is somewhat toxic to the NHDF cells. Plates transfected with $SrPO_4$ were observed to contain many rounded up cells which correlated with the large number of cells which lifted off the plate. In contrast, the lipofection technique, when used as described in Section 2.3.3, had no such effect on the NHDF cells.

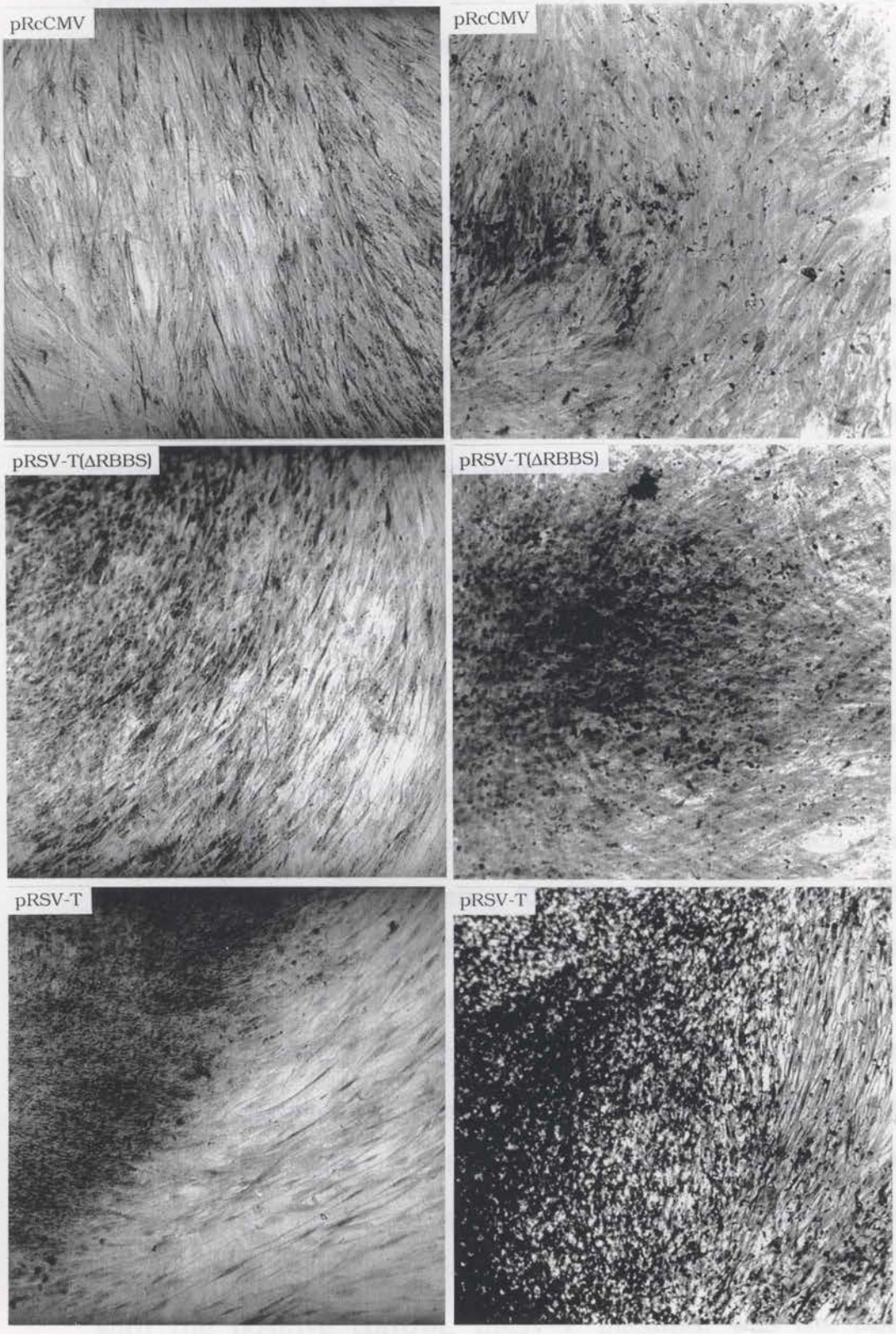
Due to the greater transfection efficiency of lipofection over $SrPO_4$-DNA co-precipitation, all focus formation experiments reported after this section utilised the lipofection technique. The suspected toxicity of $SrPO_4$ to the NHDF was thought to possibly alter the results.

The focus forming ability of the non-$p110^{RB}$-binding mutant TAg plasmids, pRSV-T(ΔRBBS) and pRSV-T(K1), was compared with that of the wt TAg plasmid, pRSV-T (focus formation with pRSV-T and pRSV-T(ΔRBBS), only, are shown in Figure 3.2). In NHDF cells, co-transfection of pRSV-T(ΔRBBS) or pRSV-T(K_1) with any of the control plasmids, pRcCMV, pRSV.3 or pSV2neo, never produced multilayered foci (Table 3.2). Occasional patches (1-2 patches per plate) of transformed cells were apparent, but only after crystal violet staining. The cells in these patches had a transformed, more cuboidal, appearance compared to the background cells which had a typical fibroblastic, fusiform, appearance (Figure 3.3). These non-

Figure 3.3 Morphological transformation of NHDF induced by non-p110^{RB}-binding mutants of TAg

Photomicrographs showing cells after fixing and staining with formaldehyde/crystal violet solution. Patches of morphologically transformed cells induced in NHDF cell strains, MRC-5 and GM03313, by the stable transfection of wt TAg plasmid pRSV-T, or non-p110^{RB}-binding mutant TAg plasmid, pRSV-T(ΔRBBS). No foci were induced by transfection with the control plasmid, pRcCMV, alone.

(Mag. X 120)


MRC-5
GM03313
pRcCMV
pRcCMV
pRSV-T(ΔRBBS)
pRSV-T(ΔRBBS)
pRSV-T
pRSV-T

p110^{RB}-binding mutant TAg-transformed cells appeared to be contact inhibited and did not pile up to form multilayered foci typical of wt TAg (Figure 3.3). The non-p110^{RB}-binding mutant TAg-induced patches thus remained undetected without staining, even after 8 weeks in culture, and so do not appear in Table 3.2.

A PCR based strategy was employed to detect integration of the non-p110^{RB}-binding mutant plasmids into the cells comprising the transformed patch. Cloning cylinders were placed on the formalin/ crystal violet stained plates of cells to isolate the foci or background cells for DNA extraction. The cells were lysed within a cloning cylinder and DNA extracted using 200 µl of Trizol, as described in Section 2.3.2. PCR using one set of primers was not sufficient to detect the TAg encoding DNA even in the pRSV-T induced foci and so a nested PCR strategy was employed (Section 2.3.5). The two sets of primers are located 3' of the region affected in the two non-p110^{RB}-binding TAg mutants. The first PCR reaction was performed at low stringency for 20 cycles. A 2 µl aliquot from this reaction was used as template for a second PCR which used nested primers at a higher stringency for 35 cycles. As seen in Figure 3.4, PCR of pRSV-T plasmid DNA as well as DNA extracted from the cells from some of the foci induced by pRSV-T, pRSV-T(ΔRBBS) and pRSV-T(K_1), gave the expected band at 407 bp, which is specific for TAg. This band was not produced in the DNA from the background cells nor the water control. A smaller band of ~250 bp also appeared in some of the lanes and probably represents non-specific amplification as it did not appear in the pRSV-T control. Also seen with the positive control, pRSV-T plasmid, was the 558 bp band generated by the first PCR reaction.

Figure 3.4 Nested PCR to detect the presence of SV40 early region DNA in foci of transfected NHDF cells

Focus formation in NHDF cell strains, GM03313 and MRC-5, stably transfected with the SV40 early region plasmids as indicated. DNA was isolated from cells within a number of induced foci or from a number of sites of the background cells. The DNA was subject to a low stringency PCR reaction for 20 cycles to generate a 558 bp fragment which was used as template for a nested reaction at higher stringency for 35 cycles to generate a 407 bp fragment. The products were separated on a 2%-agarose gel and stained with ethidium bromide to visualise the fragments. The H_2O is a PCR negative control lane and contains no bands, as do the lanes from the background cells. The pRSV-T positive control lane is pRSV-T plasmid DNA and contains both the 407 bp fragment, generated by the second set of nested PCR primers, and the 558 bp fragment, from the first set of primers. Some of the foci lanes contain the 407 bp fragment and some of these contain a faint 558 bp fragment from the first set of primers.

560bp
H2O
pRSV-T
1 2 3 4 1 2 3 4 1 2 3 1 1 2 3 4 1 2 3 4 1 1 2 3 1 2 3 4 1 2 3 4 1 2 3 4
background cells
pRSV-T foci
pRSV-T(ΔRBBS) foci
pRSV-T(K1)
background cells
pRSV-T foci
pRSV-T(ΔRBBS)
pRSV-T(K1) foci
background cells
pRSV-T foci
pRSV-T(ΔRBBS) foci
expt. 4 GM03313 cells
expt. 5 GM03313 cells
expt. 18 MRC-5 cells

The PCR reaction may have been inhibited in some of the reactions by the presence of crystal violet. The amount of crystal violet extracted from the pRSV-T-induced foci would have been greater due to the multilayered nature of these foci. This may explain why the PCR reaction on DNA from a significant number (8/12) of the pRSV-T foci did not amplify the 560 bp band.

The presence of the SV40-specific 407 bp band indicates that the cells within the pRSV-T(ΔRBBS) and pRSV-T(K1) foci contain the plasmid, suggesting that the foci were induced by the integration of, and presumed expression from, these plasmids. The cells in these foci had a transformed appearance (Figure 3.3) but did not pile up, in contrast to the cells transformed by the wt pRSV-T. The lack of piling up may indicate that the non-p110^{RB}-binding TAg plasmid transformed cells are subject to contact inhibition while the wt pRSV-T cells are not.

II Effect of RB-1 on pRSV-T induced focus formation

Despite many years of research using SV40 TAg in transformation studies, the 3D-structure of TAg is still unknown. Thus the effect of mutations on protein structure and functions are unknown. It is known, however, that the p110^{RB}-binding region of TAg also binds to p107 and p130, other members of the RB family. To verify that disruption of binding to p110^{RB} ablates TAg-induced focus formation, HFF5 and MRC-5 cells were co-transfected with pRSV-T and expression plasmids for either wt or mutant p110^{RB}, pLRbRNL or pLRb(Δ768-909)RNL, respectively. Integration and expression of an exogenous source of p110^{RB} may provide excess p110^{RB} that

would not be bound and hence not inactivated by TAg, thus decreasing focus formation in this assay.

Contrasting the focus formation with pRSV-T + pRcCMV to that with pRSV-T + pLRbRNL (Figure 3.5) demonstrates that co-transfection with pLRbRNL decreases the focus forming ability of pRSV-T to ~32%. Surprisingly, a similar degree of suppression was seen in the plates co-transfected with pLRb(Δ768-909)RNL expressing the mutant form of RB-1 (~30%). This indicates that pLRb(Δ768-909)RNL also suppresses focus formation and suggests retention of some wild type activity. These data suggest that the reduced focus formation seen with co-transfection of pRSV-T with pLRbRNL is due, at least in part, to the binding of TAg to $p110^{RB}$.

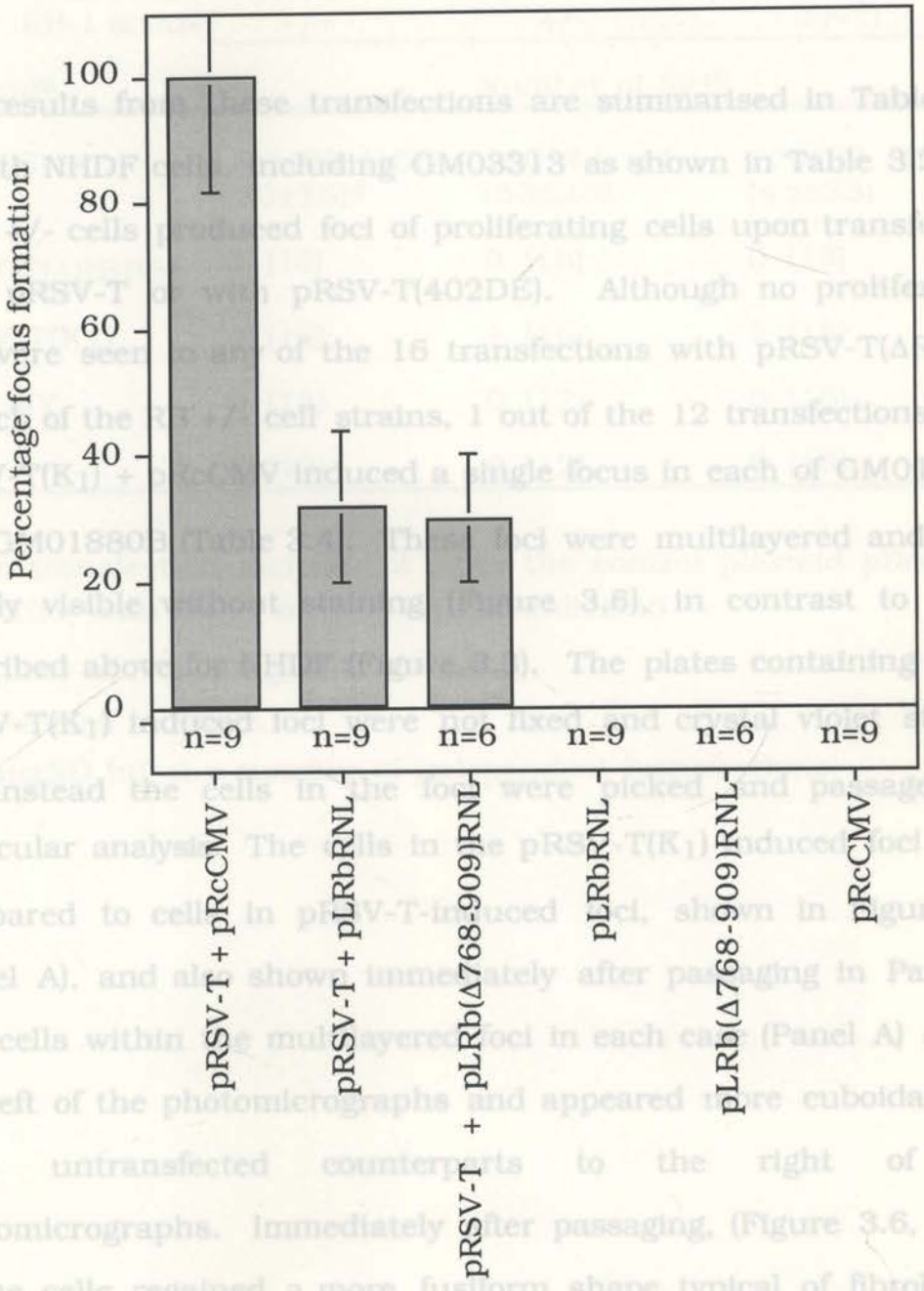
III pRSV-T(K_1)-induced proliferating foci

The non-$p110^{RB}$-binding TAg mutant plasmids were transfected into two fibroblast cell strains containing just one RB-1 allele, GM01879A and GM01880B. The lack of focus formation with the non-$p110^{RB}$-binding mutant TAg plasmids in NHDF, together with the inhibition of wt TAg-induced focus formation by pLRbRNL, indicated that binding and inactivation of $p110^{RB}$ was necessary for TAg-induced focus formation. Thus the loss of one RB-1 allele would increase the chance of loss of RB-1 function, via mutation or loss of the one remaining RB-1 allele, which may result in focus formation with the non-$p110^{RB}$-binding TAg plasmids.

The cell strains used in the following part of the study were obtained as skin fibroblast cultures from a single family with hereditary retinoblastoma (from Coriell Cell Repository). The cells

Figure 3.5 Percentage focus formation in HFF5 and MRC-5 cells

The number of foci, induced by transfection of the plasmids indicated, were counted and compared to the number induced by co-transfection of pRSV-T + pRcCMV. Focus formation expressed as a percentage of that of pRSV-T + pRcCMV which is normalised to 100%.


Percentage focus formation
100
80
60
40
20
0
n=9
n=9
n=6
n=9
n=6
n=9
pRSV-T + pRcCMV
pRSV-T + pLRbRNL
pRSV-T + pLRb(Δ768-909)RNL
pLRbRNL
pLRb(Δ768-909)RNL
pRcCMV

from the affected individuals contained only one wt RB-1 allele. The cell strains obtained were from an 11 year old (yo) female, the proband (GM01879A), the 37 yo affected mother (GM01880B), and 40 yo unaffected father (NHDF strain GM03313).

The results from these transfections are summarised in Table 3.4. As with NHDF cells, including GM03313 as shown in Table 3.2, the RB-1 +/- cells produced foci of proliferating cells upon transfection with pRSV-T or with pRSV-T(402DE). Although no proliferating foci were seen in any of the 16 transfections with pRSV-T(ΔRBBS) in each of the RB +/- cell strains, 1 out of the 12 transfections with pRSV-T(K_1) + pRcCMV induced a single focus in each of GM01889A and GM01880B (Table 3.4). These foci were multilayered and were clearly visible without staining (Figure 3.6), in contrast to those described above for NHDF (Figure 3.3). The plates containing these pRSV-T(K_1) induced foci were not fixed and crystal violet stained but instead the cells in the foci were picked and passaged for molecular analysis. The cells in the pRSV-T(K_1) induced foci were compared to cells in pRSV-T-induced foci, shown in Figure 3.6 (Panel A), and also shown immediately after passaging in Panel B. The cells within the multilayered foci in each case (Panel A) are to the left of the photomicrographs and appeared more cuboidal than their untransfected counterparts to the right of the photomicrographs. Immediately after passaging, (Figure 3.6, Panel B) the cells regained a more fusiform shape typical of fibroblasts, though these cells were wider, with a larger nucleus to cytoplasm ratio, and more refractile than the background untransfected cells shown in Panel A.

Table 3.4 Focus formation in fibroblasts from a RB-1 +/- affected family: wt TAg compared to $p110^{RB}$-binding mutants

Cell strain RB-1 status	GM03313 +/+	GM01879A +/-	GM01880B +/-
plasmid[a]	Number of foci[b]		
pRSV-T	100±69 (15)[c] {36±25}[d]	100±47 (14) {53±25}	100±73 (18) {45±33}
pRSV-T(ΔRBBS)	0 (16)	0 (16)	0 (19)
pRSV-T(K1)	0 (16)	1 (16)	1 (16)
pRcCMV	0 (12)	0 (12)	0 (15)
H_2O	0 (12)	0 (12)	0 (15)

[a]each transfection included 2 µg of the control plasmid pRcCMV together with 10 µg of the plasmid indicated

[b]{number of foci/10µgDNA/10^6 cells}

[c]normalised to 100%

[d]mean±SD (n), n = number of independent transfections

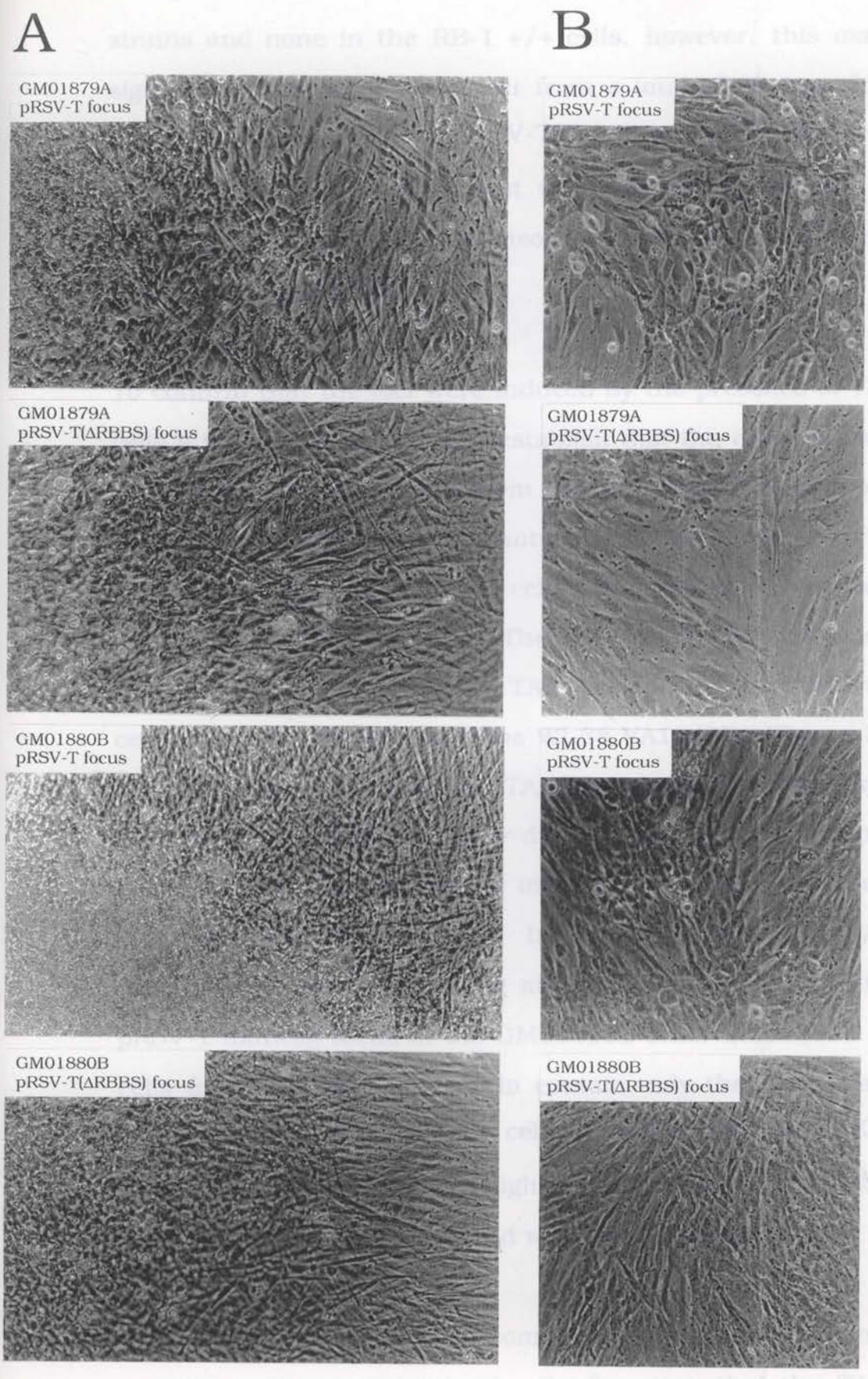
Figure 3.6 TAg-induced focus formation in RB +/- cells

Phase contrast photomicrographs showing cells from foci induced by stable transfection of the wt TAg plasmid, pRSV-T or non-$p110^{RB}$-binding plasmid, pRSV-T(ΔRBBS).

A. Foci before passaging. The transformed cells in the foci are seen tightly packed and multilayered to the left of each picture, with the normal background fusiform cells to the right, or top right.

B. Cells from each of the foci 24 hours after passaging.

(Mag. X 120)


A
B
GM01879A
pRSV-T focus
GM01879A
pRSV-T focus
GM01879A
pRSV-T(ΔRBBS) focus
GM01879A
pRSV-T(ΔRBBS) focus
GM01880B
pRSV-T focus
GM01880B
pRSV-T focus
GM01880B
pRSV-T(ΔRBBS) focus
GM01880B
pRSV-T(ΔRBBS) focus

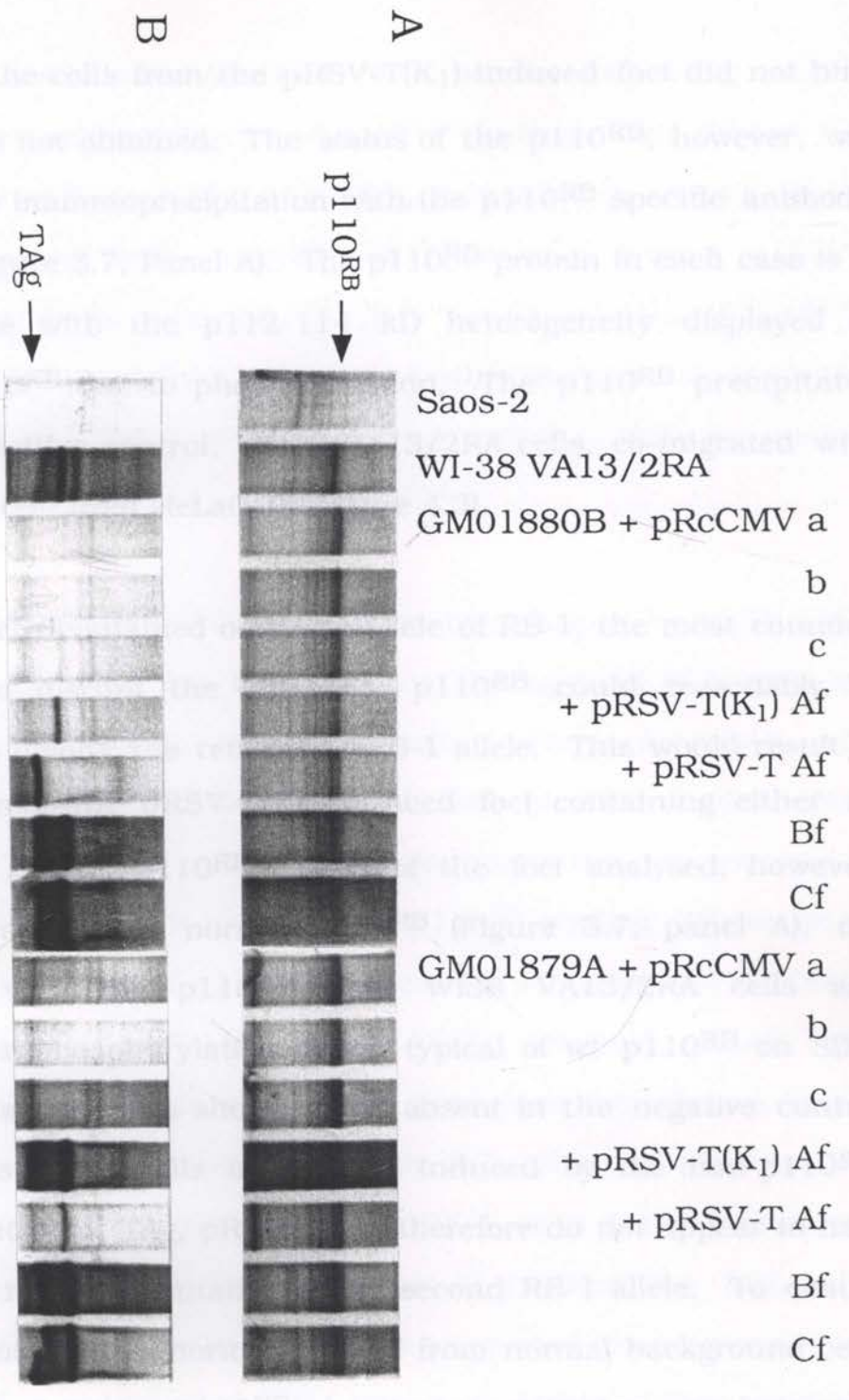
One pRSV-T(K_1) focus was induced in each of the two RB-1 +/- cell strains and none in the RB-1 +/+ cells, however, this may not be significant difference. Two foci from a total of 67 transfections of either pRSV-T(ΔRBBS) or pRSV-T(K_1) into 1 x 10^6 RB +/- cells in each transfection, is equivalent to 2 foci/6.7 x 10^7 RB +/- cells transfected. This is in comparison to 0 foci/ 7.9 x 10^7 NHDF cells transfected (Table 3.2).

To confirm that the foci were induced by the presence of TAg in the cells it was important to first establish that the cells in the foci did express TAg protein. Cells from the foci were ^{35}S-AA labelled and immunoprecipitated with an antibody to TAg, PAb108 (Figure 3.7 Panel B). TAg was detected in cells from each of the foci and not in any of the pRcCMV controls. The TAg protein detected in each case co-migrated with the 94 kD TAg present in the positive control cells, the SV40 transformed line WI-38 VA13/2RA (Figure 3.7, Panel B). The TAg in each of the TAg containing cell lines appears to migrate as a doublet, probably due to the different phosphorylated forms of TAg reported to be immunoprecipitated by the PAb108 antibody [Tack *et al.*, 1988]. In addition, WI-38 VA13/2RA also contains a "super T" migrating at ~100 kD. Exceptions include the pRSV-T induced focus in the GM01880B cells, GM01880B TAf. The cells from this focus appear to contain only the lower band of the TAg doublet. In contrast, the cells from the GM01880B K_1 Af focus appear to contain only the higher band of the TAg doublet. The reason for this is unknown and was not explored further.

Under these conditions the complex between the TAg and p110^{RB} proteins was not maintained. Confirmation that the TAg protein

Figure 3.7 Immunoprecipitation analysis of $p110^{RB}$ and TAg of TAg-induced foci in RB +/- cells

Foci (indicated by upper case letters followed by lower case f) and mass culture control cells (indicated by lower case letters) were labelled with ^{35}S-AA. The cells were then lysed using RIPA buffer and the $p110^{RB}$, **Panel A**, and TAg, **Panel B**, protein was immunoprecipitated. The precipitated proteins were then separated on an 8% SDS-polyacrylamide gel and detected by autoradiography. Control cell lines: Saos-2, contains only 95 kD mutant form of $p110^{RB}$; WI-38 VA13/2RA, contains wt $p110^{RB}$ and TAg.


A
B
p110RB
TAg
Saos-2
WI-38 VA13/2RA
GM01880B + pRcCMV a
b
c
+ pRSV-T(K1) Af
+ pRSV-T Af
Bf
Cf
GM01879A + pRcCMV a
b
c
+ pRSV-T(K1) Af
+ pRSV-T Af
Bf
Cf

present in the cells from the pRSV-T(K_1)-induced foci did not bind $p110^{RB}$ was not obtained. The status of the $p110^{RB}$, however, was analysed by immunoprecipitation with the $p110^{RB}$ specific antibody, NCL-RB (Figure 3.7, Panel A). The $p110^{RB}$ protein in each case is of correct size with the p112-114 kD heterogeneity displayed by normal $p110^{RB}$ due to phosphorylation. The $p110^{RB}$ precipitated from the positive control, WI-38 VA13/2RA cells, co-migrated with normal $p110^{RB}$ from HeLaCOT (Figure 4.3).

Since the cells contained only one allele of RB-1, the most common mutation to disrupt the action of $p110^{RB}$ could reasonably be expected to involve the remaining RB-1 allele. This would result in the cells from the pRSV-T(K_1)-induced foci containing either no $p110^{RB}$ or mutant $p110^{RB}$. Each of the foci analysed, however, contained apparently normal $p110^{RB}$ (Figure 3.7, panel A), co-migrating with the $p110^{RB}$ from WI38 VA13/2RA cells and containing a phosphorylation smear typical of wt $p110^{RB}$ on SDS-PAGE. This band was shown to be absent in the negative control Saos-2 cells. The cells in the foci induced by the non-$p110^{RB}$-binding mutant of TAg, pRSV-T(K1), therefore do not appear to have arisen as a result of mutation of the second RB-1 allele. To ensure that contamination of normal $p110^{RB}$ from normal background cells had not occurred, $p110^{RB}$ immunoprecipitation analysis was performed on the cells after passaging a further 20 PDL (data not shown). This experiment gave the same result as that shown in Figure 3.7 Panel A.

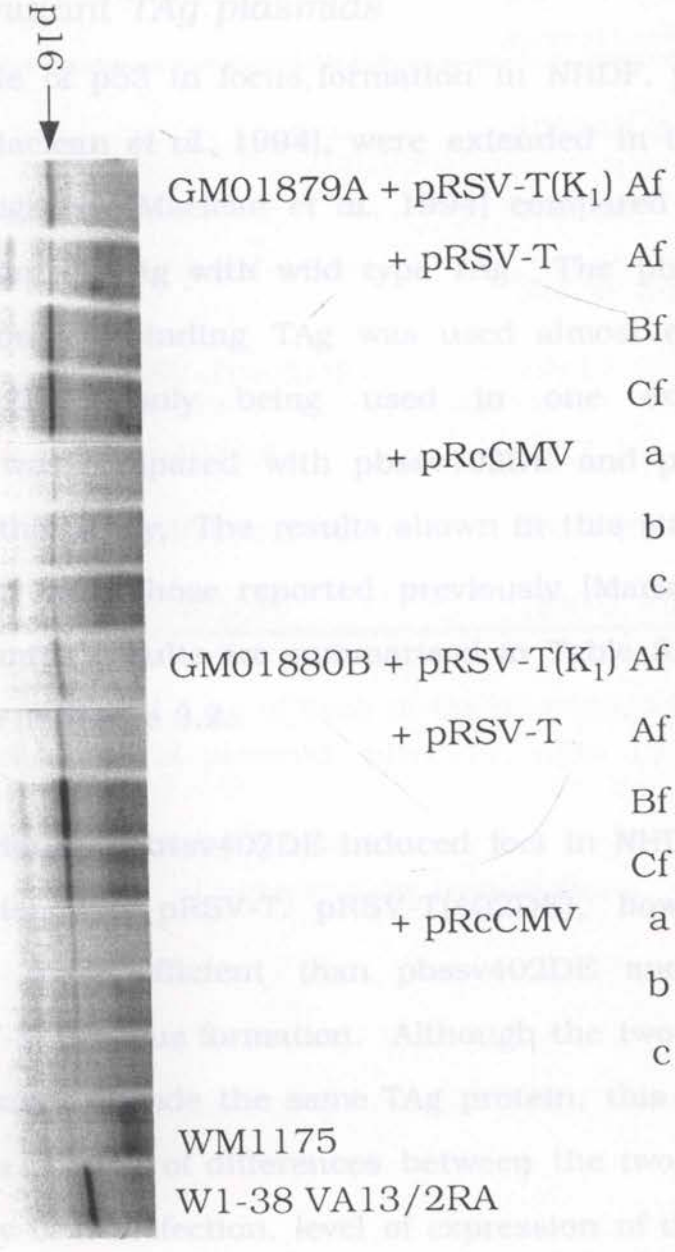
As $p110^{RB}$ was normal in each of the foci, $p16^{INK4}$ was also analysed. Lack of $p16^{INK4}$ expression has recently been suggested to be an

alternative mechanism to RB-1 mutation for bypassing $p110^{RB}$ control. Immunoprecipitation of $p16^{INK4}$ in these cells (Figure 3.8) showed that cells in each of the TAg induced foci, as well as the pRcCMV control cells, express an apparently normal $p16^{INK4}$. $p16^{INK4}$ mutations reported to date result in loss of $p16^{INK4}$ expression rather than a mutant protein [Okamoto *et al.*, 1994; Otterson *et al.*, 1994; Washimi *et al.*, 1995]. This correlation suggests these cells have normal $p16^{INK4}$.

A mechanism whereby these cells were transformed by pRSV-T(K1) to form proliferating foci in a confluent background of normal and non-proliferating cells, is not obvious. Focus formation with pRSV-T(K1) could be explained if the protein retains some undetected binding to $p110^{RB}$ or if a back mutation has occurred in the plasmid such that it has regained $p110^{RB}$ binding. There might be a decreased level of $p110^{RB}$ in these RB +/- cells that facilitated focus formation with the pRSV-T(K1) plasmid, albeit very inefficiently; a decrease in $p110^{RB}$ level was not obvious in the immunoprecipitations shown in Figure 3.7. Alternatively, mutation of other genes, possibly related to RB-1 or a pathway involving $p110^{RB}$, could induce the formation of proliferating foci. For example, cdk4 amplification has been suggested as an alternative to RB-1 mutation or loss of $p16^{INK4}$ [He *et al.*, 1994]. Other candidate genes would include D-type cyclin genes or genes involved in signalling between the membrane and the nucleus. An exploration of the status of these genes was, however, beyond the scope of this project.

Figure 3.8 Immunoprecipitation analysis of $p16^{INK4}$ from TAg-induced foci in RB +/- cells

Foci (indicated by upper case letters followed by lower case f) and mass culture control cells (indicated by lower case letters) were labelled with ^{35}S-AA. The cells were then lysed and the $p16^{INK4}$ protein was immunoprecipitated from each of the cell strains. The precipitated proteins were then separated on an SDS-polyacrylamide (4-20% gradient) gel and detected by autoradiography. Control cell lines: WM1175, contains homozygous deletion of $p16^{INK4}$; WI-38 VA13/2RA, contains normal sized $p16^{INK4}$.


p16
GM01879A + pRSV-T(K1) Af
+ pRSV-T Af
Bf
Cf
+ pRcCMV a
b
c
GM01880B + pRSV-T(K1) Af
+ pRSV-T Af
Bf
Cf
+ pRcCMV a
b
c
WM1175
W1-38 VA13/2RA

3.2.2 p53 binding is not necessary for focus formation

I Focus formation induced by non-p53-binding mutant TAg plasmids

Studies of the role of p53 in focus formation in NHDF, previously reported by us [Maclean *et al.*, 1994], were extended in this study. The initial investigation [Maclean *et al.*, 1994] compared the non-p53-binding mutant of TAg with wild type TAg. The pbssv402DE version of the non-p53-binding TAg was used almost exclusively with pRSV-T(402DE) only being used in one experiment. pRSV-T(402DE) was compared with pbssv402DE and pRSV-T in greater detail in this study. The results shown in this study are in general agreement with those reported previously [Maclean *et al.*, 1994]. Focus forming results are summarised in Table 3.5, and an example is shown in Figure 3.2.

As reported previously, pbssv402DE induced foci in NHDF at only ~2% of the efficiency of pRSV-T. pRSV-T(402DE), however, was nearly 20 times more efficient than pbssv402DE and 32% as efficient as pRSV-T in focus formation. Although the two non-p53-binding TAg plasmids encode the same TAg protein, this difference could be due to a number of differences between the two plasmids, e.g., the efficiency of transfection, level of expression of the protein and the presence of the SV40 late region genes.

Co-transfection of pRSV-T(402DE) with either of the non-p110^{RB} -binding mutants, pRSV-T(ΔRBBS) or pRSV-T(K1), did not alter focus formation and did not complement pRSV-T(402DE) to give wild type TAg efficiency (Table 3.5). Co-transfection of pbssv402DE

Table 3.5 Focus formation by non-p53-binding mutant TAg plasmids in NHDF (HFF5 and MRC-5) cells

Plasmid 1[a]	Plasmid 2	percentage focus formation[b]
pRSV-T		100±18 (9)[c]
pbssv402DE		2±3 (9)
pRSV-T(402DE)		32±28 (9)
pRSV-T(Δ)	pRSV-T(402DE)	29±17 (9)
pRSV-T(Δ)	bssv402DE	11±7 (6)
pRSV-T(K1)	pRSV-T(402DE)	28±9 (6)
pRSV-T(K1)	bssv402DE	10±12 (6)

[a]each transfection included 5 μg of each of the plasmid/s indicated together with the control plasmid, pRcCMV, upto 12 μg total plasmid DNA

[b]compared to pRSV-T in each experiment

[c]mean±SD (n), n = number of independent transfections

with pRSV-T(ΔRBBS) or pRSV-T(K1), however, did increase the efficiency of bssv402DE to nearly one-third that of pRSV-T(402DE). This demonstrates that co-transfection of two plasmids can have an effect on focus formation.

3.3 Lifespan extension

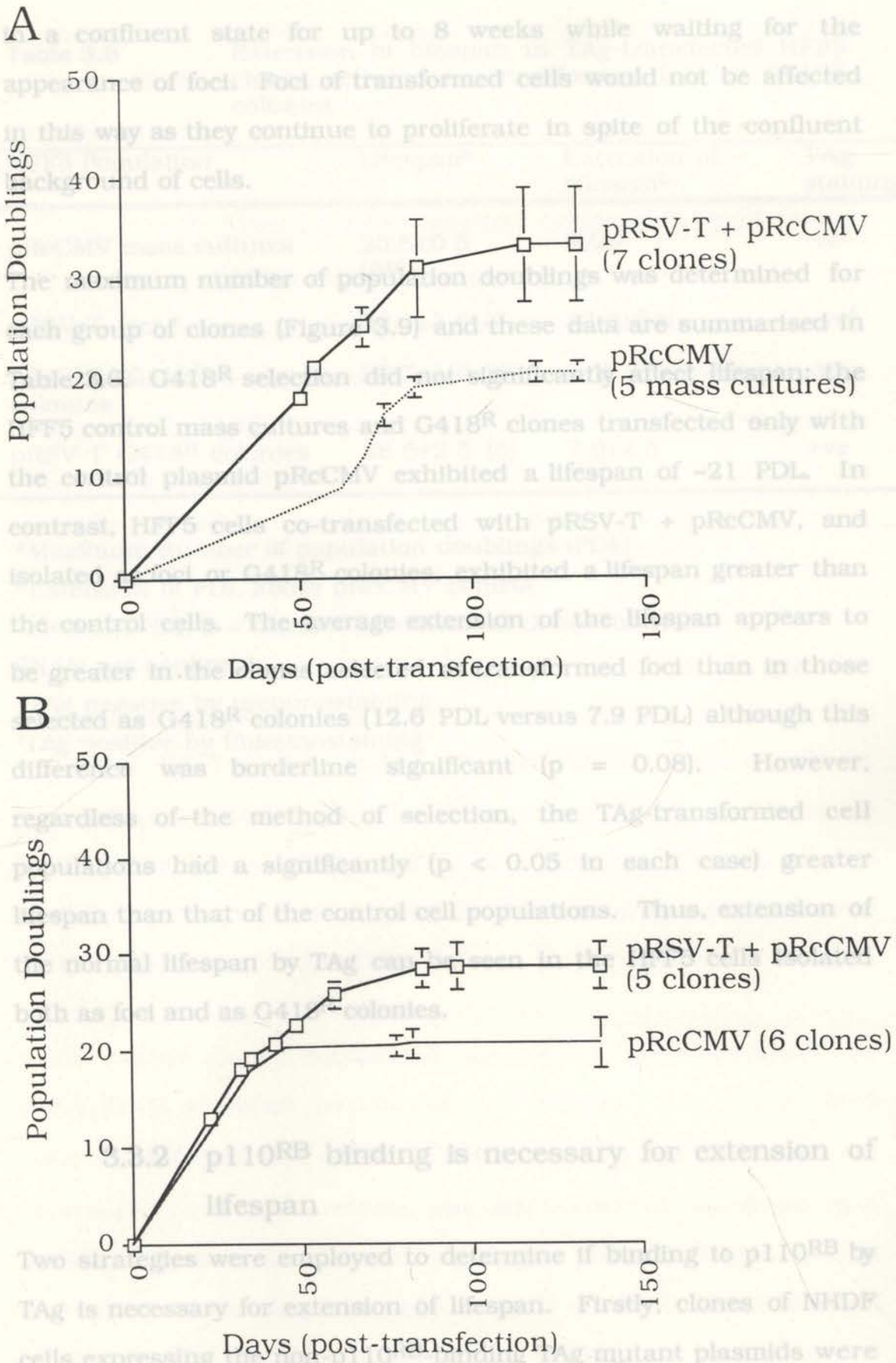
3.3.1 Effect of G418 selection on SV40-induced lifespan extension

A second effect of TAg expression in normal cells is the extension of their lifespan in culture. Foci and $G418^R$ colonies were selected and then picked using cloning cylinders as described in Section 2.3.2. The pRcCMV controls for the focus formation experiments were concurrently passaged as mass cultures. The PDL number was estimated at each passage and plotted to give a growth curve as shown in Figure 3.9. Panel A (Figure 3.9) shows the growth curves for 7 foci induced by the co-transfection of pRSV-T + pRcCMV and 5 control mass cultures transfected with pRcCMV only. For comparison, Panel B (Figure 3.9) shows the growth curves for 5 $G418^R$ resistant colonies of cells also expressing pRSV-T, and 6 control $G418^R$ colonies of cells transfected with pRcCMV only.

Generally the growth curves of the transfected HFF5 cells were exponential immediately after picking the focus or $G418^R$ colony. After a number of population doublings, however, the rate decreased and eventually leveled off. The pRcCMV control mass cultures shown in panel A (Figure 3.9) were exceptional, showing a slight inflection in the early part of the growth curve. This was probably due to the quiescence induced in the population by leaving the cells

Figure 3.9 Growth curves of A. pRSV-T-induced foci and B. pRSV-T G418^R selected clones of HFF5 cells

Cells were transfected with either pRSV-T + pRcCMV or pRcCMV alone at day 0 using lipofectamine. **A.** Clones were selected from pRSV-T induced foci and passaged individually, or control cells were passaged as mass cultures. **B.** Clones were selected as G418^R colonies. The selected clones or mass cultures were passaged individually and cumulative population doublings (PDLs) were calculated at each passage. The mean±SD of the cumulative population doublings, for each group of cells, is indicated on the growth curves.


A
Population Doublings
50
40
30
20
10
0
0
50
100
150
Days (post-transfection)
pRSV-T + pRcCMV
(7 clones)
pRcCMV
(5 mass cultures)
B
Population Doublings
50
40
30
20
10
0
0
50
100
150
Days (post-transfection)
pRSV-T + pRcCMV
(5 clones)
pRcCMV (6 clones)

in a confluent state for up to 8 weeks while waiting for the appearance of foci. Foci of transformed cells would not be affected in this way as they continue to proliferate in spite of the confluent background of cells.

The maximum number of population doublings was determined for each group of clones (Figure 3.9) and these data are summarised in Table 3.6. $G418^R$ selection did not significantly affect lifespan: the HFF5 control mass cultures and $G418^R$ clones transfected only with the control plasmid pRcCMV exhibited a lifespan of ~21 PDL. In contrast, HFF5 cells co-transfected with pRSV-T + pRcCMV, and isolated as foci or $G418^R$ colonies, exhibited a lifespan greater than the control cells. The average extension of the lifespan appears to be greater in the clones selected as transformed foci than in those selected as $G418^R$ colonies (12.6 PDL versus 7.9 PDL) although this difference was borderline significant ($p = 0.08$). However, regardless of the method of selection, the TAg-transformed cell populations had a significantly ($p < 0.05$ in each case) greater lifespan than that of the control cell populations. Thus, extension of the normal lifespan by TAg can be seen in the HFF5 cells isolated both as foci and as $G418^R$ colonies.

3.3.2 $p110^{RB}$ binding is necessary for extension of lifespan

Two strategies were employed to determine if binding to $p110^{RB}$ by TAg is necessary for extension of lifespan. Firstly, clones of NHDF cells expressing the non-$p110^{RB}$-binding TAg mutant plasmids were selected and passaged for lifespan extension. Secondly, the effect of

Table 3.6 Extension of lifespan in TAg-transfected HFF5 clones selected as transformed foci or G418^R colonies

HFF5 Population	Lifespan[a]	Extension of lifespan[b]	TAg staining
pRcCMV mass cultures	20.5±0.5 (6)[c]	N/A[d]	-ve[e]
pRSV-T foci	33.1±5.6 (7)	12.6±5.6	+ve[f]
pRcCMV G418^R colonies	20.7±1.0 (6)	N/A	-ve
pRSV-T G418^R colonies	28.6±2.5 (5)	7.9±2.5	+ve

[a]Maximum number of population doublings (PDL)

[b]Extension of PDL above pRcCMV control

[c]mean±SD (n), n = number of individual clones analysed

[d]N/A: not applicable

[e]TAg negative by immunostaining

[f]TAg positive by immunostaining

co-transfecting the RB-1 expression plasmid, pLRbRNL, with the pRSV-T plasmid on the lifespan of the selected clones was examined.

I Non-p110^{RB}-binding mutant TAg plasmids do not extend lifespan

It was not possible to determine the lifespan of the non-110^{RB}-binding TAg mutant plasmid induced foci in the NHDF cells because they were only recognised after fixing and staining. The lifespan of NHDF cells expressing either of the non-110^{RB}-binding TAg mutant plasmids, however, was determined in G418-selected clones. Selection of G418^R colonies co-transfected with either pRSV-T(ΔRBBS) or pRSV-T(K_1) proved difficult. From 9 transfections into HFF5 or MRC-5 cells, from which 56 pRSV-T G418^R colonies and 28 pRcCMV G418^R colonies were obtained, only one pRSV-T(K_1) G418^R and five pRSV-T(ΔRBBS) colonies could be successfully isolated. As for the clones shown in Figure 3.9, these colonies were also passaged to determine lifespan. The extension of lifespan, i.e. the increase beyond the lifespan of the pRcCMV-only G418^R clones, is shown in Table 3.7.

NHDF G418^R clones transfected with either pRSV-T(ΔRBBS) or pRSV-T(K_1) exhibited no extension of lifespan. The three HFF5 clones transfected with pRSV-T(ΔRBBS) had a lifespan less than the control cells, but the difference was only borderline significant ($p = 0.069$). The lack of extension of lifespan was not due to lack of expression of the TAg plasmids as each of these clones stained positive for TAg by immunostaining (not shown).

Table 3.7 Extension of lifespan in G418^R selected clones of NHDF cells, HFF5 and MRC-5, transfected with non-p110^{RB}-binding mutant TAg plasmids

G418^R colonies[a]	Extension of lifespan[b]
pRSV-T	+ 7.9±2.5 (5)[c]
pRSV-T(ΔRBBS)	- 1.6±1.7 (5)
pRSV-T(K1)	0 (1)

[a]each of the clones was TAg positive by immunostaining; each clone was co-transfected with pRcCMV

[b]Extension of population doublings above pRcCMV control (Table 3.6)

[c]mean±SD (n), n = number of individual colonies analysed

As described in Section 3.2.1, one pRSV-T(K_1)-induced focus from each of the RB+/- cell strains, GM01879A and GM01880B, was isolated. These clones were shown to have normal $p110^{RB}$ and $p16^{INK4}$ (Figures 3.7 & 3.8) and were passaged to examine lifespan extension (Figure 3.10 panels A and B). The control cell populations from each of these RB +/- cell strains exhibited an unusually long *in vitro* lifespan (40 PDL shown in Figures 3.7 & 3.8 plus ~32 PDL prior to transfection) in comparison to the NHDF in this study. The GM01879A and GM01880B clones expressing pRSV-T, as shown in Figure 3.7 (Panel B), have a lifespan similar to the pRcCMV transfected controls with two exceptions. The GM01879A + pRSV-T clones Bf and Cf (Figure 3.10, Panel A) both attained 12 more PDL than the controls (53 PDL versus 40.7±0.6 PDL). As the control cells appear to have an extended lifespan it is perhaps not surprising that no extension of lifespan was exhibited by the pRSV-T(K_1) expressing clones, for either of the cell strains. However, the lack of lifespan extension in the NHDF transfected with pRSV-T(ΔRBBS), or pRSV-T(K_1), demonstrates the need for an intact $p110^{RB}$ binding region for extension of lifespan in NHDF.

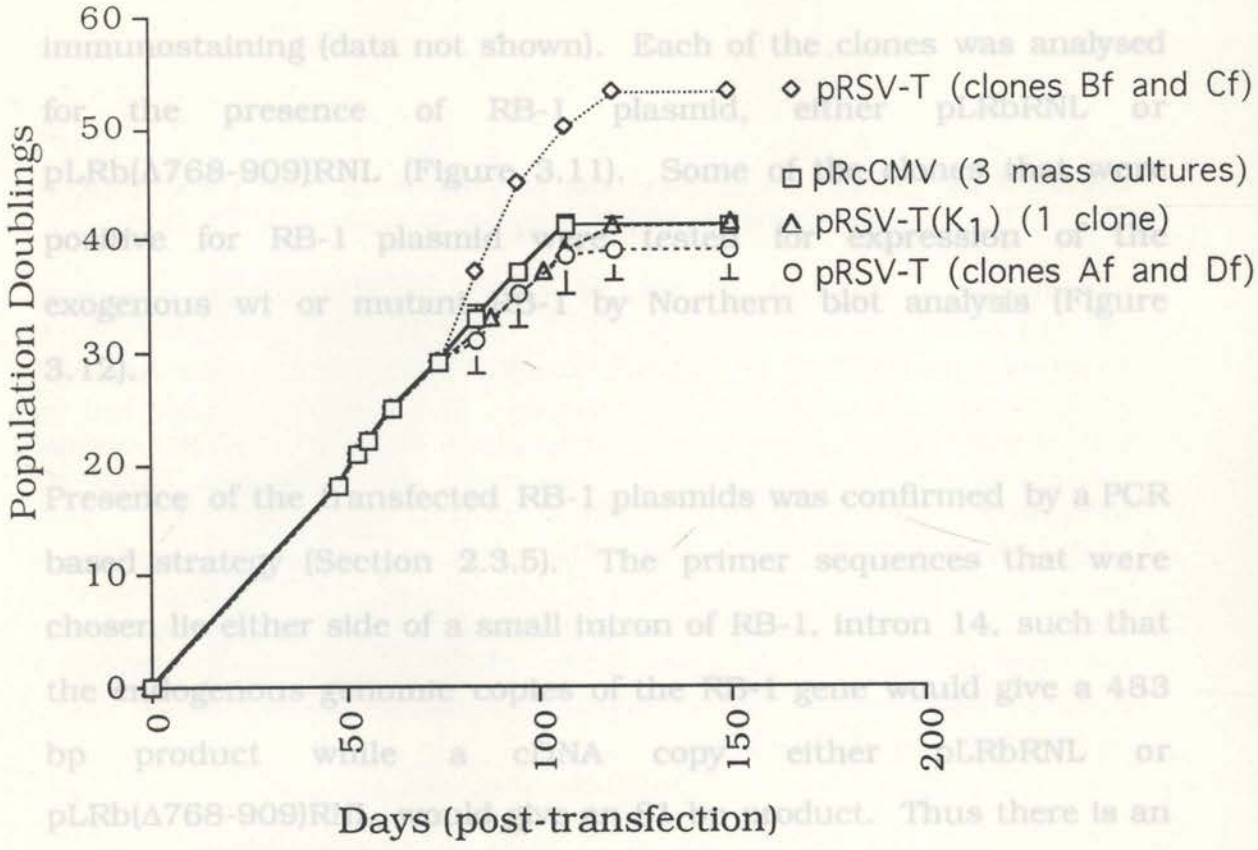
II Effect of RB-1 expression plasmids on extension of lifespan

To confirm that binding to $p110^{RB}$ is necessary for extension of *in vitro* lifespan, the effect of exogenous RB-1 on pRSV-T extended lifespan was analysed by co-transfecting pRSV-T with the wt , or mutant, RB-1 expression plasmids, pLRbRNL and pLRb(768-909)RNL respectively. The resultant foci induced in HFF5 cells were isolated and shown to express TAg by

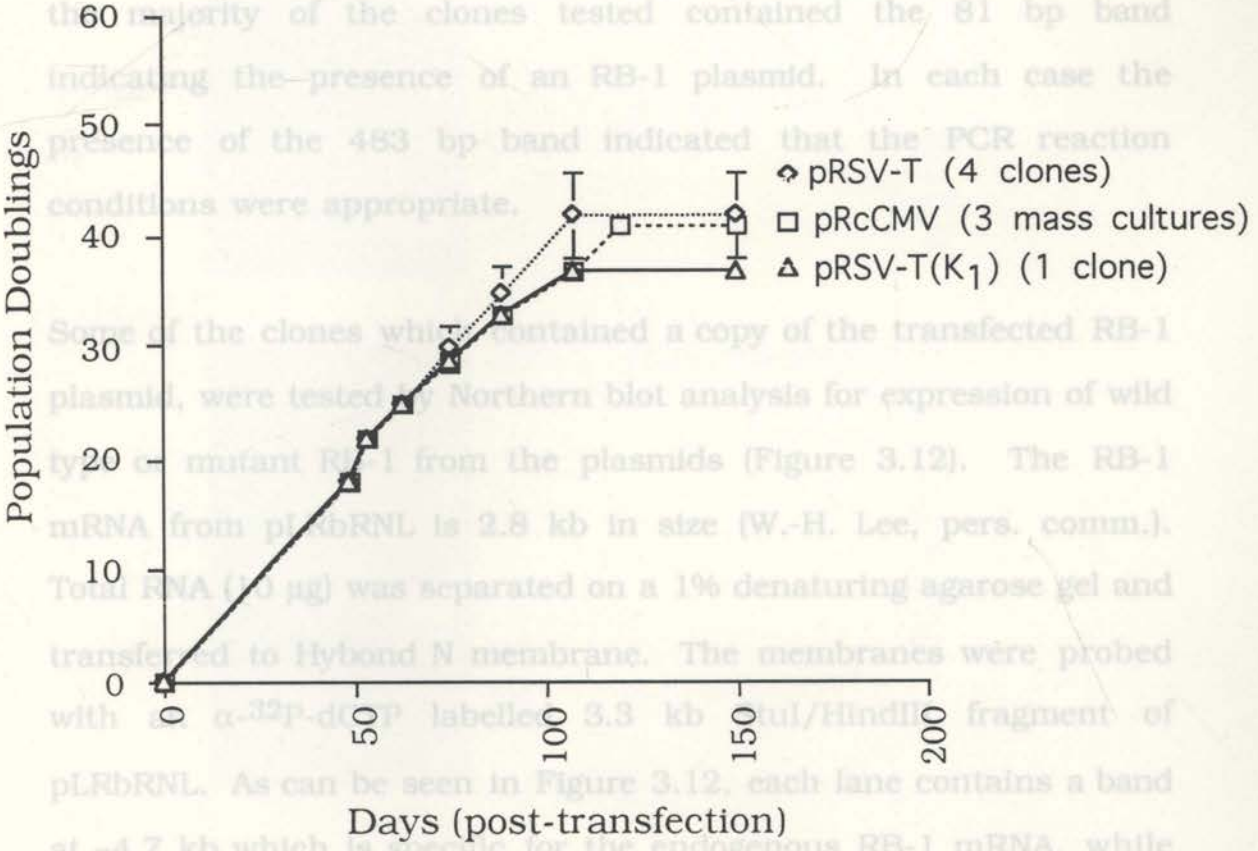
Figure 3.10 Growth curves of pRSV-T-induced and pRSV-T(K_1)-induced foci in RB +/- cells

Cell strains containing just one copy of the wild type RB-1 gene, **A** GM01879A and **B** GM01880B cells, were co-transfected with a control plasmid, pRcCMV, with either wt TAg expression plasmid pRSV-T, non-$p110^{RB}$-binding mutant TAg plasmid pRSV-T(K_1) or the pRcCMV alone at day 0 using lipofectamine. Clones were selected as individual foci or cells were passaged as mass cultures. The cells were passaged individually and cumulative population doublings (PDLs) were calculated at each passage. The mean±SD of the cumulative population doublings, for each group of cells, is indicated on the growth curves.

A


Population Doublings
60
50
40
30
20
10
0
0
50
100
150
200
Days (post-transfection)
pRSV-T (clones Bf and Cf)
pRcCMV (3 mass cultures)
pRSV-T(K1) (1 clone)
pRSV-T (clones Af and Df)

B


Population Doublings
60
50
40
30
20
10
0
0
50
100
150
200
Days (post-transfection)
pRSV-T (4 clones)
pRcCMV (3 mass cultures)
pRSV-T(K1) (1 clone)

immunostaining (data not shown). Each of the clones was analysed for the presence of RB-1 plasmid, either pLRbRNL or pLRb(Δ768-909)RNL (Figure 3.11). Some of the clones that were positive for RB-1 plasmid were tested for expression of the exogenous wt or mutant RB-1 by Northern blot analysis (Figure 3.12).

Presence of the transfected RB-1 plasmids was confirmed by a PCR based strategy (Section 2.3.5). The primer sequences that were chosen lie either side of a small intron of RB-1, intron 14, such that the endogenous genomic copies of the RB-1 gene would give a 483 bp product while a cDNA copy, either pLRbRNL or pLRb(Δ768-909)RNL, would give an 81 bp product. Thus there is an internal positive control, resulting in the amplification of the 483 bp gene specific fragment, for each reaction. As shown in Figure 3.11, the majority of the clones tested contained the 81 bp band indicating the presence of an RB-1 plasmid. In each case the presence of the 483 bp band indicated that the PCR reaction conditions were appropriate.

Some of the clones which contained a copy of the transfected RB-1 plasmid, were tested by Northern blot analysis for expression of wild type or mutant RB-1 from the plasmids (Figure 3.12). The RB-1 mRNA from pLRbRNL is 2.8 kb in size (W.-H. Lee, pers. comm.). Total RNA (10 μg) was separated on a 1% denaturing agarose gel and transferred to Hybond N membrane. The membranes were probed with an α-^{32}P-dCTP labelled 3.3 kb StuI/HindIII fragment of pLRbRNL. As can be seen in Figure 3.12, each lane contains a band at ~4.7 kb which is specific for the endogenous RB-1 mRNA, while

Figure 3.11 PCR to detect the presence of RB-1 expression plasmids in pRSV-T-induced foci of HFF5 cells

Clones were isolated as $G418^R$ selected colonies or as foci following the transfection of HFF5 cells with either pRSV-T + pLRbRNL or pRSV-T + pLRb(Δ768-909)RNL. DNA was isolated from these isolated clones for analysis. The DNA was subjected to a PCR reaction for 35 cycles to generate a 483 bp fragment across intron 14 of the RB-1 gene, and a 81 bp fragment from an integrated cDNA copy of RB-1 present in pLRbRNL and pLRb(Δ768-909)RNL plasmids. The products were separated on a 2% agarose gel and stained with ethidium bromide to visualise the fragments. Controls: HFF5, untransfected NHDF cells containing the endogenous RB-1 gene only; pLRbRNL, PCR on purified plasmid DNA; pLRb(Δ768-909)RNL, PCR on purified plasmid DNA; H_2O, water negative control.

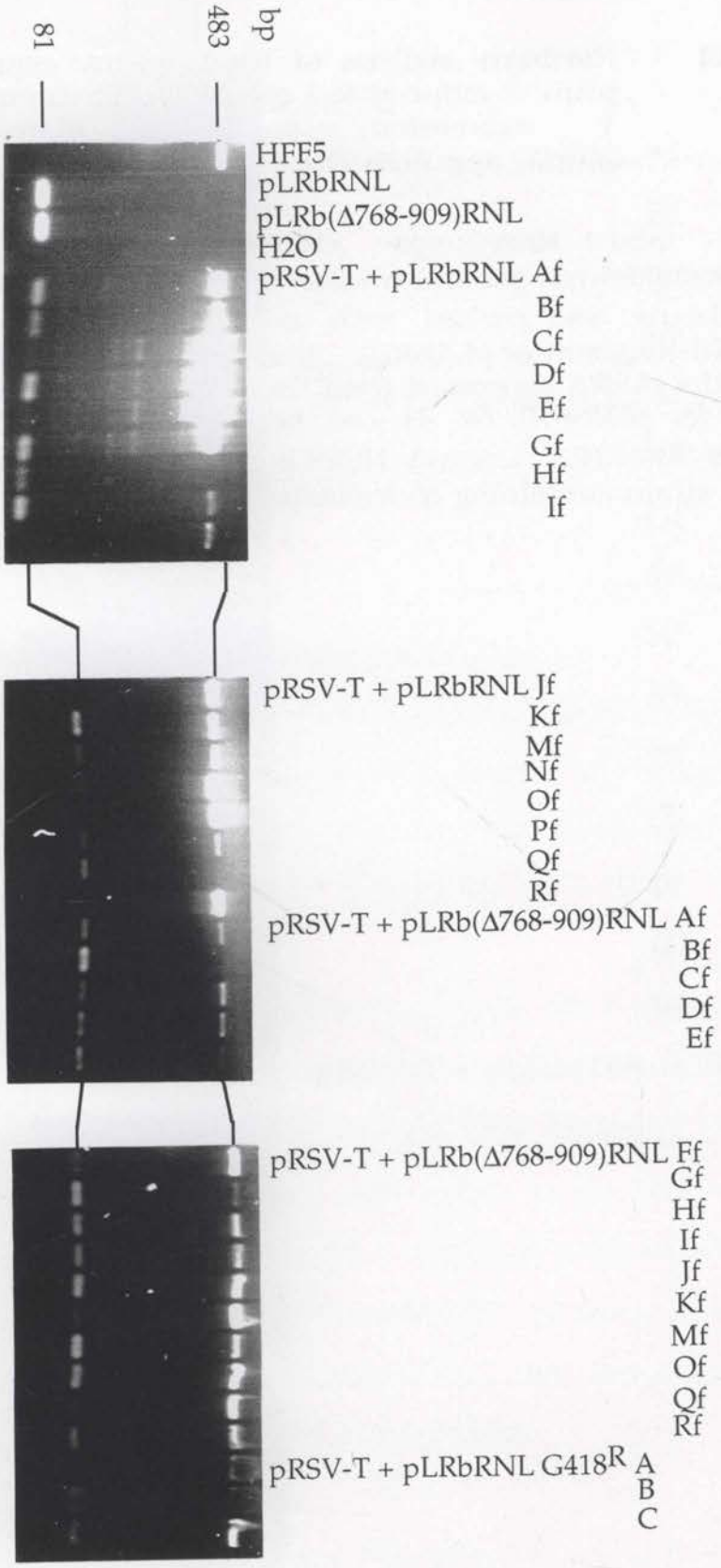
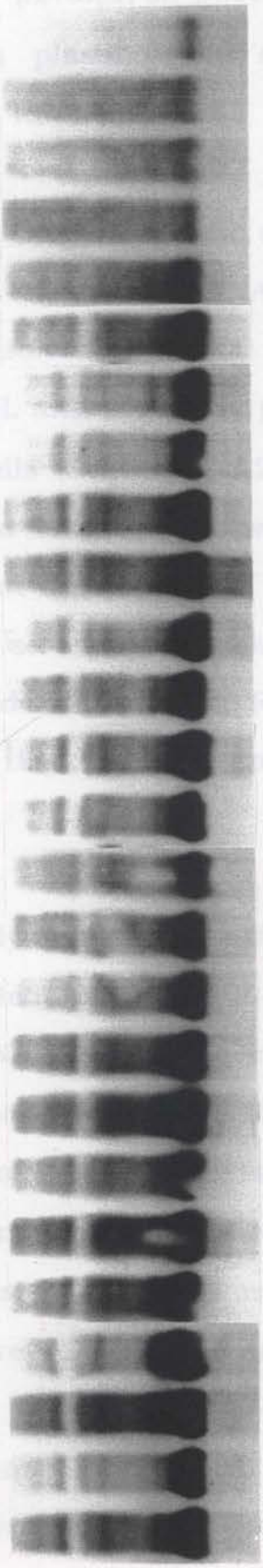

81
483
bp
HFF5
pLRbRNL
pLRb(Δ768-909)RNL
H2O
pRSV-T + pLRbRNL Af
Bf
Cf
Df
Ef
Gf
Hf
If
pRSV-T + pLRbRNL Jf
Kf
Mf
Nf
Of
Pf
Qf
Rf
pRSV-T + pLRb(Δ768-909)RNL Af
Bf
Cf
Df
Ef
pRSV-T + pLRb(Δ768-909)RNL Ff
Gf
Hf
If
Jf
Kf
Mf
Of
Qf
Rf
pRSV-T + pLRbRNL G418R A
B
C

Figure 3.12 Northern analysis of RB-1 mRNA expression in pRSV-T-induced foci of HFF5 cells containing RB-1 expression plasmids, pLRbRNL or pLRb(Δ768-909)RNL

10μg of total RNA was electrophoresed on a 1% agarose/formaldehyde gel and transferred to Hybond-N membrane. The membrane was probed with α-^{32}P-dCTP labelled 3.3 kb StuI/HindIII fragment of pLRbRNL. Endogenous RB-1 mRNA is 4.7 kb, while the mRNA expressed from the RB-1 expression plasmids is ~2.8 kb for pLRbRNL [W. -H. Lee, pers. comm.] and ~2.4 kb for pLRb(Δ768-909)RNL. Control: HFF5 is the untransfected parental NHDF cell strain containing endogenous wt RB-1 only.


kb
4.4
2.37
HFF5
expt. 15
pRSV-T + pLRbRNL Af
Bf
Df
Ff
expt. 17
pRSV-T + pLRbRNL Af
Bf
Df
Gf
Hf
Kf
Nf
Of
Pf
Qf
Rf
pRSV-T + pLRb(768-909)RNL Af
Cf
Df
Ef
Ff
Gf
Hf
Mf
Of
Qf

none contains a ~2.8 kb or ~2.4 kb mRNA encoded by either of the RB-1 plasmids. Thus the 15 pRSV-T + pLRbRNL clones, and 10 pRSV-T + pLRb(Δ768-909)RNL clones examined contain the RB-1 expression plasmid, but do not appear to be expressing the exogenous RB-1 mRNA.

To ensure that the lack of detection of exogenous RB-1 mRNA was not due to the 2.8 kb mRNA being present at very low levels, polyA$^+$ RNA was isolated from 100 μg of total RNA from 4 of each of pRSV-T + pLRbRNL and pRSV-T + pLRb(Δ768-909)RNL foci as well as HFF5 control cells (Section 2.3.2). The polyA$^+$ RNA was separated and transferred in the same way as for the total RNA Northern blots except that the amount of polyA$^+$ RNA loaded on the gel was not measured so as not to risk degrading the mRNA. The membrane was probed with the same RB-1 probe as used in Figure 3.12 and, as shown in Figure 3.13, only the ~4.7kb endogenous mRNA was detected.

This clearly demonstrates that although the RB-1 plasmids are being incorporated into the cell, as indicated by Figure 3.11, exogenous RB-1 mRNA is not being expressed. The results of these analyses are summarised in Table 3.8 . Also shown is the lifespan of some of the clones that were passaged. Although each of the foci has a lifespan greater than that of the pRcCMV controls, this is unrelated to expression of exogenous RB-1, i.e., this extension of lifespan probably represents the effect of TAg alone.

The mutant RB-1 was also not expressed in any of the isolated foci which were shown to contain the pLRb(Δ768-909)RNL plasmid.

Figure 3.13 PolyA$^+$ Northern analysis of RB-1 mRNA expression in pRSV-T-induced foci of HFF5 cells containing RB-1 expression plasmids, pLRbRNL or pLRb(Δ768-909)RNL

PolyA$^+$ RNA was isolated from 100μg of total RNA, from each clone, using the PolyATract® mRNA Isolation System III (Promega). The whole amount of polyA$^+$ RNA isolated was electrophoresed on a 1% agarose/formaldehyde gel and transferred to Hybond-N membrane. The membrane was probed with α-^{32}P-dCTP labelled 3.3 kb StuI/HindIII fragment of pLRbRNL. Endogenous RB-1 mRNA is 4.7 kb, while the mRNA expressed from the RB-1 expression plasmids is ~2.8 kb for pLRbRNL [W. -H. Lee pers. comm.] and ~2.4 kb for pLRb(Δ768-909)RNL. Control: HFF5 is the untransfected parental NHDF cell strain containing endogenous wt RB-1 only.

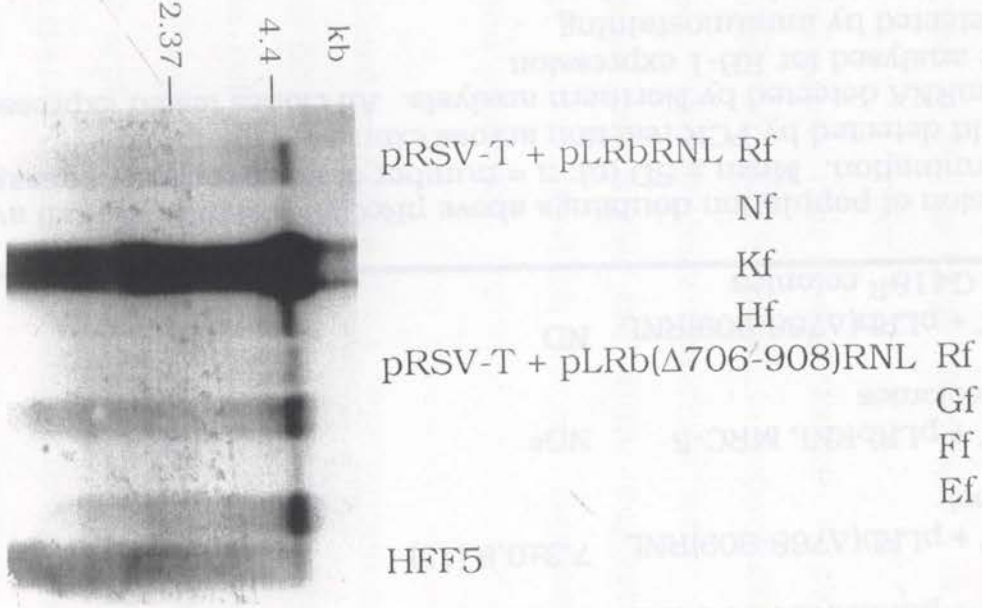

kb
4.4
2.37
pRSV-T + pLRbRNL Rf
Nf
Kf
Hf
pRSV-T + pLRb(Δ706-908)RNL Rf
Gf
Ff
Ef
HFF5

Table 3.8 Effect of co-transfecting pRSV-T with pLRbRNL or pLRb(Δ768-909)RNL in HFF5 transformed foci and MRC-5 G418 selected colonies

Clones	Extension of Lifespan[a]	Number of clones containing pLRbRNL or pLRb(Δ768-909)RNL[b]	Number of clones expressing RB-1 from pLRbRNL or pLRb(Δ768-909)RNL[c]	Number of clones positive for TAg[d]
pRSV-T + pLRbRNL HFF5 foci	8.3±1.4 (12)	13/22	0/15	22/22
pRSV-T + pLRb(Δ768-909)RNL HFF5 foci	7.3±0.8 (13)	15/15	0/9	15/15
pRSV-T + pLRbRNL MRC-5 $G418^R$ colonies	ND[e]	3/3	0/3	10/10
pRSV-T + pLRb(Δ768-909)RNL MRC-5 $G418^R$ colonies	ND	1/1	ND	15/15

[a]Extension of population doublings above pRcCMV controls. Not all available clones were passaged for lifespan determination. Mean ± SD (n); n = number of independently passaged foci

[b]Plasmid detected by PCR reaction across exon 14/15 junction

[c]RB-1 mRNA detected by Northern analysis. All clones tested expressed endogenous RB-1; not all available clones were analysed for RB-1 expression

[d]TAg detected by immunostaining

[e]ND: not determined

The lack of expression suggests this mutant retains wild type $p110^{RB}$ function with respect to being incompatible with proliferation as well as focus formation.

Figure 3.5 shows that co-transfection of pRSV-T and pLRbRNL, or pLRb(Δ768-909)RNL, resulted in suppression of TAg-induced focus formation. The analyses described here, of the cultures derived from those foci which did form, suggests that there has been selection for cells which do not express the transfected RB-1 expression plasmids.

As indicated in Figure 2.1.1, the mutant and wt RB-1 expression plasmids contain the gene and promoter which confer resistance to G418 in eukaryotic cells. It was thus possible to isolate $G418^R$ colonies from the co-transfections of pRSV-T + pLRbRNL and pRSV-T + pLRb(Δ768-909)RNL. The results of the analysis of the MRC-5 $G418^R$ clones were similar to those of the HFF5 transformed foci and these are also summarised in Table 3.8.

G418 selection of MRC-5 cells co-transfected with pRSV-T + pLRbRNL, or pRSV-T + pLRb(768-909)RNL, only rarely produced colonies. Except for one pRSV-T + pLRb(Δ768-909)RNL and three pRSV-T + pLRbRNL clones, these $G418^R$ colonies senesced before RNA or DNA could be isolated. Each of these four clones were shown by PCR to contain RB-1 plasmid DNA, as expected (three clones are shown in Figure 3.11). pRSV-T + pLRbRNL $G418^R$ clone A was shown to contain the 81 bp band with a longer exposure (not shown). Enough RNA was obtained from only two of the pRSV-T + pLRbRNL $G418^R$ clones to check for expression of the exogenous

RB-1 by Northern blot analysis (Figure 3.14, Panel A); one of these samples unfortunately was partially degraded and no more RNA could be obtained. Panel A (Figure 3.14) does demonstrate that at least one, and probably both, of the $G418^R$ selected clones tested did not express the exogenous RB-1. This is in agreement with the RB-1 mRNA expression studies with the isolated foci. As these clones were $G418^R$ selected, it was expected that they probably would still be expressing the neo^R gene. The membrane from Panel A (Figure 3.14) was stripped and re-probed with a α-^{32}P-dCTP labelled probe for neo (Panel B) which shows that one, and probably both, of the $G418^R$ clones expressed the neo^R gene. These data support of the previous finding that expression of exogenous RB-1 is incompatible with proliferation in cells transformed with TAg.

The observation that none of the foci expressed the integrated RB-1 plasmid, and that it was very difficult to isolate $G418^R$ clones from transfections with the RB-1 expression plasmids, suggests that expression of the exogenous RB-1 is detrimental to lifespan extension. These data support the results in Table 3.7 indicating that binding to, and probable inactivation of, $p110^{RB}$ is necessary for lifespan extension.

Disruption of the $p110^{RB}$-binding region of TAg ablated both the focus forming and lifespan extension ability of pRSV-T. Also, co-transfection of pLRbRNL with pRSV-T reduced the number of foci induced by pRSV-T and the exogenous RB-1 was not expressed in any clones isolated from these transfections. These data indicate that inactivation of $p110^{RB}$ by TAg is necessary for both the focus formation and lifespan extension ability of pRSV-T. This is in

Figure 3.14 Northern analysis of A. RB-1, and B. neo^R mRNA expression in $G418^R$ selected clones of HFF5 cells transfected with pRSV-T and the neo^R and RB-1 expression plasmid, pLRbRNL

10µg of total RNA was electrophoresed on a 1% agarose/formaldehyde gel and transferred to Hybond-N membrane. The membrane was probed with, **A.** α-^{32}P-dCTP labelled 3.3 kb StuI/HindIII fragment of pLRbRNL and **B.** α-^{32}P-dCTP labelled 1.0 kb SmaI fragment of pMOL-NEO. Endogenous RB-1 mRNA is 4.7 kb, while the mRNA expressed from the RB-1 expression plasmid is ~2.8 kb [W.-H. Lee pers. comm.]. Control: HFF5 is the untransfected parental NHDF cell strain containing endogenous wt RB-1 only.
Arrows on the left of the figure indicate the position of the endogenous (4.7 kb) RB-1 and neo^R (2.0 kb) mRNA. The position of the exogenous (2.8 kb) RB-1 mRNA should appear approximately 12mm below the arrow indicating the position of the genomic RB-1 mRNA.

pRSV-T + pLRbRNL G418R A
pRSV-T + pLRbRNL G418R B
pRSV-T + pLRbRNL Af
A
Rb
B
Neo

agreement with the literature indicating that $p110^{RB}$ acts to control cell cycling (Sections 1.4.2 & 1.4.3).

3.3.3 p53 binding is not necessary for TAg- induced extension of lifespan

I 402DE plasmids induce extension of lifespan in NHDF

Previous studies had indicated that non-p53-binding mutant TAg expressed from pbssv402DE did not induce extension of lifespan in NHDF cells [Lin & Simmons, 1991; Maclean *et al.*, 1994]. Lack of TAg expression in some cell populations expanded from a number of pbssv402DE-induced foci also indicated that there was no advantage for the cells to maintain expression of the non-p53-binding mutant TAg protein [Maclean *et al.*, 1994; N. Whitaker unpublished data]. Loss of TAg expression has never been reported for primary human cells transformed by wt TAg. Taken together, these data were interpreted as indicating that the functional $p110^{RB}$ binding region present in the 402DE mutant of TAg was necessary for focus formation, while the p53 binding region was necessary for extension of lifespan [Maclean *et al.*, 1994]. This study was extended by comparing the SV40 early region-only plasmid pRSV-T(402DE) with the entire SV40 genome plasmid pbssv402DE.

Induced foci were isolated from transfections with either pbssv402DE + pRcCMV or pRSV-T(402DE) + pRcCMV using cloning cylinders, and analysed for TAg specific immunostaining. Where immunostaining occurred it was typical of TAg, localised to the nucleus with nucleolar sparing. The TAg staining pattern of the

clonal populations varied from the population being completely positive (+/+), to completely negative (-/-), and in between these extremes were clones in which some or most of the population was negative for TAg staining (+/-). Examples of these staining patterns are shown in Figure 3.15 and the results are summarised in Table 3.9. In addition, there appears to be some nuclear fragmentation in the 402 +/+ panel of Figure 3.15 which may indicate apoptosis.

Consistent with our previous study [Maclean *et al.*, 1994], some of the foci induced by both bssv402DE (10/16 foci tested) and pRSV-T(402DE) (6/21 foci tested) contain cells that are negative for TAg by immunostaining (Table 3.9). In contrast to previous studies [Lin & Simmons, 1991; Maclean *et al.*, 1994], however, populations of cells from foci induced by both bssv402DE and pRSV-T(402DE) demonstrate an extension of lifespan.

The fact that the non-p53-binding mutant TAg plasmids induced foci indicates that these plasmids were initially expressed (and probably integrated) in the HFF5 cells and that the TAg negative cells had subsequently ceased expression of the mutant TAg protein. Loss of expression occurred in a significant number of foci, indicating that there is selection for cells that had shut off expression of mutant TAg or against the cells still expressing the mutant TAg. Lack of selection for continued expression of 402DE may also result in accumulation of cells not expressing the mutant TAg due to random genetic change. As seen in Table 3.9, this is not unique to the pbssv402DE plasmid and is therefore not due to the presence of the whole SV40 genome. A number of these clones were passaged for lifespan analysis and it is clear that cell

Figure 3.15 Indirect immunofluorescent staining for SV40 large and T antigens in foci induced in HFF5 cells by the non-p53-binding mutant TAg plasmids

Following transfection with a plasmid expressing the 402DE mutant TAg, cells were isolated from transformed foci. Cells from these clones were plated on a glass chamber slide and 24 hours later the cells were fixed with ice cold methanol. The cells were incubated with a monoclonal antibody, PAb108, which recognises both large and small T antigens. The cells were then incubated with an FITC-conjugated goat-anti-mouse secondary antibody. The antibody was visualised under a microscope with UV-excitation. The examples show clones with a +/- staining pattern, where only some of the cells are TAg positive, or a +/+ staining pattern where all of the cells are TAg positive and a -/- staining pattern where none of the cells is TAg positive. Controls: GM847 positive control, is an SV40-immortalised cell line; and HeLaCOT negative control, is an HPV-18 containing cell line.

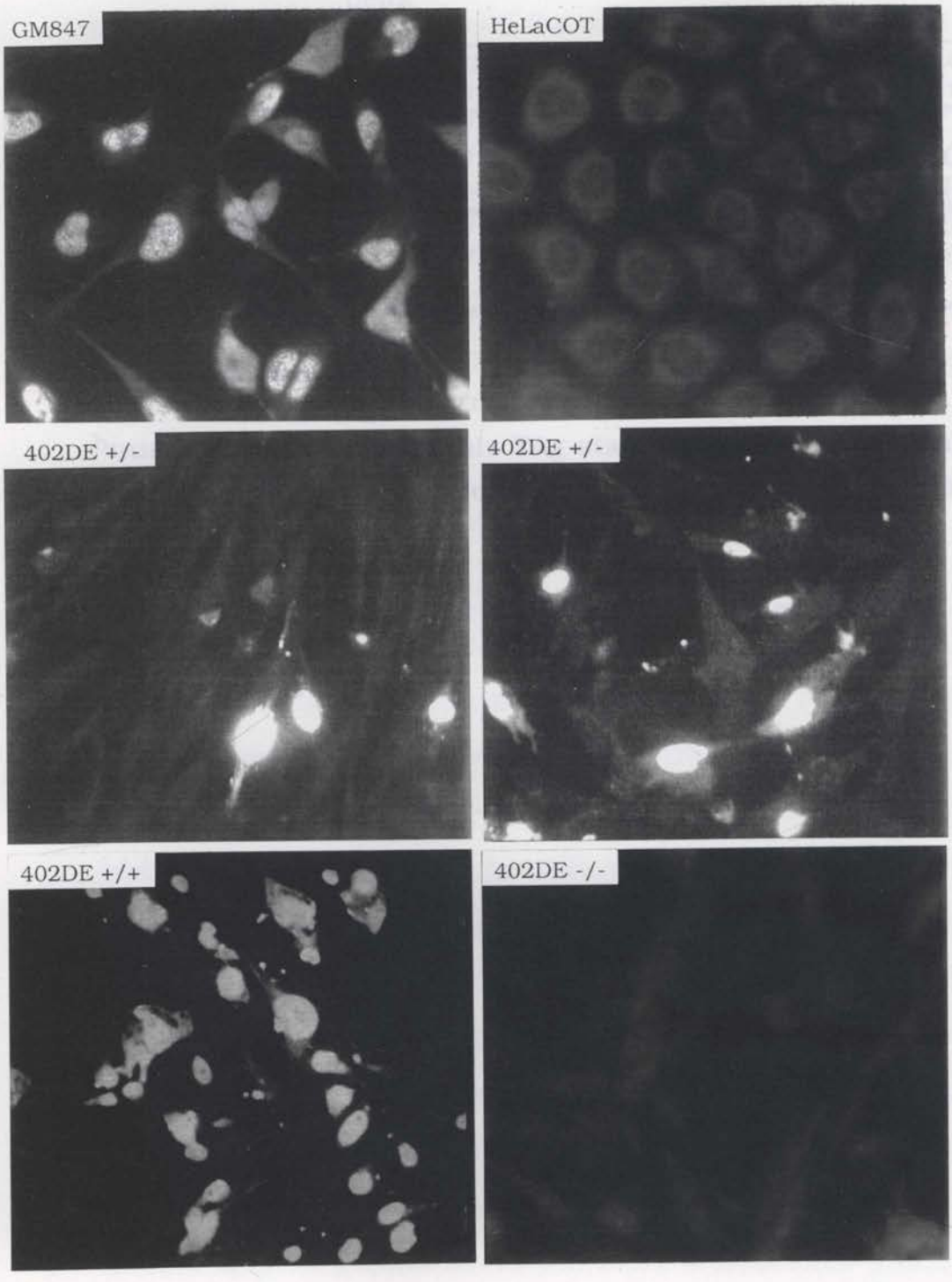

GM847
HeLaCOT
402DE +/-
402DE +/-
402DE +/+
402DE -/-

Table 3.9 Summary of TAg immunostaining and lifespan analysis of pRSV-T(402DE) induced foci in HFF5 cells

Plasmid	staining pattern[a]	number of clones[b]	extension of lifespan[c]
bssv402DE	+/+	6 (38%)	ND[d]
	+/-	9 (56%)	13.8±0.8 (2)[e]
	-/-	1 (7%)	12.5 (1)
pRSV-T(402DE)	+/+	15 (71%)	11.5±2.1 (5)
	+/-	3 (14%)	11.3 (1)
	-/-	3 (14%)	ND

[a]as shown in Figure 3.15: +/+, all cells positive; +/-, only some of cells are positive; -/-, all cells negative

[b]number of clones with this TAg staining pattern (percentage)

[c]number of population doublings above pRcCMV controls (20.7 PDL) after transfection

[d]ND: Not Determined

[e]mean±SD (n), n = number of individual clones analysed

populations expanded from pbssv402DE- and pRSV-T(402DE)-induced foci had an extended lifespan. In a number of these populations, the expression of the mutant TAg had been lost at some stage and yet the population still had an extended lifespan. This indicates that the non-p53-binding mutant TAg has the ability to induce transformed proliferating foci in NHDF cells and these populations have an extended *in vitro* lifespan regardless of the continued expression of the mutant TAg. Possible explanations of these observations are discussed in Section 3.5.

3.4 Escape from crisis

3.4.1 TAg sufficiently inactivates endogenous p110^{RB} in SV40-immortalised cells

After the extension of lifespan induced by SV40 TAg, and before the establishment of an immortal cell line, there is a period where there is no net proliferation. During this period of culture crisis, proliferation is balanced by cell death. Less than 10^{-7} of the remaining live cells may eventually give rise to an immortal population [Huschtscha & Holliday, 1983; Shay & Wright, 1989]. It is clear that further changes need to occur before the cells can escape crisis and become immortal. The low frequency of immortalisation suggests that genetic changes are required. In this part of the study, post-crisis SV40-immortalised cells were analysed to determine if a mutation of the RB-1 and/or p53 genes could be involved in this immortalisation process. An additional genetic change to either RB-1 or p53 might need to occur if binding to TAg was insufficient to eliminate all of the necessary functions of p110^{RB} or p53.

I RB-1 is normal in SV40-cells

Eleven independently derived SV40 immortalised cell lines were analysed for mutations of RB-1 by immunoprecipitation of $p110^{RB}$. Immunoprecipitation analysis of eight of these lines is shown in Figure 3.16, another two (WI-38 VA13/2RA and GM847) are shown in Figure 4.3 and MeT-5A is not shown (summarised in Table 3.10). Each of these SV40-immortalised cells contained bands ranging from 110-114 kD, specifically recognised by the $p110^{RB}$ antibody, NCL-RB. This banding pattern is typical of $p110^{RB}$ and was also present in the $p110^{RB}$ positive control line, HeLaCOT, while being absent in the negative control line, Saos-2. For comparison, the cell line, NCL-H209 contained only the underphosphorylated 110 kD band for $p110^{RB}$. NCI-H209 is known to contain a point mutation resulting in the presence of only the underphosphorylated forms of $p110^{RB}$ [Kaye *et al.*, 1990].

These immunoprecipitations were done under low stringency conditions which maintained the complex between TAg and $p110^{RB}$. Thus TAg was co-immunoprecipitated with $p110^{RB}$ in each of the SV40-immortalised cells shown in Figure 3.16. This co-immunoprecipitated TAg band co-migrated with the TAg immunoprecipitated by PAb108 in the SV40-immortalised cell line BEAS-2B/R1, and was not detected by PAb108 immunoprecipitation of HeLaCOT lysate. The $p110^{RB}$ protein in all of these cells is normal on the basis of size, phosphorylation and TAg binding. Some of the SV40-immortalised cell lines (BEAS-2B/R1, BEAS-2B, BES-1A1 and WI-38 VA13/2RA) contained a 100 kD band which was also recognised by PAb108 (Figures 3.7, 3.16 and data not shown). This 100 kD band in BEAS-2B/R1 cells was recognised by the anti TAg

Table 3.10 Status of $p110^{RB}$, $p16^{INK4}$, p53 and MDM2 in SV40-immortalised cell lines

Cell Line	Cell Type	$p110^{RB}$ Status (immuno-precipitation[a])	$p16^{INK4}$ Status (western/ northern[b])	p53 Status	MDM2 Status (Southern/ northern[c])
GM847	Fibroblast	WT	present	WT[d]	normal
WI38 VA13/2RA	"	WT	present	WT[d]	normal
BET-1A	Bronchial epithelial	WT	present	WT[e]	normal
BET-2A	"	WT		ND[g]	
HB56B/5T	"	WT		WT[f]	
BEAS-2B	"	WT		WT[f]	
BEAS-2B/S6	"	WT		WT[f]	
BEAS-2B/R1	"	WT		WT[f]	
BES-1A1	"	WT		WT[f]	
BES-1A1.6	"	WT		ND	
MeT-5A	Mesothelial	WT		WT[f]	

[a]Indicated by the size of the protein, presence of phosphorylation bands and binding to TAg
[b]Indicated by the presence of a normal sized protein recognised by a $p16^{INK4}$ specific antibody
[c]Indicated by lack of amplification (Figure 4.8) and overexpression (Figure 4.5)
[d]Indicated by binding to p53 conformation specific antibodies (Figure 4.7)
[e]Reddel *et al.*, 1995
[f]Lehman *et al.*, 1993
[g]ND: not determined

Figure 3.16 Immunoprecipitation analysis of $p110^{RB}$ in SV40-immortalised cell lines

^{35}S-AA labelled cells were lysed with either a low stringency lysis buffer for anti-$p110^{RB}$ immunoprecipitation, with monoclonal antibody NCL-RB, or RIPA lysis buffer for anti-TAg immunoprecipitation, with monoclonal antibody PAb108. The immunoprecipitate was separated on SDS-PAGE and the protein bands were visualised with autoradiography. Control cell lines: NCI-H209 contains a point mutation resulting in $p110^{RB}$ which is hypophosphorylated; HeLaCOT, HPV-18 immortalised cell line which is TAg negative and contains wt $p110^{RB}$; Saos-2 contains only a 95 kD $p110^{RB}$ protein.

69.8 K
105 K
MW
NCI-H209
HeLaCOT
Saos-2
BET-2A
BET-1A
BEAS-2B
BES-1A1
BES-1A1.6
HB56B/5T
BEAS-2B/R1
BEAS-2B/S6
BEAS-2B/R1
HeLaCOT
NCL-RB
PAb108
TAg
p110RB

antibody and is therefore a "super T Ag" as has been described previously in other cell lines [Chen & Pollack, 1985, Kao *et al.*, 1993a].

All (11/11) of the SV40-immortalised cell lines express normal $p110^{RB}$, indicating that there is no selection for mutation of RB-1 during escape from crisis. The presence of normal $p110^{RB}$ in these cell lines also suggests that inactivation of $p110^{RB}$ by binding to TAg is sufficient to eliminate the relevant functions of $p110^{RB}$.

II $p16^{INK4}$ is normal in SV40-immortalised cells

It was important also to rule out mutation of $p16^{INK4}$ as an alternative mechanism for escape from crisis since there is evidence that $p16^{INK4}$ mutation is an alternative to RB-1 mutation (Section 4.1). As shown in Figure 4.4 (Panel A) $p16^{INK4}$ was analysed by immunoprecipitation and western analysis in three of the SV40 cell lines listed in Table 3.10 (WI-38 VA13/2RA, GM847 and BET-1A) and each of these cell lines was found to contain $p16^{INK4}$. This was confirmed by northern analysis (Figure 4.5 Panel A). Although only a small number of cell lines were analysed in this way, it supports the notion that SV40 TAg is sufficient to disrupt the growth inhibitory functions of $p110^{RB}$ and there is no selection for mutation of RB-1, or $p16^{INK4}$, in escape from crisis. Therefore, escape from crisis must involve other cellular changes.

3.4.2 SV40-TAg inactivates endogenous p53 sufficiently for immortalisation

I p53 is normal in SV40-immortalised cells

p53 status has been determined by sequence analysis in a large number of cells, including seven of the SV40-immortalised cell lines listed in Table 3.10. Immunoprecipitation analysis of p53 was performed on a further two SV40-immortalised cell lines (Figure 4.7). These analyses show clearly that p53 is normal in all SV40-immortalised cell lines examined, suggesting that TAg inactivates p53 function sufficiently to allow immortalisation.

II MDM2 is not overexpressed in SV40-immortalised cells

It has recently been shown that MDM2 overexpression can overcome the p53-induced block in the G1/S transition in the cell cycle. It has therefore been suggested that overexpression of MDM2, usually by amplification, is an alternative to p53 mutation. I therefore examined the status of the MDM2 gene and/or mRNA in three of the SV40-immortalised cell lines. The Southern analysis (Figure 4.8; and summarised in Table 3.10) shows that there is no amplification of the MDM2 gene in two of the SV40-immortalised cell lines, WI38 VA13/2RA and BET-1A. The Northern analysis (Figure 4.5, Panel B) supports the Southern data and clearly shows that there is no overexpression of MDM2 in the three SV40-immortalised cell lines, GM847, WI38 VA13/2RA and BET-1A. Although this is only a small sample it is in support of the notion that p53 function, which might otherwise conflict with SV40 transformation and immortalisation, is fully inactivated by binding to TAg.

3.5 Discussion

Inactivation of p110^{RB} induces focus formation

Disrupting the p110^{RB} binding region of TAg in pRSV-T to produce pRSV-T(ΔRBBS) and pRSV-T(K1) clearly ablates the focus forming ability of TAg (Table 3.2). Apart from disruption of the p110^{RB}-binding region, the plasmids are otherwise comparable to pRSV-T. The resultant proteins are recognised by the TAg specific monoclonal antibody, PAb108, and are localised to the nucleus (Figure 3.1 Panel A). The expressed proteins are as stable as the wild type protein (Figure 3.1 Panel B). This clearly implicates the necessity for a functioning p110^{RB}-binding region in focus formation.

Transfection of the non-p110^{RB}-binding mutant TAg plasmids did induce patches of cells with a transformed morphology, but these cells did not pile up (Figure 3.3), in contrast to the foci induced by wt TAg in which the cells piled up to form clearly visible multilayered (Figure 3.2). The change in morphology (Figure 3.3) indicates the presence of SV40 early region genes and is probably due in part to dissolving of the actin cables by tAg [Graessmann *et al.*, 1980]. At least some of the non-p110^{RB}-binding TAg induced patches consisted of cells which contained the transfected TAg plasmid (Figure 3.4) which is consistent with these plasmids altering the cells' morphology. These cells did not pile up which suggests that the cells were contact inhibited, indicating a role for the p110^{RB}-binding region of TAg in release from this constraint.

This same region of TAg, however, has also been shown to be responsible for binding other members of the RB-1 family, p107 and

p130. Thus two approaches were used to determine if binding to $p110^{RB}$ was necessary for focus formation. Firstly, pRSV-T was co-transfected with the $p110^{RB}$ expression plasmid, pLRbRNL, into NHDF to see if pLRbRNL had an effect on pRSV-T-induced focus formation. Secondly, the non-$p110^{RB}$-binding plasmids were introduced into cells containing just one wt allele of RB-1, GM01879A and GM01880B. The presence of just one wt RB-1 allele would increase the chance of an event inactivating $p110^{RB}$ function. If inactivation of $p110^{RB}$ is necessary for focus formation then any foci induced by the non-$p110^{RB}$-binding mutant TAg plasmids might contain a detectable inactivation of the single wt RB-1 allele.

Co-transfection of pLRbRNL with pRSV-T significantly decreased focus formation to 32±12% (n=9) of that obtained with pRSV-T + pRcCMV (Figure 3.5). The decrease in focus formation suggests that pLRbRNL provides a pool of active $p110^{RB}$, which is not bound to TAg, and is consistent with inactivation of $p110^{RB}$ by binding to TAg being necessary for focus formation. Co-transfection of pLRb(Δ768-909)RNL with pRSV-T also decreased the focus forming ability of pRSV-T to a similar level, 30±10% (n=6). This can be interpreted to mean that the result obtained with pLRbRNL is not significant or the pLRb(Δ768-909)RNL plasmid retains some wild type functions of $p110^{RB}$. The comparison of co-transfection of pRSV-T + pRcCMV with pRSV-T + pLRbRNL, however, suggests that the inhibition of focus formation is specific.

Deletion of AAs 768-909 of $p110^{RB}$ has previously been shown to ablate binding to TAg and E1A [Hu *et al.*, 1990], which is known to correlate with E2F-binding and cell cycle regulation functions of

$p110^{RB}$ (Section 1.4.2). There were no growth inhibition data on this specific mutation in RBΔ768-909 [Hu *et al.*, 1990], however, it appears that in this focus forming assay, pLRb(Δ768-909)RNL retains some wild type activity. An explanation for the growth inhibitory activity of mutant $p110^{RB}$ protein encoded by pLRb(Δ768-909)RNL could be regulation of other transcription factors. Although it is not known which regions are critical for these activities of $p110^{RB}$, candidate factors include Sp1 associated factors including $TAF_{II}250$ [Shao *et al.*, 1995] and SP1-I [Chen *et al.*, 1994], ATF-2 [Kim *et al.*, 1992], MyoD and myogenin [Gu *et al.*, 1993], Elf-1 [Wang *et al.*, 1993] and TBF [Rustgi *et al.*, 1991; Hagemeier *et al.*, 1993].

The above discussion indicates that inactivation of $p110^{RB}$ by TAg is necessary for focus formation and suggests that TAg binding to $p110^{RB}$ disrupts contact inhibition, allowing the cells to pile up to form the multilayered foci. This is the first time, to my knowledge, that inactivation of $p110^{RB}$ by binding to TAg has been implicated in contact inhibition. A mechanism which may include $p110^{RB}$ in contact inhibition might be inhibition of $p110^{RB}$ activity by induction of kinase inhibitors. An attractive candidate for such a kinase inhibitor is $p27^{Kip1}$ which has been shown to be induced by quiescence [Firpo *et al.*, 1994; Kato *et al.*, 1994; Slingerland *et al.*, 1994], TGF-β treatment as well as cell-cell contact [Polyak *et al.*, 1994; Slingerland *et al.*, 1994].

pRSV-T(K_1)-induced focus formation in RB +/- cells

Transfection of two cell strains containing just one allele of RB-1, GM01879A and GM01880B, with the non-$p110^{RB}$-binding mutant

plasmid pRSV-T(K1) resulted in 1 focus in each cell strain. Although pRSV-T(K1) did induce two foci in these cells, the two most obvious candidate genes for mutation, RB-1 and $p16^{INK4}$, were normal (Figures 3.7 and 3.8). This suggests that a change has occurred in another gene to allow focus formation. Genes that might be involved could include the D-type cyclins, cdk4, cdk6, $p15^{INK4B}$ or a back mutation in the plasmid DNA. Mutation of these genes has not yet been examined in these cells.

Induction of two foci out of 67 separate transfections of RB +/- cells is not significantly different from 0 foci out of 79 separate transfections of NHDF cells (P = 0.134). This is clearly a different situation to that in LFS fibroblasts which contain just one wt copy of p53, where focus formation induced by the non-p53-binding TAg plasmids (10% of wt TAg) was significantly greater ($P \leq 0.05$) than focus formation in NHDF cells (0.4% of wt TAg) [Maclean *et al.*, 1994]. This difference suggests that there is a dosage effect with the loss of one wt copy of p53 which is not apparent with the loss of one RB-1 gene. Alternatively, there is selection for retaining wt RB-1 in cycling cells and it is, therefore, somehow "easier" to lose a copy of wt p53 that to lose wt RB-1.

A total of 6.7×10^7 RB +/- cells and 7.9×10^7 NHDF cells were transfected with either of the two non-$p110^{RB}$-binding mutant TAg plasmids in 67 and 79 separate transfections, respectively. This is a large number of cells, however, not every cell is successfully transfected, as indicated by the efficiency of expression in Table 3.1. Also, the efficiency of stable integration is thought to be even less. Assuming the number of foci induced by pRSV-T in NHDF is equal to

the number of cells which stably integrate the plasmid (average ~45 foci/10^6 cells transfected) and assuming that the mutant TAg plasmids are stably integrated at approximately the same frequency, then ~3000 RB +/- cells and ~3200 NHDF cells stably integrated either of the non-p110^{RB}-binding mutant TAg plasmids. Although two foci were obtained, it is apparent that the transfection efficiency is too low to reliably see the putative mutational event required to cooperate with non-p110^{RB}-binding mutant TAg plasmids to induce foci in fibroblasts. It would be very interesting to repeat this experiment using retroviral infection, which results in a higher frequency of stable integration.

Inactivation of p53 is not necessary for focus formation

Table 3.1 shows that transiently transfected HT1080 cells express pbssv402DE (3.0% of cells positive by immunostaining) less efficiently than pRSV-T(402DE) (8.1% positive). The reason for this difference is unclear but could be related to the presence of the late region of SV40 and/or the SV40 promoter region, as the plasmids are otherwise comparable. In support of this, TAg has been shown to inhibit its own expression (Section 1.3.3) from the SV40 promoter. Alternatively, the presence of the late region, or expressed proteins, might be detrimental to the short-term survival of the transfected cells.

The number of foci obtained with the non-p53-binding mutant TAg plasmids, bssv402DE and pRSV-T(402DE), was ~2% and ~38% of that for pRSV-T, respectively. Induction of focus formation by each of the non-p53-binding TAg plasmids suggests that binding to p53 is unnecessary for focus formation although it increases the efficiency.

This is consistent with the results we reported previously [Maclean *et al.*, 1994]. The reason for the difference in focus forming efficiency may be accounted for, at least in part, by the difference in efficiency of expression from transient transfection discussed above. Other differences in the efficiency of focus formation probably stem from the presence of the late region and the use of different promoters.

pRSV-T and pBR/SV (SV40 genome cloned into pBR322) have been reported to give similar focus formation efficiency [Ke *et al.*, 1989], indicating that the late region *per se* does not decrease focus formation. It is possible that the level of expression from the two non-p53-binding mutant TAg plasmids was different, favouring expression from pRSV-T(402DE). The SV40 promoter, present in pbssv402DE, is known to be down-regulated by TAg [Rio & Tijan, 1983]. Although this potential difference in expression level of TAg may have no measurable effect on focus formation with wt TAg, it may affect focus formation induced by TAg containing a 402DE point mutation. A reason for this may be that 402DE, although displaying reduced binding to p53 (compared to pRSV-T) as shown in Figure 3.1, still appears to retain some binding (indicated by the presence of a TAg band in the immunoprecipitation of pRSV-T(402DE) with the α–p53 antibody). Thus, if there is a higher level of expression from pRSV-T(402DE), compared with pbssv402DE, it could be expected that the amount of p53 bound by this mutant TAg would increase, resulting in an increased efficiency of focus formation. Levels of expression from the two 402DE plasmids, however, were not compared.

The binding to p53 by TAg expressed from pRSV-T(402DE) is unexpected as the TAg expressed by pbssv402DE demonstrated no detectible binding to p53 [Lin & Simmonds, 1991]. The reason for this is unclear but my be due to a higher level of expression from the RSV LTR, or a back mutation to wild type TAg. Alternatively this may be a cell specific effect. It would be of considerable interest, therefore, to both, sequence the TAg region of pRSV-T(402DE), and to analyse binding to p53 in other cells.

We have previously examined focus formation with another non-p53-binding TAg plasmid, dl1137, containing a deletion more extensive than the p53-binding region of TAg. In MRC-5 cells, this plasmid failed to induce focus formation, although it did induce 1-2 foci in transfections into LFS fibroblasts [Maclean *et al.*, 1994], supporting the notion that p53-binding is not essential for focus formation. To verify this hypothesis it would be valuable to compare focus formation of other non-p53 binding TAg plasmids. Other, less conservative, substitutions of codon 402 have been made [Lin & Simmons, 1991] and may further disrupt binding of TAg to p53.

Complementation of mutant TAg for focus formation

Co-transfection of the non-p110^{RB}-binding mutant TAg plasmids, pRSV-T(ΔRBBS) or pRSV-T(K_1), with pRSV-T(402DE) had no effect on the focus forming ability of pRSV-T(402DE) alone which was ~30% of pRSV-T. This lack of complementation could be due to failure to co-transfect both of the plasmids. This is unlikely however, as the results of co-transfection of the non-p110^{RB}-binding mutant TAg plasmids with pbssv402DE (Table 3.5) and co-transfection of pLRbRNL with pRSV-T (Table 3.8) clearly indicate

that co-incorporation of two plasmids is frequent. Three possible explanations for the lack of complementation of pRSV-T(402DE), with the non-p110^{RB}-binding mutant TAg plasmids, in focus formation could be: i) other functions of TAg might be disrupted by the mutations in each of the mutant TAg plasmids, ii) balance among the transforming domains of TAg, and iii) balance of the amount of TAg and tAg is important.

It is unlikely that the same functions of TAg would be disrupted by each of the three mutations of TAg used in this study, although this cannot be ruled out (Section 1.3.3). It is possible, however, that some of the functions of TAg require the regions involved to be actually present on the same molecule, for example, binding to two different cellular proteins. Thus, having all of the functions of TAg present, but on separate molecules, may decrease the focus forming ability of TAg.

It is possible that the balance between the various transforming domains of TAg is inappropriate in the co-transfection situation. There is evidence for transforming activity in the N-terminus of TAg (Section 1.3.3). The balance between this function and the p110^{RB}- and p53-binding activities, for example, would be perturbed by co-transfection of the mutant TAgs.

Results obtained by D. Zahra [unpublished] in this laboratory indicate that disrupting tAg while leaving the TAg coding sequence intact severely decreases focus formation, regardless of the level of TAg expression. The lack of focus formation by TAg alone indicates a role for tAg, however, complementation was not seen between TAg-

only and tAg-only expression plasmids. This lack of complementation suggests that the relative amounts of Tag and tAg are important and tightly regulated by being encoded on the same DNA, which is alternatively spliced to give mRNA encoding each of the two proteins. The results of co-transfection of non-p110^{RB}-binding mutant TAg plasmids with pRSV-T(402DE) may also indicate that the balance of tAg and TAg is important. Having one plasmid each for two different mutants of TAg and, thus, two sources of tAg may disrupt the balance of tAg and TAg and may reduce focus formation.

Co-transfection of a non-p110^{RB}-binding mutant TAg plasmid, pRSV-T(ΔRBBS) or pRSV-T(K_1), with bssv402DE increased focus formation from ~2% to ~10% of pRSV-T. The apparent complementation between pbssv402DE and the non-p110^{RB}-binding mutant TAg plasmids suggests that an inhibitory effect of the SV40 genome, outside the early region, is partially overcome by the presence of the non-p110^{RB}-binding mutant TAg plasmids. This may be due to the presence of p53 binding function in the non-p110^{RB}-binding mutant TAg or may be just due to an increase in the amount of TAg in the cells.

Effect of transfection method on focus formation

The focus formation efficiency of pRSV-T was significantly greater in transfections using Lipofectamine compared to $SrPO_4$/DNA co-precipitation. As discussed in Section 3.2.1, this difference appeared to be due to the toxicity of $SrPO_4$/DNA co-precipitation or induction of DNA damage. It was shown recently that $CaPO_4$/DNA co-transfection induced p53 expression and a p53-dependent

transient growth arrest [Renzing and Lane, 1995]. Since the $CaPO_4$/ and $SrPO_4$/DNA co-precipitation methods are very similar, it might be expected that the $SrPO_4$ procedure will also induce p53, perhaps associated with DNA damage. In the presence of TAg the p53 induced G1 block would be overridden and the cell may attempt DNA synthesis with damaged DNA, which may lead to apoptosis. Alternatively, the fact that the cells enter S-phase in the presence of excess p53 may be sufficient to induce apoptosis.

Binding of TAg to p110^{RB} is necessary for lifespan extension

G418^R-selected colonies expressing pRSV-T displayed an extension of lifespan of ~8 PDL greater than the G418^R-selected colonies transfected with pRcCMV only (Table 3.6). This amount of lifespan extension is not significantly different from that for the pRSV-T-induced foci (~13 PDL) ($P = 0.108$). There is perhaps a trend, however, for the foci to have a greater lifespan than the G418 selected colonies, and this might be explained by the different selection pressures. Formation of foci would appear to select for proliferation and loss of contact inhibition, which is dependent on TAg expression, while G418 selection depends only on expression of the neo^R gene.

Only four G418^R colonies transfected with either of the non-p110^{RB}-binding mutant TAg plasmids could be isolated from 9 transfections. This is significantly less than the 56 colonies obtained containing pRSV-T + pRcCMV and 28 containing pRcCMV only. The low number of G418^R colonies with co-transfection with non-p110^{RB}-binding mutant TAg plasmids suggests that there is selection against expression of non-p110^{RB}-binding mutant TAg. In

the colonies that were isolated there was no extension of lifespan associated with the non-p110^{RB}-binding mutant TAg plasmids, even though each was TAg positive by immunostaining. This is a clear indication that the p110^{RB} binding region of TAg is necessary for extension of lifespan.

Interestingly the RB-1 +/- cell strains, GM01879A and GM01880B, had unusually long *in vitro* lifespans, comparable to the lifespan of the pRSV-T transfected clones. This suggests that these cells already had an extended lifespan which was not further extended by the presence of the SV40 early region genes. This could be explained by a gene dosage effect with RB-1, the absence of one wt copy partially transforming the cells or making the cells more susceptible to changes. There is, however, no precedent for this in the literature and mutations in hereditary retinoblastoma patients occur at a frequency that can probably be fully accounted for by the spontaneous mutation rate without invoking a dosage effect.

Disruption of p53 in the presence of wt p110^{RB} is incompatible with cell growth

The fact that there were so few G418^{R} colonies from the transfection with the non-p110^{RB}-binding mutant TAg plasmids suggests that expression of mutant TAg which binds p53 but not p110^{RB} is detrimental to the cell. This is perhaps due to the requirement of the p53-induced cell cycle block for DNA damage repair, which presumably is significant immediately after transfection. It was recently reported that the introduction of mutant p53 by amphotropic retroviral infection was sufficient to extend the *in vitro* lifespan of "pre-aged" fibroblasts [Bond *et al.*,

1994]. The difference between the results presented in this study and those presented by Bond *et al.* may indicate that other functions of TAg, and not p53 inactivation, are detrimental to cell proliferation in the presence of wt $p110^{RB}$. Alternatively, this difference may also indicate that retroviral infection induces less DNA damage than transfection and thus there is not the same selection against inactivation of p53 in proliferating cells.

The situation in LFS fibroblasts appears to be different. LFS fibroblasts have been reported to have a spontaneous increase in lifespan associated with the loss of the remaining copy of wt p53, but in the presence of wt $p110^{RB}$ and $p16^{INK4}$ [Rogan *et al.*, 1995]. A major difference is that LFS fibroblasts, despite containing one wt copy of p53, appear to be karyotypically less stable than normal cells. Thus, these LFS cells are able to grow normally with one copy of wt p53 while accumulating genetic changes. Perhaps these pre-existing changes explain how the cells are able to continue to proliferate beyond their normal lifespan after loss of the wt copy of p53.

Alternatively, lack of lifespan extension may be an artifact of $G418^{R}$ selection. This may occur if the non-$p110^{RB}$-binding mutant TAg prevents expression of the neo^{R} gene in pRcCMV. The fact that it was not possible to obtain clones co-expressing exogenous RB-1 from pLRbRNL with TAg from pRSV-T, however, would suggest that inactivation of $p110^{RB}$ is necessary for extension of lifespan.

Each of the clones, co-transfected with pRSV-T + pLRbRNL and isolated by focus formation or G418 selection was TAg positive by

immunostaining and the majority had integrated pLRbRNL (Figure 3.8). However, all were negative for expression of RB-1 mRNA from pLRbRNL (Figures 3.9 & 3.11). This suggests that there is selection against expression of exogenous RB-1, similar to that demonstrated for p53 [Noble *et al.*, 1992]. This is consistent with exogenous expression of RB-1 being incompatible with proliferation of normal cells and suggests that binding and inactivation of $p110^{RB}$ by TAg is necessary for the extended lifespan, and probably proliferation, of cells.

Binding of TAg to p53 is not necessary for lifespan extension

Cells isolated containing the non-p53-binding mutant TAg plasmid bssv402DE have been reported not to display an extended lifespan [Lin & Simmons, 1991; Maclean *et al.*, 1994], indicating that TAg binding to p53 is necessary for extension of lifespan. However, analysis of lifespan of five pRSV-T(402DE) and two bssv402DE clones indicated that the non-p53-binding mutant TAg plasmids clearly did have an extended lifespan (Table 3.9). This extension of lifespan would appear to be comparable to that induced by pRSV-T although the number of clones is quite small. This is consistent with either, p53 binding by TAg being unnecessary for extending the lifespan of NHDF cells, or 402DE mutant TAg retaining some p53 binding. In order to verify that TAg binding to p53 is dispensable for extension of lifespan, it would be interesting to examine the TAg expression levels and amount of 402DE mutant TAg binding to p53 in the clones with an extended lifespan and compare with clones already isolated [Maclean *et al.*, 1994] which did not exhibit an extended lifespan.

The fact that inactivation of $p110^{RB}$ is necessary while inactivation of p53 appears to be not necessary for extension of lifespan of cells is entirely consistent with the antisense experiments of Hara *et al.* [1991]. Treatment of NHDF with antisense p53 had no effect on lifespan while the treatment of the cells with antisense RB-1 increased their lifespan by ~10 PDL. Interestingly, the combination of antisense RB-1 and antisense p53 resulted in an increase of ~20 PDL, and this increase was dependent on continued treatment with the antisense oligonucleotides over the extended lifespan.

Approximately 63% of the bssv402DE-induced foci and 29% of the pRSV-T(402DE)-induced foci analysed contained cells that were negative for TAg by immunostaining. This was not seen in the pRSV-T-induced foci. Presumably the foci arose from cells which were originally TAg positive, suggesting that expression of the plasmid is lost in some of the cells and that there is no selective advantage for the cells to continue expressing the mutant TAg. Alternatively, there could be a carry-over of normal cells from the time at which the foci were isolated, but the fact that the 402DE TAg- and wt TAg-induced foci were isolated using the same method argues against this. Interestingly, some of the foci (n=3) which contained only some TAg positive cells and one focus which contained no TAg positive cells (Table 3.9) still exhibited an extended lifespan. This suggests that the lifespan of the cells is extended by the initial expression of the non-p53-binding mutant TAg and the continued expression of the mutant TAg is not necessary for the maintenance of the extended lifespan. It appears that the non-p53-binding mutants of TAg instigated changes that

extended the lifespan of the cells, and perhaps caused other changes not analysed here such as chromosomal instability.

It has been clearly shown that there is a continued requirement for inactivation of both p53 and $p110^{RB}$ by TAg for the proliferation of SV40-immortalised cells post crisis (Section 1.3.2). It has also been shown that wt TAg expression is maintained in all pre-crisis clones examined in this study and another [Maclean *et al.*, 1994]. It is therefore surprising that expression is lost from a significant number of the non-p53-binding mutant TAg clones (Table 3.9). This suggests that a function of wt TAg, probably p53 binding, is continuously selected for during the extended lifespan of the cells, or alternatively, there is selection against continued expression of the 402DE mutant TAg.

A question of balance of p53 and $p110^{RB}$

These data obtained with the 402DE plasmids indicate that there is a requirement for a balance of p53 and $p110^{RB}$ which results in non-proliferation of cells when the balance is upset. A mechanism that may in part account for this is apoptosis. As discussed in Section 1.4.4, p53-induced apoptosis is well documented in cells which inappropriately enter S-phase with damaged DNA in the presence of wild type $p110^{RB}$ [Morgenbesser *et al.*, 1994; Almasan *et al.*, 1995]. This may reflect the situation in the cells transfected with non-p53-binding mutant TAg plasmids, the encoded proteins of which do not inactivate p53 but may induce cells to form foci and to proliferate by binding to $p110^{RB}$. These cells may be expected to undergo apoptosis in the presence of DNA damage, which might induce p53 expression. Thus there would be selection against

continued expression of the 402DE mutant TAg but not the wt TAg from pRSV-T. This is analogous to the results reported by White *et al.* [1992], in which E1A expression without E1B induced cell death. The DNA tumour viruses normally circumvent this problem by inactivating both p53 and $p110^{RB}$.

$p110^{RB}$ and p53 are normal in SV40-immortalised cells

All of the SV40-immortalised cell lines were shown to express TAg (Figure 3.16), indicating a continued requirement for expression of TAg. This conclusion is consistent with the results reported previously indicating the requirement for the expression of TAg after immortalisation [Wright *et al.*, 1989; Radna *et al.*, 1989]. The cell lines WI38 VA13/2RA, BEAS-2B, BEAS-2B/R1 and BES-1A1 also express a TAg species of approximately 100 kD in addition to the normal sized protein (Figure 3.16). TAgs of increased size (so called "super-T antigens") have been observed in SV40-transformed murine [Chen & Pollack, 1985] and human [Kao *et al.*, 1993a] cell lines. Since this super-TAg was present in only one of the BEAS-2B sub-lines, it does not appear to be required for immortalisation of these cells.

It is evident from the occurrence of crisis that while the ability of TAg to bind and inactivate p53 and $p110^{RB}$ proteins is required for the first phase of SV40's effect on human cells, *i.e.* extension of lifespan, it is insufficient for immortalisation [reviewed in Bryan & Reddel, 1994]. The frequency of immortalisation is consistent with a single mutation event in the host genome [Huschtscha & Holliday, 1983; Shay & Wright, 1989]. Presumably its occurrence is facilitated by the chromosomal changes induced by the SV40 genes.

It is possible that immortalisation-competent clones have already lost a copy of a dominantly acting gene before crisis, so that loss of function of the remaining allele is all that is required for escape from crisis to occur. It was therefore considered possible that mutation of p53 and RB-1 genes might confer an advantage on the cells that exceeded that of binding of their protein products to TAg, and might contribute to escape from crisis.

p53 sequence data were available for most of these cell lines [Lehman *et al.*, 1993] and 2 cell lines were analysed by immunoprecipitation. The use of immunoprecipitation analysis to indicate RB-1 status is justified as protein analysis has proven a very sensitive method of detecting mutant RB-1 [Reissmann *et al.*, 1993]. The proteins encoded by these mutants are apparently all altered in their size and/or phosphorylation pattern [reviewed in Goodrich & Lee, 1993]. In addition, RB-1 mutations usually result in inability of the protein to bind to TAg [Bignon *et al.*, 1990; Hu *et al.*, 1990]. The presence of normal $p110^{RB}$ (Figure 3.16) and normal p53 sequence (Table 3.10) in all SV40-immortalised cells (no sequence data were available for GM847 or WI38 VA13/2RA) clearly indicates that TAg is sufficient for the inactivation of these proteins.

The presence of wt $p110^{RB}$ and p53 sequence is consistent with a report by Kao *et al.* [1993b] which found no evidence of p53 or RB-1 mutations in SV40-immortalised human uroepithelial cells. The presence of the wt p53 and $p110^{RB}$ presumably accounts at least in part for the continuing requirement for TAg expression.

Chapter 4

Changes of RB-1, p53 and p16INK4 genes in cells of the immortalisation complementation groups

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Chapter 4

Changes of RB-1, p53 and p16INK4 genes in cells of the immortalisation complementation groups

4.1 Introduction

The isolation of mortal hybrids from fusion studies between normal and immortal cell lines [Bunn & Tarrant, 1980; Muggleton-Harris & DeSimone, 1980] indicates that immortalisation, rather than senescence, is due to loss of normal gene function. These findings were extended by the studies of Pereira-Smith and Smith [Pereira-Smith & Smith, 1983; Pereira-Smith & Smith, 1987], in which fusion of different immortalised human cell lines with each other often resulted in mortal hybrids. This indicated that the cell lines have become immortalised via different genetic events and at least four complementation groups for immortalisation, referred to as groups A, B, C and D, have since been identified (Pereira-Smith & Smith, 1988). Identification of complementation groups is an important development as it will allow the identification of the critical changes associated with immortalisation.

Microcell-mediated chromosome transfer studies have tentatively localised genes critical for senescence to individual chromosomes. Senescence has been induced by the introduction of single chromosomes into cell lines in each of the four complementation groups: chromosome 6q into group A cells [Sandhu *et al.*, 1994], chromosome 4 into group B cells [Ning *et al.*, 1991b], chromosome 1q into group C cells [Hensler *et al.*, 1994] and chromosome 7 into group D cells [Ogata *et al.*, 1995]. The specific genes associated with the induction of senescence of these cell lines are yet to be elucidated.

Studies of the transforming proteins of DNA tumour viruses, such as SV40, have indicated that the inactivation of both p53 and $p110^{RB}$ proteins is an early and essential step in the SV40-immortalisation of human cells. It is not known, however, if inactivation of p53 and $p110^{RB}$ is also necessary for the immortalisation of human cells by means other than the introduction of DNA tumour viruses. The inactivation of these two proteins by mutation in a wide variety of tumours suggests that they are important. It is reasonable to expect, therefore, that the tumour suppressor genes, p53 and RB-1 would be mutated, or otherwise inactivated, in at least a subset of immortal cell lines.

Other genes that might be involved in the control of proliferative potential include the $p16^{INK4}$ gene [Serrano *et al.*, 1993] which has been shown to be deleted or mutated in many cell lines derived from human tumours of diverse histological origin [Kamb *et al.*, 1994; Nobori *et al.*, 1994]. Deletion of chromosome 9 is a common genetic alteration in human bladder cancer, however,

deletion or mutation of $p16^{INK4}$ was found to be three times more common in human bladder carcinoma derived cell lines than in uncultured tumours [Spruck *et al.*, 1994]. This suggests that establishment of cell lines is more easily achieved with those tumours that have mutated or deleted $p16^{INK4}$, or that loss of wt $p16^{INK4}$ occurs during establishment of cells in culture. Phosphorylation of $p110^{RB}$, which inhibits its growth suppressive properties, can be inhibited by $p16^{INK4}$ binding to cdk4 (Figure 4.1) [Serrano *et al.*, 1993]. Thus, loss of $p16^{INK4}$ expression may result in the loss of at least some functions of $p110^{RB}$, possibly including its normal role in senescence. This suggests that loss of $p16^{INK4}$ might be equivalent to mutation of RB-1 and may occur in a subset of immortal cell lines containing wt $p110^{RB}$.

RB-1–induced growth arrest has been shown to be reversed by overexpression of cyclins A, E [Hinds *et al.*, 1992], D2 and mutant, but not wild type, D1 [Ewen *et al.*, 1993; Dowdy *et al.*, 1993]. The complex responsible for phosphorylation of $p110^{RB}$ includes cdk4 and a cycD (Figure 4.1) [Serrano *et al.*, 1993], while the cycE:cdk2 and cycA:cdk2 complexes are involved in the G1/S transition (Figure 1.4). It is thus conceivable that overexpression of a cycD or of cdk4 may have effects on cellular proliferative potential that are equivalent to loss of wt $p110^{RB}$.

MDM2 has been shown to bind both mutant and wild type p53 (Figure 4.2). In binding to p53, MDM2 has been shown to inhibit some of the functions of p53, such as binding to specific DNA sequences and thus specific induction of expression of some genes. It has also been shown that MDM2 overexpression is able to

Figure 4.1 Braking the cell cycle

Cyclin D and cdk4 may stimulate cell division by adding phosphate to $p110^{RB}$ and releasing transcription factors (TF; most notably E2F) that turn on genes in the nucleus. $p16^{INK4}$ prevents the activity of cdk4 (Section 1.4.3)

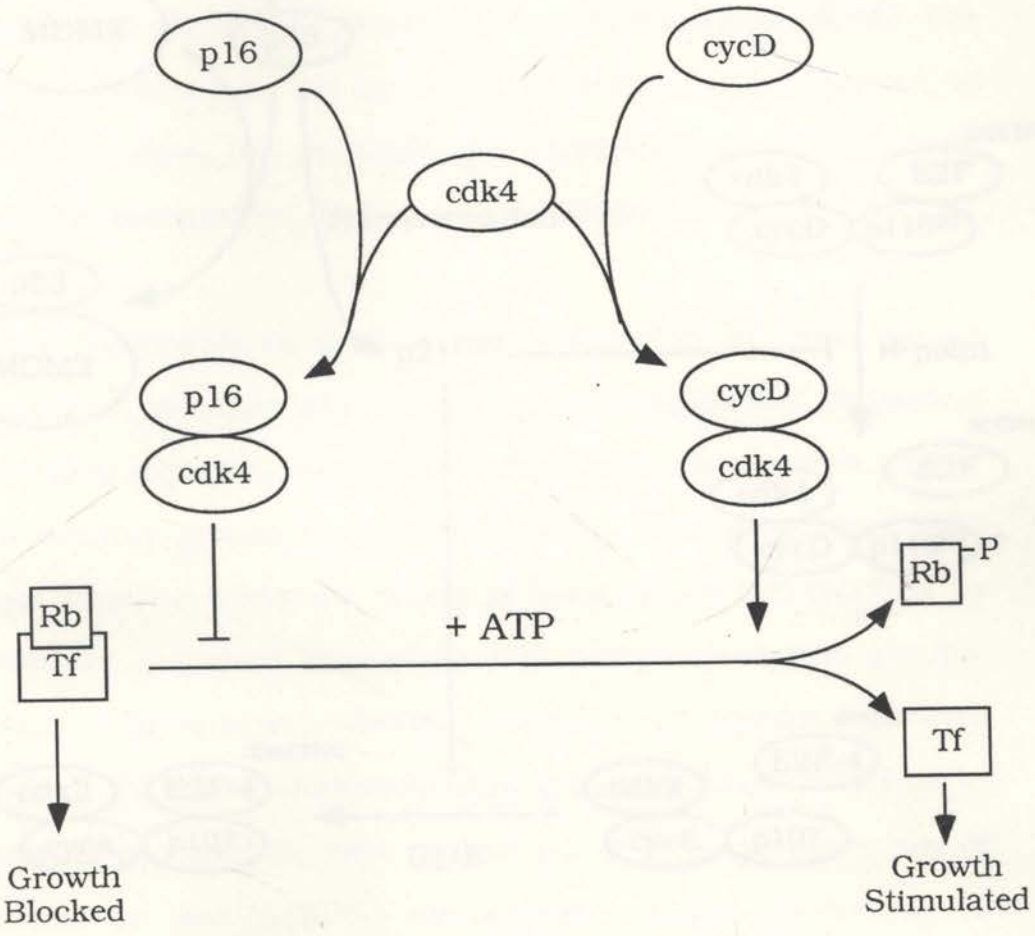
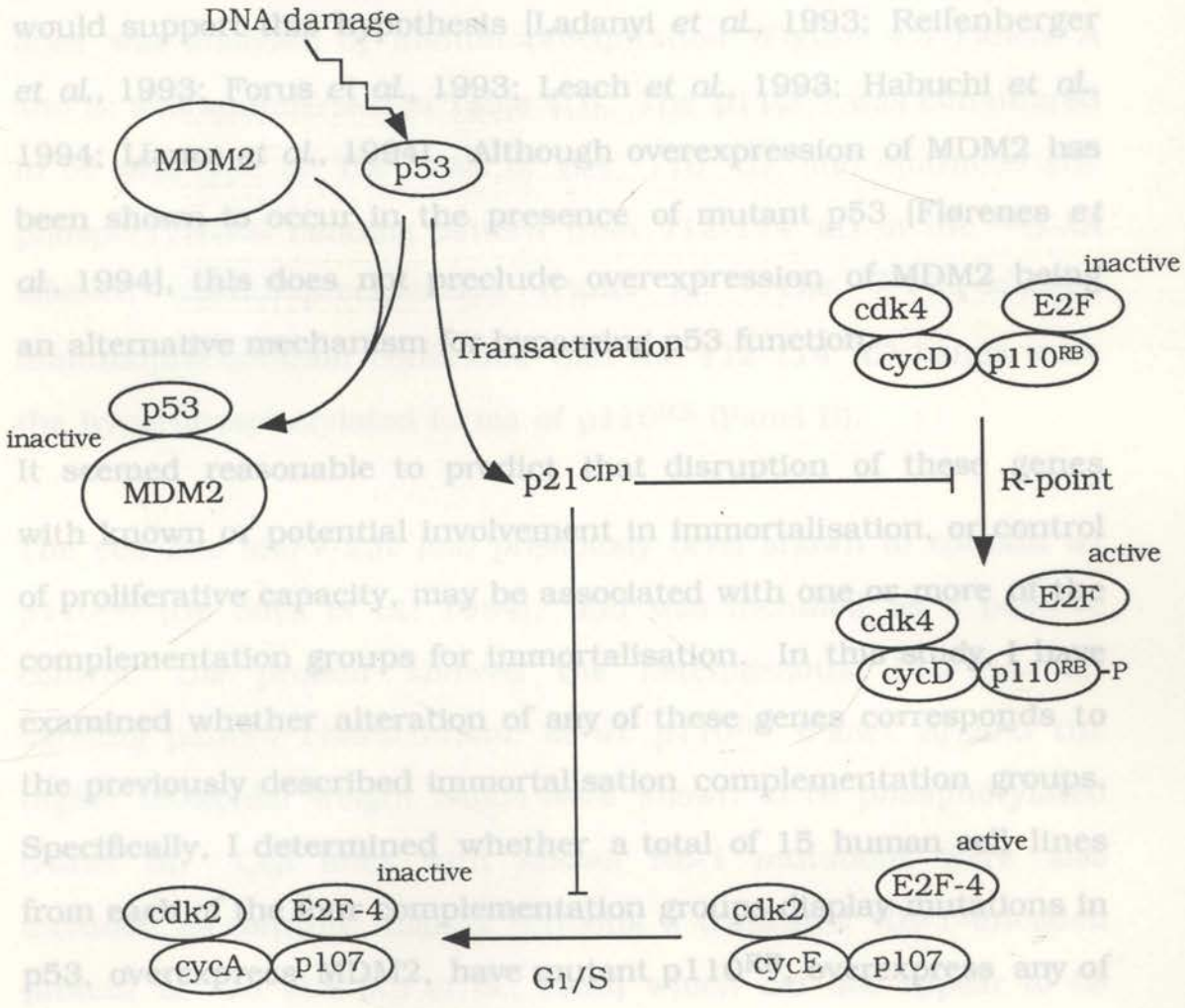

p16
cycD
cdk4
p16
cdk4
cycD
cdk4
Rb
Tf
+ ATP
Rb
P
Tf
Growth
Blocked
Growth
Stimulated

Figure 4.2 Blocking p53-mediated check points

Binding of MDM2 to the transactivation domain of p53 blocks the transactivation of $p21^{CIP1}$ and inhibits p53-mediated growth arrest. $p21^{CIP1}$, a non-specific cyclin dependent kinase inhibitor, may prevent the activity of cdk4 at the R-point and cdk2 at the G1/S boundary. $p21^{CIP1}$ may also have activity at the G2/M check point (Section 1.4.3).


DNA damage
MDM2
p53
Transactivation
inactive
cdk4
E2F
cycD
p110RB
p53
inactive
MDM2
p21CIP1
R-point
active
E2F
cdk4
cycD
p110RB-P
active
E2F-4
inactive
cdk2
E2F-4
cdk2
cycA
p107
cycE
p107
G1/S

overcome the p53 induced G1 block [Khatib *et al.*, 1993; Reifenberger *et al.*, 1993; 1994]. It is therefore thought that MDM2 overexpression is an alternative to mutational inactivation of p53 [Oliner *et al.*, 1992] and the inverse correlation of MDM2 overexpression and the presence of mutant p53 in some tumours would support this hypothesis [Ladanyi *et al.*, 1993; Reifenberger *et al.*, 1993; Forus *et al.*, 1993; Leach *et al.*, 1993; Habuchi *et al.*, 1994; Lianes *et al.*, 1994]. Although overexpression of MDM2 has been shown to occur in the presence of mutant p53 [Flørenes *et al.*, 1994], this does not preclude overexpression of MDM2 being an alternative mechanism for bypassing p53 function.

It seemed reasonable to predict that disruption of these genes with known or potential involvement in immortalisation, or control of proliferative capacity, may be associated with one or more of the complementation groups for immortalisation. In this study, I have examined whether alteration of any of these genes corresponds to the previously described immortalisation complementation groups. Specifically, I determined whether a total of 15 human cell lines from each of the four complementation groups display mutations in p53, overexpress MDM2, have mutant $p110^{RB}$, overexpress any of the cyclins, or lack $p16^{INK4}$ expression. Since telomerase is activated in many immortalised cell lines (Section 1.2), the presence of telomerase activity in these cell lines is also discussed.

4.2 Status of RB-1 in complementation group cell lines

Each of the 15 cell lines from the four complementation groups, shown in Table 4.1, were labelled with ^{35}S-AA or $^{32}PO_4$ and lysed with a low stringency lysis buffer. p110^{RB} from each of the cell lines was analysed by immunoprecipitation (Figure 4.3 Panels A and B; and summarised in Table 4.1). The p110^{RB} was considered to be wild-type on the basis of size, 110 kD, and characteristic phosphorylation banding pattern from 112-114 kD in the ^{35}S-AA labelled immunoprecipitation (Panel A). The $^{32}PO_4$-labelled immunoprecipitation confirmed that the 112-114 kD bands were the hyperphosphorylated forms of p110^{RB} (Panel B).

The cell line MePV-23I had previously been shown to contain wt p110^{RB} (De Silva *et al.*, 1994), and was included as a positive control; the protein showed the heterogenous 110-114 kD banding pattern characteristic of wt p110^{RB} (Panel A) and the higher molecular weight bands were shown to be phosphorylated (Panel B). Cell lines with known RB-1 mutations were also included as controls: Saos-2 contains a truncated RB-1-encoded protein of ~95 kDa [Xu *et al.*, 1989] which did not appear to be phosphorylated; NCI-H209 has a point mutation at codon 706 of RB-1 resulting in a hypophosphorylated protein of wt molecular weight, i.e., only the 110 kDa band was present [Kaye *et al.*, 1990] which also did not appear to be phosphorylated (Panel B); and C-33A has a small in-frame deletion at the 5' end of exon 20 resulting in a protein that is slightly smaller than the wild type size and hypophosphorylated [Scheffner *et al.*, 1991]. It also appeared to be not phosphorylated in Panel B (Figure 4.3).

Figure 4.3 Immunoprecipitation of $p110^{RB}$

$p110^{RB}$ protein from cell lines from each of the complementation groups for immortalisation indicated was analysed by immunoprecipitation. ^{35}S-amino acid (**Panel A**) and $^{32}PO_4$ (**Panel B**) pulse labelled cells were lysed with a low stringency buffer that maintains complexes between $p110^{RB}$ and SV40 large T antigen. The cell lysates were immunoprecipitated with anti–human $p110^{RB}$ antibody, NCL-RB, and the proteins were separated on an SDS-8% polyacrylamide gel and detected by autoradiography. Controls: Saos-2, containing only a 95 kD mutant form of $p110^{RB}$; C-33A and NCI-H209 containing only hypophosphorylated $p110^{RB}$; MePV-23I, containing wild type $p110^{RB}$.

A
B
WI-38 VA13/2RA
T24
HT1080
GM847
T98G
J82
HeLaCOT
CMV-Mj-HEL-1
143BTK-
SUSM-1
A1698
A549
A2182
BET-1A
293
Saos-2
C-33A
NCI-H209
MePV-23I
Group
A
B
C
D
p110RB
T Ag
p110RB
T Ag

Table 4.1 Status of RB-1 and $p16^{INK4}$ in cell lines from each of the four complementation groups for immortalisation

Cell Line	DNA Tumour Virus Sequences[1]	RB-1 status[2]	$p16^{INK4}$ status[2,3]
Group A			
GM847	SV40	wild type	present
WI-38	SV40	wild type	present
VA13/2RA			
HT1080	-	wild type	absent
T24	-	wild type	absent
Group B			
HeLaCOT	HPV-18	wild type	present
J82	-	truncated	present
T98G	-	wild type	absent
Group C			
143BTK-	-	wild type	absent
CMV-Mj HEL-1	-	wild type	absent
Group D			
293	Adenovirus 5	wild type	present
BET-1A	SV40	wild type	present
A1698	-	wild type	absent
A2182	-	wild type	absent
A549	-	wild type	absent
SUSM-1	-	wild type	absent

[1]DNA tumour virus sequences present, known to encode proteins that bind to $p110^{RB}$ and p53

[2]Determined by immunoprecipitation

[3]Determined by western analysis

With the exception of J82 which had previously been shown to contain a truncated RB-1 protein [Horowitz *et al.*, 1989], all other cell lines representative of immortalisation complementation groups contained wt $p110^{RB}$. The $p110^{RB}$ bands in these cell lines, compared with the non-hyperphosphorylated $p110^{RB}$ shown for the NCI-H209 and C-33A cell lines, each contained the p112-114 kD bands indicative of normally hyper-phosphorylated $p110^{RB}$. Immunoprecipitation of $^{32}P0_4$-labelled $p110^{RB}$ confirmed that the higher molecular weight bands are phosphorylated (Panel B). The relative amounts of the various phosphorylated forms of $p110^{RB}$ varied among these cell lines, presumably due at least in part to the growth rate of the cells at the time of metabolic labelling.

The low stringency lysis buffer maintained the complex between TAg and $p110^{RB}$. TAg co-immunoprecipitated with $p110^{RB}$ from the three SV40-immortalised cell lines WI-38VA13/2RA, GM847 and BET-1A, which is consistent with the $p110^{RB}$ being wild type in these cell lines (Panel A). TAg is also a phosphoprotein and, therefore, also appears in Panel B (Figure 4.3). Confirmation that $p110^{RB}$ and TAg from these cell lines co-immunoprecipitated was obtained from Western blot analyses (data not shown).

Cell lines from each of the complementation groups contained wt $p110^{RB}$ and, as there were non-virally immortalised cell lines with wt $p110^{RB}$ in each of the four complementation groups, it can be concluded that inactivation of RB-1 by mutation or by binding of $p110^{RB}$ to DNA tumour viral proteins does not account for any of the four immortalisation complementation groups.

4.3 Status of $p16^{INK4}$ and cyclin expression in complementation group cell lines

4.3.1 Cyclin-dependent kinase inhibitor $p16^{INK4}$

The majority of cell lines examined in this study contained wt $p110^{RB}$, which was unexpected considering the importance of $p110^{RB}$ binding by TAg in SV40-induced immortalisation. It is possible, however, that an alternative mechanism to RB-1 mutation may inactivate the growth suppression activity of $p110^{RB}$. The ability of $p16^{INK4}$ to inhibit the phosphorylation of $p110^{RB}$ (Figure 4.1), and the frequent occurrence of $p16^{INK4}$ mutations in cell lines [Spruck *et al.*, 1994] suggested that loss of $p16^{INK4}$ expression might be such a mechanism. $p16^{INK4}$ protein was analysed both by Western blot (Figure 4.4 Panel A) and by ^{35}S-AA labelled immunoprecipitation analysis (Panel B). $p16^{INK4}$ protein was detected in the normal cell strain WI-38, but not in WM1175 cells which are known to have a homozygous deletion of the $p16^{INK4}$ gene (G. Mann, pers. comm.). $p16^{INK4}$ was also readily detected by this method in each of four other normal cell strains (data not shown).

Analysis of the 15 cell lines from each of the four complementation groups, as well as the $p110^{RB}$ mutant control cell lines Saos-2, C-33A and NCI-H209 (Figure 4.4 Panels A and B), revealed that $p16^{INK4}$ protein was detected only in cell lines containing mutant $p110^{RB}$ or DNA tumour virus proteins. Due to the inability to detect $p16^{INK4}$ protein in some cell lines, some of the cell lines were also analysed for expression of the mRNA by Northern blot analysis (Figure 4.5 Panel A). In all but three of the cell lines, expression of the mRNA correlated with presence of the $p16^{INK4}$

Figure 4.4 $p16^{INK4}$ protein status

$p16^{INK4}$ protein from cell lines from each of the complementation groups for immortalisation indicated was subjected to Western or immunoprecipitation analysis. Controls: WI-38 normal human diploid cell strain, containing wild type $p16^{INK4}$; WM1175, containing homozygous deletion of $p16^{INK4}$.

Panel A: Western analysis of $p16^{INK4}$ protein. 30μg of protein from each cell type was separated on an SDS-polyacrylamide (4-20% gradient) gel. The proteins were transferred to a PVDF membrane and probed with a rabbit-polyclonal anti-$p16^{INK4}$ antibody followed by an alkaline phosphatase-linked goat anti-rabbit antibody. The antibody was then detected by colour reaction.

Panel B: Immunoprecipitation analysis of $p16^{INK4}$ protein. ^{35}S-amino acid pulse labelled cells were lysed with RIPA buffer and the $p16^{INK4}$ protein was immunoprecipitated from each of the cell lines. The precipitated proteins were then separated on an SDS-polyacrylamide (4-20% gradient) gel and detected by autoradiography.

A
B
WI-38
WM1175
WI-38 VA13/2RA
T24
HT1080
GM847
T98G
J82
HeLaCOT
CMV-Mj-HEL-1
143BTK-
SUSM-1
A1698
A549
A2182
BET-1A
293
Saos-2
C-33A
NCI-H209
Group
A
B
C
D
p16INK4
p16INK4

Figure 4.5 Northern analysis of $p16^{INK4}$ and MDM2

10μg of total RNA, isolated from cell lines from each of the complementation groups for immortalisation indicated, was separated on a 1%-formaldehyde gel The RNA was transferred to nylon membrane and hybridised with an α^{32}-P-dCTP labelled EcoRI/XhoI fragment from plasmid pcDNA3/p16, **Panel A**. The membrane was then stripped and hybridised with an α^{32}-P-dCTP labelled A201 oligonucleotide probe for MDM2, **Panel B**. In each case, the 5.5 kb MDM2 mRNA was detected by autoradiography. Controls: MRC5 normal human diploid cell strain, containing wild type $p16^{INK4}$ and MDM2; WM1175, containing homozygous deletion of $p16^{INK4}$.

B
A
MRC-5
WM1175
WI-38 VA13/2RA
HT1080
GM847
T98G
J82
HeLaCOT
CMV-Mj-HEL-1
143BTK-
SUSM-1
A1698
A549
BET-1A
A
B
C
D
Group
MDM2
p16INK4

protein. That is, the mRNA and protein were both present, and apparently normal, in cell lines WI-38 VA13/2RA, GM847, J82, HeLaCOT and BET-1A, while both mRNA and protein were absent in cell lines HT1080, T98G, 143BTK$^-$ and A549.

Three of the cell lines, CMV-Mj-HEL-1, SUSM-1 and A1698, contained no detectable $p16^{INK4}$ protein (Figure 4.4, Panels A and B) yet expressed $p16^{INK4}$ mRNA (Figure 4.5, Panel A). As $p16^{INK4}$ protein was detected in normal control cells, WI-38, the presence of the $p16^{INK4}$ mRNA but not protein suggested that post-transcriptional regulation or mutation resulted in the mRNA not being translated. $p16^{INK4}$ sequence data are not available for these cell lines except for exon 2 in T24 which was normal [Spruck *et al.*, 1994]. Other mutations could also result in no protein being made. This demonstrates the advantage of analysing the gene at the protein level rather than the DNA or RNA level.

The data summarised in Table 4.1 demonstrate that each immortalised cell line either lacked detectable $p16^{INK4}$ or contained inactive $p110^{RB}$ (mutant or bound to viral protein), but not both. It is important to note that this includes virally and non-virally immortalised cell lines from different histological origins. $p110^{RB}$ thus appears to be inactivated in each of the cell lines by one of the following: the presence of RB-1 mutation (J82); presence of viral proteins known to inactivate $p110^{RB}$ (WI-38 VA13/2RA, GM847, HeLaCOT, 293 and BET-1A); or loss of $p16^{INK4}$ protein expression (HT1080, T24, T98G, 143BTK$^-$, CMV-Mj-HEL-1, A1698, A2182, A549 and SUSM-1).

4.3.2 Cyclins

Expression levels for each of the cyclins, A, B1, D1-3 and E, were examined in two cell lines from each complementation group (HT1080, WI-38 VA13/2RA, J82, T98G, CMV-Mj-HEL-1, 143BTK-, A549 and BET-1A) by Northern analysis by E. Musgrove (Figure 4.6). Cyclin D2 mRNA was undetectable except in 143BTK- cells; cyclin D2 expression, however, is also often undetectable in normal cells (Won *et al.*, 1992). The mRNA for each of the other cyclins was of the same size and expressed at similar levels in each cell line tested. Overexpression of the cyclins therefore did not appear to play a role in immortalisation of any of the complementation groups. As each of the cell lines contained inactivated RB-1, by means of mutation or presence of a tumour viral protein or absence of $p16^{INK4}$ protein, it was decided not to further examine the cyclins at the protein level.

4.4 Status of p53 and MDM2

4.4.1 p53

The p53 status for 14 cell lines from the four complementation groups was determined by ^{35}S-AA labelling and immunoprecipitation with conformation specific anti-p53 antibodies (Figure 4.7; and summarised in Table 4.2). The monoclonal antibodies used were: PAb122 that recognises p53 protein independent of conformation; PAb1620, wild type conformation only [Milner *et al.*, 1987]; and PAb240, mutant conformation only [Gannon *et al.*, 1990]. In addition, during the course of this study, p53 sequence data became available for many of these cell lines (Table 4.2). The immunoprecipitation data are

Figure 4.6 Northern analysis of the cyclins

10μg of total RNA, isolated from cell lines from each of the complementation groups for immortalisation indicated, was separated on a 1%-formaldehyde gel. The RNA was transferred to nylon membrane and hybridised with an α^{32}-P-dCTP cyclin cDNA probe, and subsequently with an oligonucleotide probe complementary to a highly conserved region of the 18S ribosomal RNA.

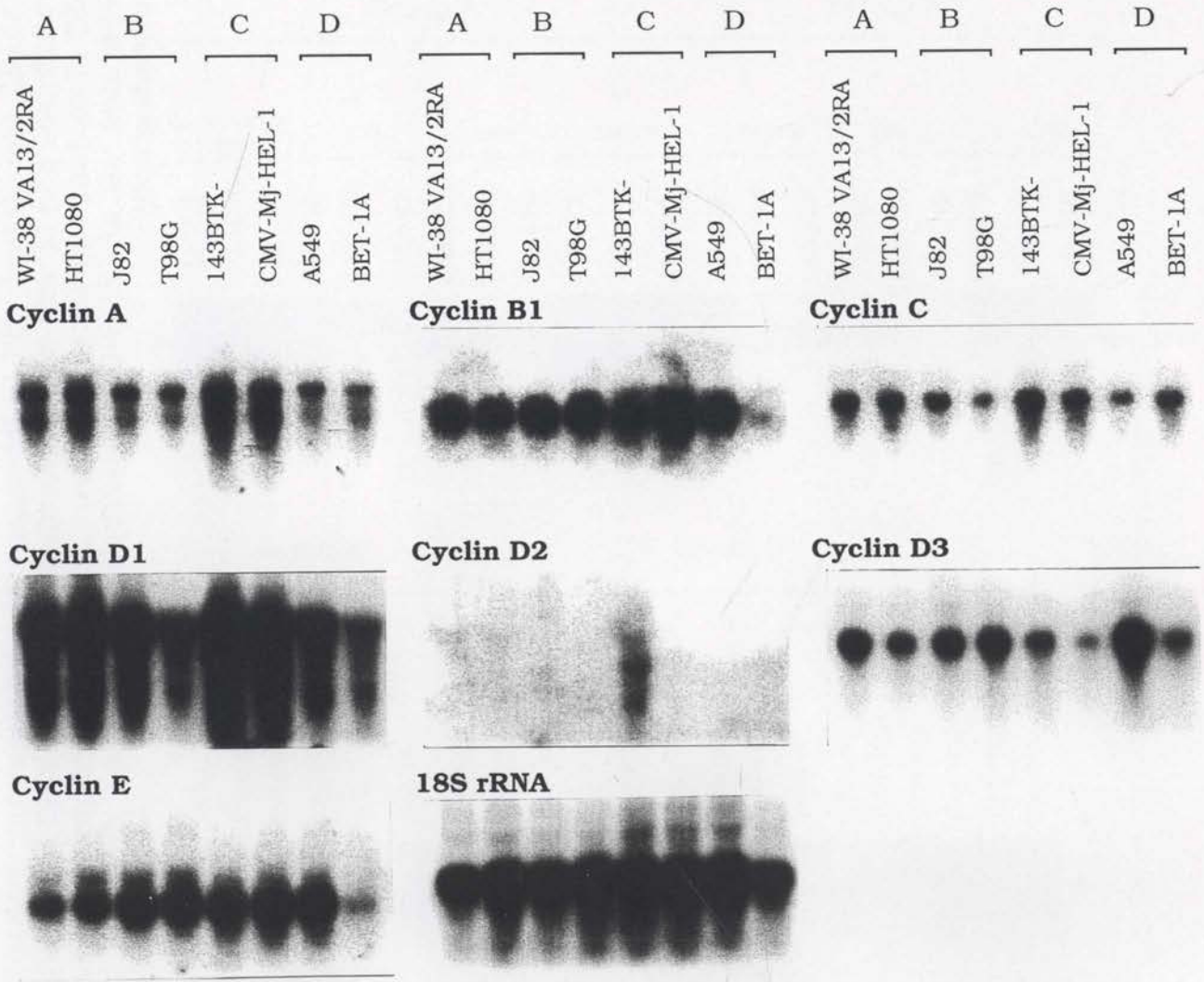

A
B
C
D
WI-38 VA13/2RA
HT1080
J82
T98G
143BTK-
CMV-Mj-HEL-1
A549
BET-1A
Cyclin A
Cyclin B1
Cyclin C
Cyclin D1
Cyclin D2
Cyclin D3
Cyclin E
18S rRNA

Figure 4.7 Immunoprecipitation analysis of p53

p53 protein from cell lines from each of the complementation groups for immortalisation was analysed by immunoprecipitation. ^{35}S-amino acid pulse labelled cells were lysed with RIPA buffer and the lysates were immunoprecipitated with: N, non-immune serum; PAb122 (recognises p53 independent of conformation); PAb1620 (recognises p53 of wild-type conformation only); PAb240 (recognises p53 of mutant conformation only). The precipitated proteins were separated on an SDS-8% polyacrylamide gel and detected by autoradiography.

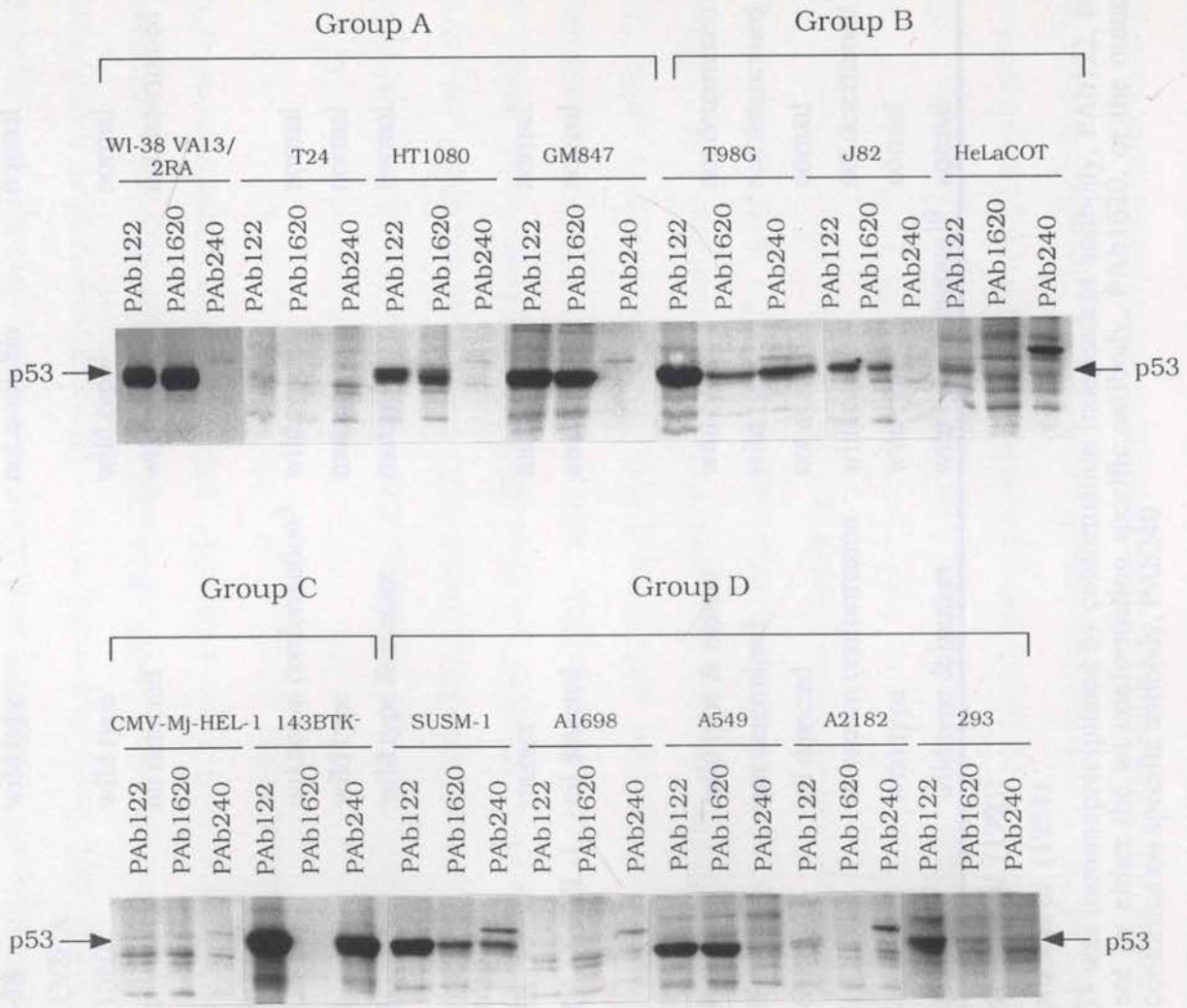

Group A
Group B
WI-38 VA13/ 2RA
T24
HT1080
GM847
T98G
J82
HeLaCOT
PAb122
PAb1620
PAb240
p53
Group C
Group D
CMV-Mj-HEL-1
143BTK-
SUSM-1
A1698
A549
A2182
293

Table 4.2 Status of p53 and MDM2 in cell lines from each of the four complementation groups for immortalisation

Cell Line	p53 Immunoprecipitation	p53 published sequence	MDM2 mRNA level
Group A			
GM847	wild type	not available	normal
WI-38	wild type	not available	normal
VA13/2RA			
HT1080	wild type	wild type[1]	normal
T24	nil detected	wild type[2]	not determined
Group B			
HeLaCOT	unknown conformation[3]	wild type[4]	normal
J82	wild type	mutant[2]	normal
T98G	wild type & mutant	mutant[5]	normal
Group C			
143BTK-	mutant	mutant[6]	normal
CMV-Mj HEL-1	nil detected	mutant[7]	normal
Group D			
293	wild type & mutant	wild type[8]	not determined
BET-1A	not determined	wild type[9]	not determined
A1698	nil detected	not available	normal
A2182	unknown conformation	wild type[8]	not determined
A549	wild type	wild type[8]	normal
SUSM-1	wild type & mutant	wild type & mutant[10]	normal

[1]Sharma *et al.* (1993)

[2]Spruck *et al.* (1994)

[3]p53 was immunoprecipitated by conformation independent antibody, PAb122, but not by either the wt conformation specific antibody, PAb1620, or the mutant conformation specific antibody, PAb240

[4]Scheffner *et al.* (1991)

[5]Ullrich *et al.* (1993)

[6]Romano *et al.* (1989)

[7]Whitaker *et al.* (1995)

[8]Lehman *et al.* (1991)

[9]Reddel *et al.* (1995)

[10]M. Namba *et al.*, unpublished sequence data

presented here and discussed, and compared with published sequence data in the discussion (Section 4.5).

The presence of a band upon immunoprecipitation with PAb122 and PAb1620, but not with PAb240, in cell lines GM847, WI-38 VA13/2RA, HT1080, J82 and A549 indicated that the p53 protein in these cell lines was wild type. The presence of a band upon immunoprecipitation with PAb122 and PAb240, but not with PAb1620, in cell line 143BTK- indicated the presence of only mutant p53 protein.

No p53 was detected in T24, CMV-Mj-HEL-1 and A1698 cells, suggesting that the p53 in these cells was not recognised by any of the anti-p53 antibodies or that these cells were p53 null. In contrast, each of PAb122, PAb1620 and PAb240 antibodies immunoprecipitated p53 from T98G, 293 and SUSM-1 cells; this could indicate either the presence of a mutant p53 protein detectable by both of the conformation specific antibodies, or the presence of two different alleles for p53.

PAb122 immunoprecipitation in HeLaCOT and A2182 cells resulted in a faint band but p53 was not immunoprecipitated by either of the conformation specific antibodies PAb1620 or PAb240. This may indicate that the p53 protein was non-functional in these cells.

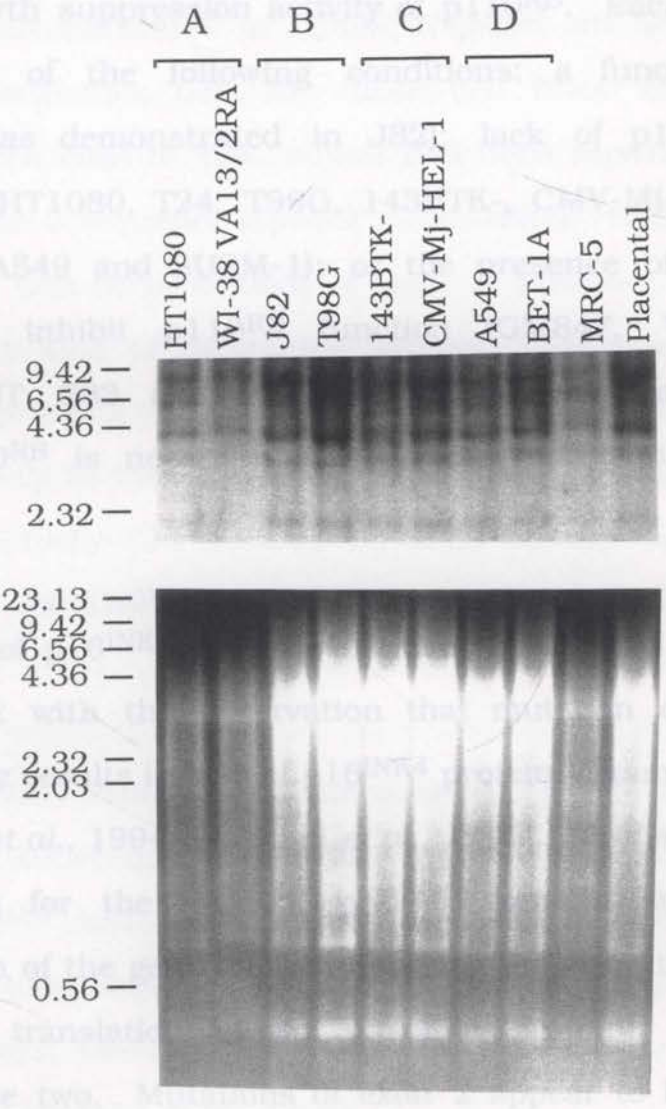
4.4.2 MDM2

As indicated in Figure 4.2, binding of MDM2 to p53 can inhibit the transactivation functions of wt p53 and this has been shown to relieve the G1/S inhibiting functions of p53. A 5-50 fold overexpression of MDM2, usually by amplification, has therefore been suggested as an alternative to mutational inactivation of p53 [Oliner *et al.*, 1992]. Amplification was examined in two of the cell lines from each of the complementation groups by Southern analysis (Figure 4.8). The expected bands at approximately 6.6, 4, 3 and 1.8 kb can be seen in the NHDF cell strain, MRC-5 and the placental DNA lanes. The extra band greater than 9 kb in size possibly resulted from incomplete digestion and appears in each of the lanes. The same bands seen in the normal controls can also be seen in each of the cell lines, indicating that there were no gross rearrangements. Also, these bands appeared to be at a similar intensity, indicating that no amplification of the MDM2 gene had occurred in any of these cell lines.

mRNA expression levels were also examined by Northern blot analysis in most of the cell lines listed in Table 4.2. The membrane originally probed for $p16^{INK4}$ mRNA in Figure 4.5 (Panel A) was stripped and reprobed with an α-^{32}P-dCTP-labelled oligo probe (Table 2.4) for MDM2 mRNA as shown in Figure 4.5 (Panel B; and summarised in Table 4.2). Each of the cell lines had a similar level of expression of 5.5 kb MDM2 mRNA as the NHDF cell strain control, WI-38. Overexpression of MDM2 was not apparent in any of the cell lines examined and would thus appear not to account for immortalisation of cells in any of the complementation groups.

Figure 4.8 Southern blot analysis of MDM2

5µg of DNA extracted from two cell lines from each of the four complementation groups was digested with EcoRI, separated on a 0.7% agarose gel and transferred to a nylon membrane. The membrane was probed for MDM2 DNA with the α-^{32}P-dCTP-labelled 900 bp Xho1 fragment from MDMC14-2 plasmid. Placental DNA and DNA from the NHDF strain, MRC-5, were included as normal controls. Also shown is ethidium bromide stained DNA in the gel before transfer.


A
B
C
D
HT1080
WI-38 VA13/2RA
J82
T98G
143BTK-
CMV-Mj-HEL-1
A549
BET-1A
MRC-5
Placental
9.42
6.56
4.36
2.32
23.13
9.42
6.56
4.36
2.32
2.03
0.56

4.5 Discussion

Inverse correlation between p110^{RB} and p16^{INK4} abnormalities

In all cell lines examined, including cell lines from each of the four complementation groups, there existed a mechanism for inactivating the growth suppression activity of p110^{RB}. Each cell line contained one of the following conditions: a functional mutation of RB-1 (as demonstrated in J82); lack of p16^{INK4} protein expression (HT1080, T24, T98G, 143BTK-, CMV-Mj-HEL-1, A1698, A2182, A549 and SUSM-1); or the presence of viral protein known to inhibit p110^{RB} function (GM847, WI-38 VA13/2RA, HeLaCOT, 293 and BET-1A). This suggests that inactivation of p110^{RB} is necessary for the immortalisation of human cells.

Lack of expression of p16^{INK4} proteins in many of the cell lines tested is consistent with the observation that mutation of the p16^{INK4} gene usually results in loss of p16^{INK4} protein [Okamoto *et al.*, 1994; Otterson *et al.*, 1994; Washimi *et al.*, 1995]. Mechanisms that could account for the lack of p16^{INK4} protein include homozygous deletion of the gene, point mutations resulting in lack of translation (or translation of an unstable protein), or a combination of these two. Mutations of exon 2 appear to be the most common mutations of p16^{INK4} [Spruck *et al.*, 1994]. Recently, it was also demonstrated that silencing of p16^{INK4} mRNA expression can occur by methylation of a CpG island identified in the non-coding exon 1 of the p16^{INK4} gene [Merlo *et al.*, 1995].

In cell lines without $p16^{INK4}$ protein expression, $p16^{INK4}$ mRNA was also usually undetectable. However, three of the cell lines tested, CMV-Mj-HEL-1, SUSM-1 and A1698, expressed $p16^{INK4}$ mRNA but expressed no detectable protein. Presumably these cell lines contain mutations of $p16^{INK4}$ which resulted in expressed mRNA which was not translated or which encoded an unstable protein. $p16^{INK4}$ sequence data for these cell lines are not available except in the case of T24, which had been reported to contain a normal exon 2 sequence [Spruck *et al.*, 1994]. Mutations of the $p16^{INK4}$ gene that occur outside of exon 2, however, could still account for the lack of detectable protein. Interestingly, preliminary finger-printing data suggest that the subclone of A1698 used exclusively in this study, $A1698^{DM}$ (provided by O. M. Pereira-Smith), is actually T24 [E. Duncan unpublished]. This is also consistent with the $p16^{INK4}$ protein analyses for each of these cell lines.

While the report of this study was in preparation I learnt that an inverse correlation between the presence of wt RB-1 and the expression of $p16^{INK4}$ has also been shown in cell lines and tumours from the lung, oesophagus, liver, pancreas and colon [Okamoto *et al.*, 1994; Otterson *et al.*, 1994; Tam *et al.*, 1994; Shapiro *et al.*, 1995; Aagaard *et al.*, 1995].

Immortalisation of each of the human cell lines tested appeared to involve inactivation of $p110^{RB}$ function, indicated by the presence of mutant RB-1, absence of $p16^{INK4}$ protein, or presence of viral proteins which inactivate $p110^{RB}$ in these lines. This is consistent with the established role of $p110^{RB}$ in control of the cell cycle.

Although p110^{RB} function appeared to be inactivated in the cell lines tested by either one of these three mechanisms, alternative mechanisms are also possible. A correlation of overexpression of cycD1 [Schauer *et al.*, 1994], or cdk4 [He *et al.*, 1994], with normal RB-1 suggests that overexpression of these genes may also provide alternative mechanisms for inactivating the proliferation control of p110^{RB}. Overexpression of cyclins A, B1, C, D1, D2, D3 or E was not detected at the mRNA level in any of the cell lines (Figure 4.6), and the cdk4 expression level was not examined.

Inactivation of p53 is a common occurrence in cell lines

Published sequence data for the cells in Table 4.2 would indicate that the status of p53 is normal in most of the immortalised cell lines assigned to a complementation group. This is unexpected given that p53 mutations occur in approximately 50% of all tumours. It should be noted, however, that the cell lines listed in Table 4.2 are only a small sample. The presence of viral proteins known to bind p53 in some of these cells, indicates that cell lines containing functionally inactive p53 appear in each of the complementation groups (Table 4.2). Furthermore, the p53 immunoprecipitation data indicate p53 is functionally mutant in some of the cell lines listed.

Immunoprecipitation data (Figure 4.7) for cell lines HT1080 and A549 indicated the presence of only wt p53 in these cell lines. 143BTK- cells appeared to contain only mutant p53 while SUSM-1 appeared to contain p53 protein of both mutant and wt conformation. These data are consistent with previously published sequence data (Table 4.2). CMV-Mj-HEL-1, on the other hand,

contained no immunoprecipitable p53 protein. Exons 2 to 9 of p53 from CMV-Mj-HEL-1 were sequenced by A. Chang in this laboratory [Whitaker *et al.*, 1995]. CMV-Mj-HEL-1 was found to have a nonsense p53 mutation at codon 126, altering this codon from TAC (tyrosine) to TAG which is consistent with the absence of detectable p53 protein.

The presence of a band upon immunoprecipitation with PAb122 and PAb1620, but not with PAb240, in cell lines GM847, WI-38 VA13/2RA and J82, indicated that the p53 protein in these cell lines is wild type (Figure 4.7). No sequence data were available for cell lines GM847, WI-38 VA13/2RA, but the presence of wt p53 protein is in agreement with studies showing that cells containing TAg contained wt p53 [Kao *et al.*, 1993b; Lehman *et al.*, 1993]. J82 would appear to contain only wt p53 protein indicating that these cells contain wt p53 protein and presumably at least one copy of wt p53 sequence. The available sequence data for J82 cells would indicate that J82 contains at least one mutant p53 allele [Spruck *et al.*, 1994]. The lack of detection of a conformationally mutant protein may indicate that the mutant mRNA is not translated or that the mutant protein is unstable or not recognised by the PAb240 antibody.

PAb122 immunoprecipitation of A2182 and HeLaCOT cell lysates resulted in a band but p53 was not immunoprecipitated by either of the conformation specific antibodies PAb1620 or PAb240 (Figure 4.7). HPV-18 encoded E6 protein, however, is known to induce the ubiquitin dependent degradation of p53 [Scheffner *et al.*, 1990], making immunoprecipitation of p53 difficult in these

cells and also possibly affecting the conformation of the p53 protein. The published sequence data [Scheffner *et al.*, 1991] and the wild type transactivation activity of p53 in HeLa cells [Butz *et al.*, 1995], however, would indicate that p53 is wild type in these cells (Table 4.2). A2182 cells are also reported to contain wild type p53 sequence [Lehman *et al.*, 1991]. The low level of p53 protein, which was not recognised by the conformation specific antibodies, suggests that these cells are functionally p53 null, although this would not be predicted from the sequence data (Table 4.2). Situations in which the sequence obtained may not reflect the encoded protein may include: mutations outside the sequenced region; sequence obtained from just one allele where the two alleles are different; errors in sequencing; and also, changes in other proteins which affect p53 expression or modify p53 function by interacting with p53 protein.

Each of the PAb122, PAb1620 and PAb240 antibodies immunoprecipitated p53 from cell lines T98G and 293 (Figure 4.7); this could indicate either the presence of a mutant p53 protein detectable by both of the conformation specific antibodies or the presence of two different alleles for p53. T98G has been reported to contain a mutation of Ile^{237} in p53 [Ullrich *et al.*, 1993] and in an earlier report the endogenous p53 was shown to be functionally defective in inhibiting growth [Mercer *et al.*, 1990a]. Although T98G cells may express both mutant and wt p53 protein, the cells would therefore appear to be functionally mutant for p53 (Table 4.2). 293 cells may contain both wt and mutant p53 protein encoded by two different alleles. Alternatively, binding to E1B encoded by Adenovirus 5 in 293 cells, may alter the conformation

of wild type protein so that it was recognised by both conformation specific antibodies, similar to the effect of mutant p53 binding to wt p53 [Milner & Medcalf, 1991]. The fact that E1B binds and presumably inactivates p53 [Lane & Crawford, 1979] and that 293 cells are reported to contain wild type sequence for p53 [Lehman *et al.*, 1993] would indicate that p53 is normal in these cells but functionally inactivated by binding to E1B. This is indicated for p53 status in Table 4.2.

No p53 was detected in T24 or A1698 cells. No sequence data are available for A1698. T24 cells, however, are reported to contain wild type p53 sequence for exons 5 to 8 [Spruck *et al.*, 1994]. The low level of p53 protein, which is not recognised by the conformation specific antibodies, suggests that these cells are functionally p53 mutant although this would be not predicted from the sequence data for the same reasons discussed for the A2182 p53 results above (Table 4.2). As mentioned in the $p16^{INK4}$ discussion above, preliminary DNA fingerprinting data suggest that the subclone of A1698 used in this study is actually T24. The $p110^{RB}$ and p53 immunoprecipitation data (Figures 4.3 & 4.7) are consistent with this.

As shown in Table 4.2, most of the cell lines within each of the complementation groups contain functionally mutant or inactive p53. Functional inactivation of p53 is either by mutation, which is indicated by mutant sequence or immunoprecipitation of the protein with conformational specific antibodies, or by the presence of viral proteins known to bind p53. The only cell lines which appeared to have functionally wt p53 protein are HT1080 (group

A), J82 (group B) and A549 (group D). Northern (Figure 4.5 Panel B) and Southern (Figure 4.8) analysis of MDM2 in these cell lines provided no evidence for overexpression of MDM2. It is therefore unlikely that p53 function is inhibited by excessive MDM2 in these cells.

p53 and p110^{RB} may be inactivated in all cell lines

It has recently been shown that cells lacking functional p110^{RB} undergo apoptosis in the presence of wild-type p53 [Howes *et al.*, 1994; Pan & Griep, 1994; Morgenbesser *et al.*, 1994; Almason *et al.*, 1994]. If loss of p16^{INK4} expression has the same effect as loss of p110^{RB}, then it could be predicted that those cell lines lacking functional p110^{RB}, but with functional p53, will be found to have other genetic alterations that result in inactivation of p53 function. This would therefore suggest that loss of functional p110^{RB} and p53 (or equivalent genetic events) occur in all immortalised cells regardless of the immortalisation complementation group to which they have been assigned.

The cell lines, HT1080, J82 and A549, appear to have a functionally wild type p53 status with a functionally mutant, or inactive, RB-1 status. This would indicate that p53 is inactivated by mutations of, as yet unidentified, genes. p21^{CIP1} is an obvious candidate but appears not to be targetted for mutation, as indicated by the lack of reports of mutant p21^{CIP1} in tumours and cell lines. Other candidate genes could include p27^{Kip1} and as yet unidentified proteins which bind to p53.

p53 and p110^{RB} are normal in virally transformed cell lines

In the cell lines known to contain DNA tumour virus proteins (SV40 TAg, Human Papillomavirus type 18 E6 and E7, or Adenovirus 5 E1A and E1B), the p53, RB-1 and p16^{INK4} proteins (Tables 4.1 and 4.2) all appeared to be normal. Mutations in these genes presumably confer no selective growth advantage in the presence of viral proteins that bind to p53 and p110^{RB}. This is consistent with results reported for SV40-immortalised cell lines [Kao *et al.*, 1993b; Lehman *et al.*, 1993], and consistent with the actions of the viral proteins being sufficient for the inactivation of both p53 and p110^{RB}.

Inactivation of p53 and p110^{RB} is insufficient for immortalisation

It has been known for several years that inactivation of both p53 and p110^{RB} is insufficient for immortalisation of human cells containing DNA tumour virus proteins. The presence of virally encoded proteins which bind to p53 and p110^{RB} results in an increased proliferative potential but usually not in immortalisation without a period of crisis. The somatic cell hybridisation studies using the same cell lines examined in this study indicate that p110^{RB} and p53 inactivation is also insufficient for the immortalisation of non-virally immortalised cell lines, including tumour derived cell lines.

Identification of additional genes involved in senescence and immortalisation is therefore necessary in order to understand these processes.

Chapter 5

Conclusions

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Chapter 5

Conclusions

It has long been known that inactivation of both $p110^{RB}$ and p53 is important, if not necessary, for SV40-induced immortalisation (Section 1.3.3). This is also consistent with the known biochemical functions of these two tumour suppressor genes. Furthermore, the prevalence of mutations of these genes in tumours, and the fact that many tumourigenic cells are also immortal, indicate the importance of their inactivation in the immortalisation of all human cells. This, and the data presented in Chapters 3 and 4, suggest the general scheme for the immortalisation of normal human diploid cells shown in Figure 5.1, which serves to focus the conclusions of this study. In this scheme, inactivation of $p110^{RB}$ results in an extension of the normal *in vitro* lifespan of the cell population (Section 3.3.2).

Inactivation of $p110^{RB}$ by TAg also leads to the loss of contact inhibition, as indicated by focus formation (Section 3.2.1). Inactivation of $p110^{RB}$, however, would appear to be insufficient for focus formation as the introduction of HPV-16 E7 or Adenovirus-5/12 E1a genes does not result in focus formation [N. Whitaker unpublished data; Gallimore *et al.*, 1986]. Inactivation of p53 does not appear to be necessary for focus formation and lifespan extension but its inactivation potentially further extends the replicative lifespan of the cells (Section 3.5). In addition, and perhaps more importantly, p53 inactivation protects the cells from

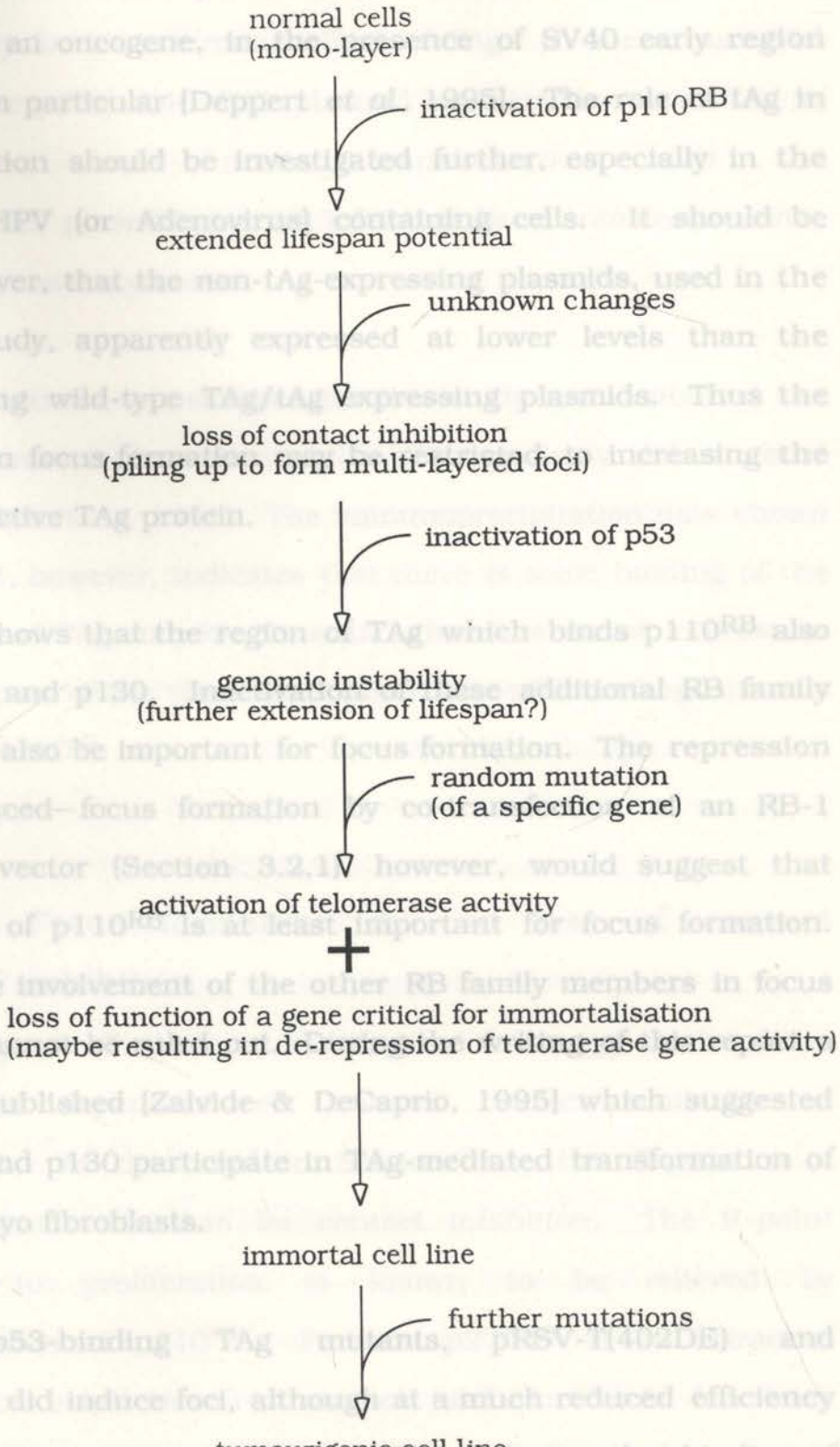
apoptosis and lack of wt p53 permits the accumulation of mutations, resulting in destabilisation of the genome (Section 1.4.1). This increased mutational rate leads to an increased chance of inactivation of other genes necessary for immortalisation. In this scheme, inactivation of p53 is essential for this mutational event which results in the immortalisation of the cells.

The assignment of cell lines to as few as four complementation groups suggests that the inactivation of as few as four critical genes is responsible for immortalisation of human cells. Although maintenance of telomeres would appear to be necessary for immortality, it appears that telomerase activity together with inactivation of p53 and $p110^{RB}$ is not sufficient for immortality. Inactivation of other genes is therefore required for immortalisation.

5.1 Focus formation requires inactivation of p110RB

In contrast to the wild type TAg expression plasmid pRSV-T, which reliably induced focus formation, the non-$p110^{RB}$-binding TAg mutants, (pRSV-T(Δ) and pRSV-T(K1)), never induced foci. As mentioned, HPV-16 E7 genes or Adenovirus-5 encoded E1a genes also do not induce focus formation and this suggests that while inactivation of $p110^{RB}$ is necessary, its inactivation is not sufficient for focus formation. Functions which are unique to TAg may indicate other functions necessary for focus formation.

Figure 5.1 Scheme for immortalisation of normal human diploid cells



Work by D. Zahra [unpublished] in this laboratory, has indicated the presence of tAg is important for this SV40-induced focus formation. It has recently been suggested that p53 is stabilised, and acts as an oncogene, in the presence of SV40 early region genes, tAg in particular [Deppert *et al.*, 1995]. The role of tAg in focus formation should be investigated further, especially in the context of HPV (or Adenovirus) containing cells. It should be noted, however, that the non-tAg-expressing plasmids, used in the D. Zahra study, apparently expressed at lower levels than the corresponding wild-type TAg/tAg expressing plasmids. Thus the role of tAg in focus formation may be restricted to increasing the amount of active TAg protein.

Figure 1.3 shows that the region of TAg which binds $p110^{RB}$ also binds p107 and p130. Inactivation of these additional RB family genes could also be important for focus formation. The repression of TAg-induced focus formation by co-transfection of an RB-1 expression vector (Section 3.2.1), however, would suggest that inactivation of $p110^{RB}$ is at least important for focus formation. The possible involvement of the other RB family members in focus formation cannot be ruled out. During the writing of this report a study was published [Zalvide & DeCaprio, 1995] which suggested that p107 and p130 participate in TAg-mediated transformation of mouse embryo fibroblasts.

The non-p53-binding TAg mutants, pRSV-T(402DE) and bssv402DE, did induce foci, although at a much reduced efficiency than the wt TAg expression plasmid. This indicates that binding of p53 is unnecessary for focus formation. There is further evidence

that binding to p53 is unnecessary for focus formation. Although in a different species, rat embryo fibroblast (REF) transformation studies [Hansen *et al.*, submitted] indicate that binding to p53 is not necessary for focus forming effect of TAg. In these immortal REF cells, the pRSV-T(402DE) plasmid was as active as wt TAg in transformation and further, transformation with the pRSV-T(402DE) plasmid was not inhibited by co-transfection with a wt p53 expression plasmid.

The 402DE mutant was selected for this study as it contained the most conservative substitution which still reportedly did not bind p53 [Lin & Simmons, 1991]. The immunoprecipitation data shown in Figure 3.1, however, indicates that there is some binding of the 402DE mutant TAg to p53. It would therefore be of interest to repeat this part of the study with other non-p53-binding mutants of TAg, e.g. 402DN.

5.1.1 Focus formation due to loss of contact inhibition

The piling up of cells to form multilayered foci suggests that focus formation is dependent on loss of contact inhibition of proliferation. As discussed in Section 3.5, the R-point is a candidate as a mechanism for contact inhibition. The R-point restriction to proliferation is known to be relieved by phosphorylation of $p110^{RB}$. Further, $p27^{Kip1}$ is an attractive candidate for involvement in contact inhibition as it has been shown to be induced by cell-cell contact and its expression is associated with quiescence [Firpo *et al.*, 1994; Kato *et al.*, 1994;

Polyak *et al.*, 1994; Slingerland *et al.*, 1994]. Expression of kinase inhibitors could be responsible for inhibiting phosphorylation of $p110^{RB}$ and subsequent R-point release. If inhibition of $p110^{RB}$-phosphorylation is involved in contact inhibition, then it could be predicted that $p110^{RB}$ would be predominantly hypophosphorylated in confluent normal cell strains and predominantly hyperphosphorylated in confluent cell populations which will go on to form foci.

As $p27^{Kip1}$ is a non-specific inhibitor of kinases, its induction would also presumably result in the inhibition of other kinases such as cycA/cdk2 and cycE/cdk2 kinase at the G1/S boundary. Perhaps this pathway provides a clue to the other activities of SV40-early region genes necessary for focus formation, functions which appear to be missing in the E1a/E1b combination [Gallimore *et al.*, 1986]. This differential focus forming ability provides an excellent opportunity to elucidate other functions necessary for focus formation. Co-transfection of E1a/E1b with deletion mutants of the SV40 early region may localise regions that, in addition to the $p110^{RB}$-binding region, are necessary for focus formation.

5.2 Inactivation of $p110^{RB}$ induces an extension of normal *in vitro* lifespan

As in the case of the focus formation data, the non-$p110^{RB}$-binding mutant TAg expression plasmids failed to induce an extension of lifespan (Section 3.3.2). This suggests that $p110^{RB}$ has a role in determining the lifespan of cells, perhaps via its role in the R-point of the cell cycle. The extension of lifespan was inseparable

from focus formation in the experiments presented here, perhaps suggesting that inactivation of $p110^{RB}$ is necessary for both.

Interestingly, binding to p53 appeared to be not required for extension of lifespan, as indicated by the extended lifespan of NHDF cells expressing the non-p53-binding mutant TAg from the bssv402DE and pRSV-T(402DE) plasmids. The small number of clones examined in this study had a lifespan comparable to that of the wt TAg expressing clones. This is in contrast to the report by Hara *et al.* [1991] in which inactivation of p53, by the introduction of p53 anti-sense oligonucleotides, had a synergistic effect on the $p110^{RB}$ antisense oligonucleotide-induced extension of lifespan. These results reported by Hara *et al.* [1991] would predict that inactivation of p53 would further increase the extension of lifespan, i.e., greater extension of *in vitro* lifespan induced by wt TAg than by the non-p53-binding mutant TAg. It would be important to address this question by analysing the lifespan of more clones from pRSV-T(402DE)- and bssv402DE-induced foci. Such clones are available for analysis.

Co-transfection of the RB-1 expression plasmid, pLRbRNL, with TAg appeared to ablate the TAg-induced extension of lifespan (Section 3.3.2). This supports the idea that inactivation of $p110^{RB}$ is necessary for extension of lifespan. The mutant $p110^{RB}$ expression plasmid, pLRb(Δ768-909)RNL, also appeared to have wild type activity in suppression of TAg-induced focus formation and extension of lifespan. Although a region shown to be required for E2F binding had been deleted in this mutant $p110^{RB}$ plasmid, it is possible that other regions/functions of $p110^{RB}$ are

responsible for this activity. The region of $p110^{RB}$ required for inhibiting extension of lifespan has not been mapped. An extension of this type of study, using other mutants of $p110^{RB}$, would be useful for mapping this activity of $p110^{RB}$.

5.3 Role for p53-binding

It is interesting to speculate on the role of p53-inactivation in this SV40-induced immortalisation model. The focus formation and lifespan extension data suggest that binding to p53 is dispensable, at least for the early effects of TAg. This is in agreement with a study reported by Bond *et al.* [1994], in which inactivation of p53, by transfection of young NHDF with a dominant mutant of p53, had no effect on lifespan.

Loss of expression of TAg from the pRSV-T(402DE) and pbssv402DE plasmids in growing cells (Section 3.3.3), indicates either, selection against expression of non-p53-binding TAg in the proliferating cells or, selection for the cells which no longer express this mutant TAg. This is in contrast to wild type TAg, whose expression has never been reported to be lost when introduced into normal human cells, indicating that there is no apparent selection against its expression. This indirectly suggests that there is a role for p53-binding in TAg-induced immortalisation. The study reported by Hara *et al.* [1991], (as discussed above) indicates that inactivation of p53 has a role in cooperating with inactivation of $p110^{RB}$ to further induce proliferation of normal cells. Also, while mutant p53 had no effect on the proliferative potential of young fibroblasts, transfection of a

dominant mutant p53 into near senescent NHDF induced an extension of lifespan in these cells [Bond *et al.*, 1994]. These two reports [Hara *et al.*, 1991; Bond *et al.*, 1994] indicate a role for inactivation of p53 in extension of lifespan, and that this is dependent on other factors, such as inactivation of $p110^{RB}$ and/or the proliferative potential of the cells. The mechanism for this, however, remains unclear.

Another obvious role for p53 inactivation is in maintenance of genomic stability. It has been reported that the N-terminal 147 AA of TAg is sufficient for destabilisation of the genome [Woods *et al.*, 1994]. Although this region does not bind p53, lack of wt p53 permits the accumulation of mutations, resulting in destabilisation of the genome (Section 1.4.1). Thus, inactivation of p53, by binding to TAg, could also be expected to destabilise the genome, leading to loss and mutation of genes. Perhaps this is sufficient for the additional extension of lifespan as discussed above. An increased mutational rate could also be expected to increase the chance of escape from crisis in cells expressing TAg. In this context, cells expressing the non-p53-binding mutant TAg plasmids may be incapable of immortalisation. Immortalisation of cells expressing either pRSV-T(402DE) or bssv402DE, however, was not examined in this study. Examination of mutation and immortalisation rates of cells transformed with pRSV-T(402DE) or bssv402DE, compared to cells transformed with pRSV-T, may address this.

5.4 $p110^{RB}$ and p53 are disrupted in all immortal cell lines

The transforming proteins of a number of DNA tumour viruses that have been shown to immortalise human cells, bind both $p110^{RB}$ and p53. This binding implicated inactivation of these two cellular proteins as being important for virally-induced immortalisation of human cells. Cell lines from each of the four complementation groups for immortalisation, which included non virally-immortalised cell lines, were examined to see if this observation could be extended to include other cell lines. Examination of the $p110^{RB}$ and p53 status in cell lines from each of the four complementation groups indicates that inactivation of these two genes, or their protein products, is common in cell lines. The data summarised in Table 4.1, suggest that $p110^{RB}$ is inactivated in all immortal cell lines. Inactivation of $p110^{RB}$ occurs either by mutation of RB-1, presence of viral binding proteins or loss of $p16^{INK4}$.

It appears that p53 is also functionally inactive in nearly all cell lines (Table 4.2). For some of these cell lines the protein data and published sequence data are in apparent conflict, but as discussed in Section 4.5, these discrepancies can often be accounted for. From Table 4.2, it would appear that p53 is functionally inactive, by mutation or presence of transforming proteins, in all but two cell lines examined. The exceptions, HT1080 and A549, would provide an excellent opportunity to find whether there are alternative mechanisms for inactivating p53. MDM2 overexpression appeared not to be an alternative mechanism for inactivation of p53 in the cell lines examined in this study.

5.4.1 Significance of p110^{RB} inactivation

It is interesting to speculate on the significance of the different mechanisms for the inactivation of p110^{RB}; either by mutation, binding to viral proteins or loss of p16^{INK4}. It is possible that phosphorylated p110^{RB} has a function in S- or M-phase, as suggested by its continued presence through these phases of the cell cycle. Perhaps this function of p110^{RB} is maintained in cells in which p110^{RB} is inactivated by mechanisms other than mutation of RB-1, and replaced by further changes in cells containing mutant p110^{RB}. This potential role for phosphorylated p110RB could be examined in immortal cell lines which contain wt, non-mutated, RB-1.

Mutation of RB-1 in the presence of wt p53 is known to result in apoptosis. It would be important to establish if this is also the case when p110^{RB} is inactivated by loss of p16^{INK4} or overexpression of cdk4. Although alteration of these genes has been suggested as an alternative mechanism in immortalisation, it has not been established whether this extends to other parameters such as apoptosis, cell cycle rate, contact inhibition and differentiation. Perhaps alteration of these genes is restricted to subsets of tumours, due to the different types of mutation-inducing agents the cells are exposed to as well as the constraints on the cells due to histological origin and/or cellular environment. As mentioned in Section 1.4.3, it is thought that the role of p16^{INK4} is to extinguish cdk4 activity to prevent the growth suppressive functions of p110^{RB} being overridden or by-passed. Perhaps the status of cdk4 is important for the decision to undergo apoptosis. This decision may also be dependent on the presence of DNA damage, thus, it

would be interesting to compare $p16^{INK4}$ null cells with mutant RB-1 containing cells, with and without DNA damage.

5.5 Role for telomerase

Evidence is accumulating that telomerase activity is a common feature of the immortal phenotype (Morin, 1989; Counter *et al.*, 1992; 1994; Klingelhutz *et al.*, 1994; Kim *et al.*, 1994). Work in this laboratory by T. Bryan [Whitaker *et al.*, 1995] has shown that, while most cell lines in each complementation group contained telomerase activity, 3/15 immortalised cell lines were telomerase negative. Two other examples of telomerase negative cell lines have been found by others (Kim *et al.*, 1994) and the significance of this is unknown at present. Preliminary analyses indicate that these three cell lines (and another twelve subsequently identified) have an alternative mechanism for prevention of excessive telomere shortening (Bryan *et al.*, 1995). The data therefore suggest that loss of functional $p110^{RB}$, and probably p53 (or equivalent genetic events), together with activation of telomere maintenance processes occur in all, or most, immortalised cells regardless of the immortalisation complementation group to which they have been assigned. This indicates that other genes must be involved in immortalisation.

As discussed in Section 1.3.2, it has been known for several years that loss of functional p53 or $p110^{RB}$ is insufficient for immortalisation of human cells. The combination of inactivation of these proteins with telomerase activity is also insufficient for immortalisation, since the fusion of two telomerase-positive virally

immortalised cell lines, BET-1A (which expresses SV40 TAg) and HeLa (which expresses HPV-18 E6 and E7), yields hybrids with finite proliferative capacity (Whitaker *et al.*, 1992). The combination of p53 and $p16^{INK4}$ mutations together with the presence of telomerase activity is also insufficient for immortalisation. This is illustrated by the fact that the cell lines T98G and 143BTK- both contain telomerase activity [Whitaker *et al.*, 1995] as well as mutant p53, and have no detectable $p16^{INK4}$ expression (Table 4.1) yet are in different complementation groups (Pereira-Smith & Smith, 1988). The identification of additional genes involved in senescence and immortalisation is therefore necessary in order to understand these processes.

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