A Yeast-Based Assay for Detection of Mutations in the Human p16 Gene.

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Hereby I declare that the script submitted towards a M.Med.Sc degree at the University of the Orange Free State is my original and independent work and has never been submitted to any other university or faculty for degree purposes.

All the sources I have made use of or quoted have been acknowledged by complete references.

C. Botha
May 1999

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Above all, I thank Him who gave me strength to start and finish this.

List of Abbreviations

ARF:	alternative reading frame
ATP:	adenosine triphosphate
CAK:	CDK-activating kinase
CKI:	cyclin-dependent kinase inhibitor
CDK:	cyclin-dependent kinase
CDK4I:	cyclin-dependent kinase 4 inhibitor
DEPC:	diethyl pyrocarbonate
DNA:	deoxyribonucleic acid
dNTP:	nucleic triphosphate
DTT:	dithiothreitol
E. coli:	*Escherichia coli*
EDTA:	ethylenedinitrilo tetraacetic acid
EtOH:	ethanol
G:	gravity
Gal:	galactose
Glc:	glucose
GM-CSF:	human granulocyte-macrophage colony-stimulating factor
GMRα:	alpha-subunit of the human GM-CSF receptor
HIS:	histidine gene
kb:	kilobase
kDa:	kilodalton
LiAc:	lithium acetate
M:	molar
$MgCl_2$:	magnesium chloride
µl:	microlitre
ml:	mililitre
mM:	milimolar
Mr:	relative molecular weight
mRNA:	messenger RNA

MTS:	multi-tumour suppressor
NaAc:	sodium acetate
NaCl:	sodium chloride
Ng:	nanogram
PCR:	polymerase chain reaction
PEG:	polyethylen glycol
Pg:	picogram
PI:	propidium iodide
pYes:	Yeast expression plasmid
Rb:	Retinoblastoma gene
RNA:	ribonucleic acid
Rpm:	revolutions per minute
RT-PCR:	reverse transcript PCR
S. cerevisiae:	*Saccharomyces cerevisiae*
SDS:	sodium dodecyl sulfate
SS-DNA:	single stranded DNA
TGF:	transforming growth factor
YNB:	yeast nitrogen base

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Chapter 1

The Life and Death of Cells

1. Introduction

In order for an organism to grow and develop, the cells of the body have to reproduce. This is done by duplicating their contents and then dividing this content to form two new cells. The cell cycle is the means by which this is accomplished. Cells undergo the cell cycle in order for a new individual to develop, to replace cells that were lost by wear and tear or to replace cells that underwent programmed cell death or apoptosis. From this it can be seen that if cell division were to be halted for whatever reason, the stricken individual would face imminent death (Alberts *et al.*, 1994).

The finer detail of the cell cycle may vary among different organisms, but the basic steps are universal. Step one requires replication of the DNA, followed by the segregation of the DNA into two separate cells (Alberts *et al.*, 1994).

A cell cycle control system has been discovered that controls the cycle as a whole. This system is evolutionarily well-preserved, so well in fact, that some components will function even when transferred from mammalian cells to yeast cells (Alberts *et al.*, 1994). This enables us to study the cell cycle control system for a variety of cells.

1.1 The cell cycle: a basic strategy

The cell cycle consists of two interacting components (Sorrentino, 1996). Firstly, the mechanical component, which refers to DNA replication, mitosis and cytokinesis, and secondly, the regulatory component which include events in G_1 that control entrance into the S phase and those in G_2 that regulate entry into mitosis. The accuracy of the cell cycle events depends primarily on accurate replication of the chromosomes as well as their segregation. This is achieved by the strict controlling of preceding steps, ensuring that they are completed before the next step begins. In other words, cells have to complete DNA replication before they enter mitosis and must accurately align all chromosomes on the mitotic spindle before segregating them (Sorrentino, 1996). If these mechanisms become damaged, resulting in failure to accurately control the

cell cycle, this could cause alterations and mutations that may cause cell death or cancer (Sorrentino, 1996).

1.2 Controlling the cell cycle

The two interacting components of the cell cycle can be described as one in which the decision to proliferate is made and the other in which this decision is executed. When the decision is made, information concerning the extracellular environment and intracellular state of a cell is integrated through a number of regulatory pathways which can cause a cell either to cease growth or to enter the division cycle (Carnero & Hannon, 1998). As soon as the cell cycle has started, extracellular signals have no more effect on the cell, although intracellular checkpoints can still stop cell division (Carnero & Hannon, 1998).

The key to controlling the cell cycle lies with two families of proteins. The first family consists of the cyclin-dependent kinases or CDKs. These proteins are responsible for inducing downstream processes by phosphorylating selected proteins on serine and threonine residues.

The second family, the cyclins, named for their cyclic synthesis and degradation in each division of the cell cycle, bind to the CDKs and control their ability to phosphorylate appropriate target proteins.

Due to the assembly, activation and disassembly of cyclin-CDK complexes, the cell cycle can move from one phase to the next. This is controlled by two classes of cyclin/CDK enzymes namely cyclin D/CDK4(6) and cyclin E/CDK 2. The cyclin D/CDK4 enzyme acts first and it is thought that this enzyme is the key downstream recipient of positive and negative extracellular signals (Sherr, 1993), but recent discoveries proved that cyclin E/CDK 2 also plays a role in reception of signals such as those which ensure appropriate cell-matrix contacts (Fang, *et al.*, 1996; Zhu *et al.*, 1996).

1.3 The role of inhibitors

The activity of the cyclin-CDK complexes is strictly controlled by a number of regulatory mechanisms. The first level of control of the kinase activity is the

availability of the cyclin subunit. CDK4 and CDK6 associate exclusively with D-type cyclins (D1, D2 and D3). Expression of the D-cyclins is controlled by extracellular signals (Sherr, 1993). Once cyclin D and CDK4 subunits are available, their association appears to require an assembly factor whose activity may also respond to extracellular growth stimuli (Matsushime *et al.*, 1994). When these complexes have been assembled, they are subject to both activation phosphorylation by CDK-activating kinase (CAK) and possible inhibitory phosphorylation of a tyrosine residue in the ATP binding site (Draetta, 1990). It appears as if CAK phosphorylation is constitutive, whereas inhibitory phosphorylation may constitute a regulatory mechanism (King *et al.*, 1994).

The first CDK-inhibitors were identified in yeast, where they function not only to mediate cell cycle arrest in response to antimitogenic factors, but also to ensure that certain cell cycle events do not initiate before others are completed (Nigg, 1995).

These CDK-inhibitors also play a major role in cell cycle events of vertebrates (Nigg, 1995). Up to now, two classes of CDK-inhibitors have been identified. The first class can be defined by p21. P21 was identified as a CDK-binding protein, a protein that is up-regulated in senescent cells, as well as a gene product that can be induced by the tumor-suppressor p53 (Hunter & Pines, 1994; Peter & Herskowitz, 1994; Elledge & Harper, 1994). An interesting matter however is the fact that p21 can also be found in active CDK/cyclin complexes (Zhang *et al.*, 1994). Thus, maybe p21 plays a dual part and so might function as an assembly factor, or an inhibitor, depending on the stoichiometry of the CDK/cyclin/p21 complex (Zhang *et al.*, 1994).

The closely related proteins p15 and p16 define the second class. Their structure and function (Serrano *et al.*, 1993; Hannon & Beach, 1994) can differentiate the two classes from each other. P15 and p16 exclusively target CDK4 and CDK6 and by this prevent their binding to cyclins (Serrano *et al.*, 1993; Hannon & Beach, 1994). The two genes are located in a region found to be frequently mutated in a large number of cancers, namely on chromosome 9p21 (Kamb *et al.*, 1994).

1.4 The interaction between p16 and CDK4

The p16 gene, also known as INK4a, MTS1 (multi tumor suppressor 1), CDK4I (cyclin-dependent kinase 4 inhibitor) and CDKN2, is known as a tumour suppressor

gene. Named for the protein's apparent molecular weight, the acronym p16 is the most widely used and refers to the gene product (protein). This nomenclature will also be used throughout this study. When referring to the gene itself, it will be written as "p16 gene" to distinguish it from the protein.

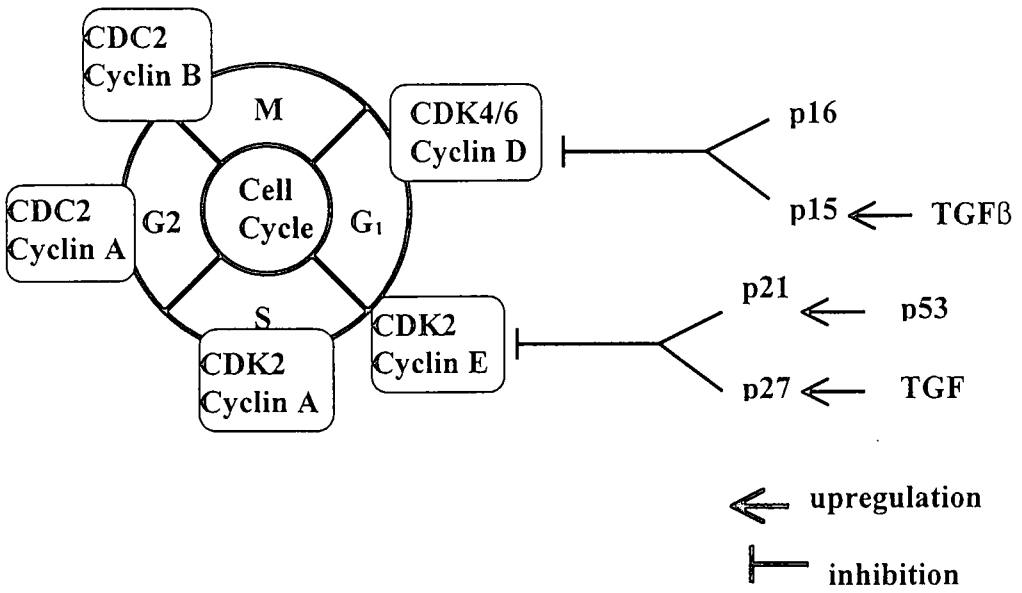


Figure 1.1. The mammalian cell cycle, its cyclins, CDKs and CDK inhibitors.

The p16 protein was first noticed in a study to identify differences in the cell cycle regulators of normal and transformed cells (Fig. 1.1). CDK4 was not found in association with cyclin D, as expected, but rather bound to a protein with a Mr of 16 kDa. The p16 gene was cloned by using CDK4 as the interacting target in the yeast two-hybrid approach (Carnero & Hannon, 1998). The results suggested that p16 was an inhibitor of CDK4 kinase (Carnero & Hannon, 1998). The p16 protein consists of 167 amino acids in mouse and 156 amino acids in humans (Quelle *et al.*, 1995a). The sequence consists almost entirely of four ankyrin repeat units, suggesting that the protein might be folded from helix-β-turn-helix motifs (Kalus *et al.*, 1997). P16 binds specifically to CDK4 and CDK6 in a 1:1 association (Serrano *et al.*, 1993; Hannon & Beach, 1994). This binding causes loss of CDK4 kinase activity toward its physiological substrate, Rb. P16 binds CDK4 and CDK6 in the absence of cyclin D, and purified p16 can promote dissociation of the CDK4-cyclin D complex (Hall *et al.*, 1995; Serrano *et al.*, 1993).

1.5 P16, Rb and CDK4

Early on, it has been shown that the p16 gene is overexpressed in tumor cell lines that lack a functional Rb gene (Serrano *et al.*, 1993; Parry *et al.*, 1995; Ueki *et al.*, 1996). A reasonable assumption concerning p16 function is that it is responsible for maintaining Rb in a functional state, since p16 can inhibit only CDK4 and CDK6 and their only known substrate is the Rb protein. So, in cells lacking Rb, p16 has to maintain CDK4 in an inactive state and so occurs in abundance in the cell (Chellappan *et al.*, 1998). This also indicates that p16 -mediated inhibition of cell cycle progression is completely dependent on the presence of a functional Rb protein (Lukas *et al.*, 1995).

Experiments done with human fibroblasts show that, after microinjection of the p16 gene into these cells, those that received the wild type p16 gene and had a functional Rb gene resulted in an enrichment of cells in G_1 phase, whereas cells with no Rb gene showed no effect (Lukas *et al.*, 1995; Tam *et al.*, 1994). Mouse cell lines were used as well and showed p16 inhibiting mouse embryo fibroblasts from dividing, but $Rb^{-/-}$ cell lines showed no G_1 phase arrest. Since p16 mainly contributes to maintaining Rb function, these two genes have never both been found inactivated in the same tumour. This is logical since there is no need to mutate two genes that function in the same pathway to regulate the cell cycle (Chellappan *et al.*, 1998). Mutating the p16 gene will lead to inactivation of Rb by CDK4 / CDK6, which would be equivalent to a mutation of the Rb gene, and a mutated Rb would make the presence of a functional CDK4 unnecessary and so p16 would not even come into the picture.

1.6 P16 and $p19^{ARF}$

The locus of the p16 gene can give rise to two distinct transcripts under two different promoters. These two transcripts have identical 3' sequences but unique 5' ends (Duro *et al.*, 1995; Stone *et al.*, 1995; Quelle *et al.*, 1995b). The two transcripts are named INK4a and $p19^{ARF}$ (alternative reading frame). $P19^{ARF}$ is derived from a distinct first exon (exon 1β) that is 13 - 20 kb centromeric to the first exon of INK4a (exon 1α). Exon 1β is spliced to exon 2, which is shared with the p16 gene, but this occurs in a different reading frame and hence p16 and $p19^{ARF}$ show no similarity in their amino

acid sequence (Fig. 1.3) (Duro *et al.*, 1995; Stone *et al.*, 1995; Quelle *et al.*, 1995b). The same arrangement of the p16 gene and $p19^{ARF}$ is found in the mouse genome.

The $p19^{ARF}$ transcripts' open reading frame is terminated within exon 2, with exon 3 comprising an untranslated 3' exon. The polypeptide encoded by $p19^{ARF}$ has no sequence homology to other known proteins and shows about 50% identity overall between human and mouse, whereas p16 shows about 65% amino acid similarity. Expression of the $p19^{ARF}$ transcript is ubiquitous in postnatal tissues, in contrast to the more restricted expression pattern of the p16 gene and it seems as if its occurrence is elevated in cells that lack functional p53 (Duro *et al.*, 1995; Stone *et al.*, 1995; Quelle *et al.*, 1995b). The $p19^{ARF}$ gene product does not bind to CDKs and it doesn't inhibit the cyclin-CDK activity either, but in some way it has a cell cycle arresting affect (Quelle *et al.*, 1995b).

It seems that the $p19^{ARF}$ gene may play a role in the p53 pathway (Fig 1.2). P53 induces growth arrest and apoptosis. MDM2 acts as its inhibitor by ubiquitinating p53, which leads to p53 degradation, thus allowing cell growth to continue. Apparently $p19^{ARF}$ prevents this interaction and so stabilizes p53. $P19^{ARF}$ is induced by oncogenic

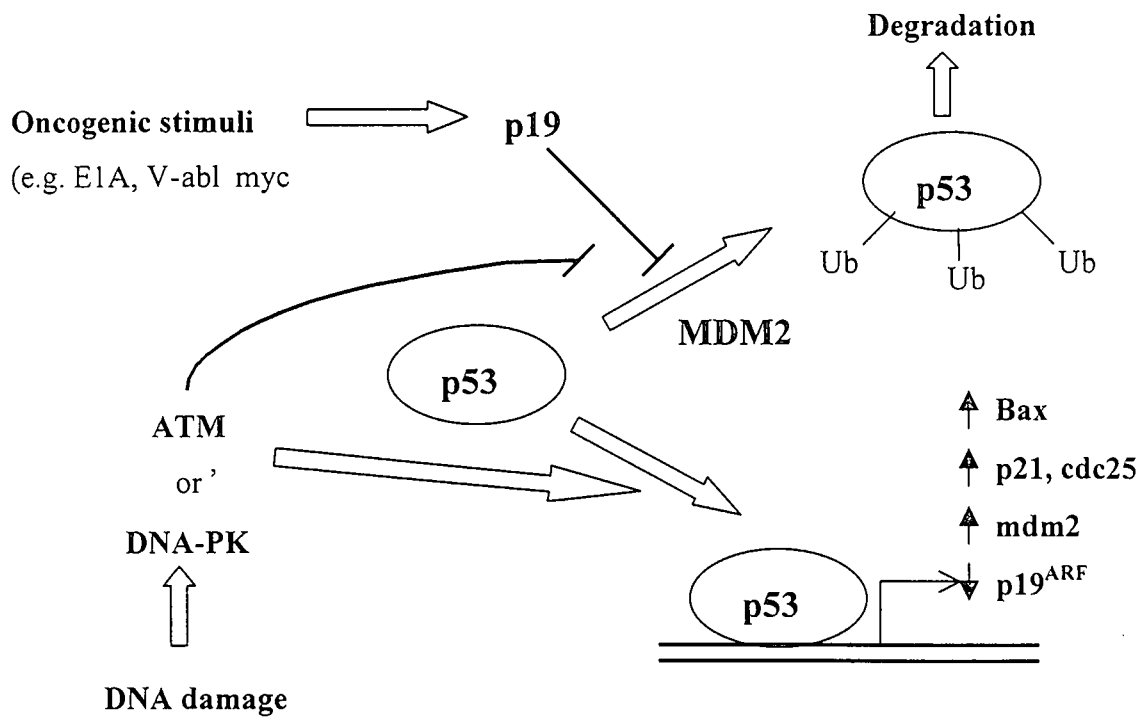


Figure 1.2. The proposed interaction of $p19^{ARF}$ with the p53 pathway. Sharpless & DePinho, 1999)

stimuli such as E1a, v-abl or myc. When p53 is stabilized, it allows induction of genes important for growth arrest (p21, cdc24) and apoptosis (bax). P53 can also be induced by DNA damage, but it seems as if $p19^{ARF}$ does not play a role here (Sharpless & DePinho, 1999).

There have been no reports on mutations in the $p19^{ARF}$ gene in human cancer. The unique exon 1β seems to be mutation free and any mutations in the shared exon 2 disrupt p16 as well. So far no mutations were found that disrupt $p19^{ARF}$ alone and thus it seems as if it plays no role in development of cancer.

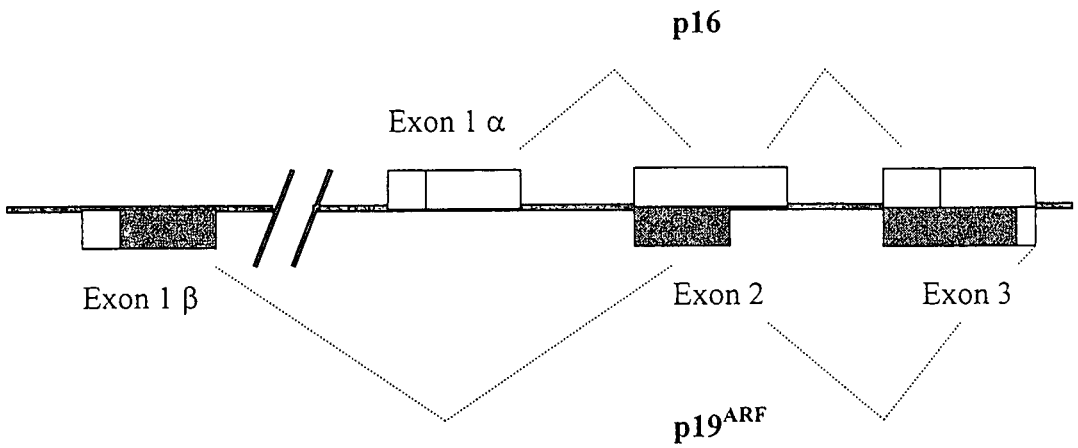


Figure 1. 3. The genomic organization of the human CDKN2A gene (the p16 gene and $p19^{ARF}$).The darker regions indicate the p19 reading frame and the lighter regions the p16 gene reading frame. White regions are untranslated parts of the exons.

1.7 The p15 gene

The $p15^{INK4b}$ gene was discovered during a cytogenetically-based cloning of the p16 gene (Kamb *et al.*, 1994). A genomic segment, closely linked to the coding sequence of the p16 gene exon 2 but with no homology to exon 1 showed no alterations in tumor cell lines. At first, it was thought that the orphan exon 2 homolog was a probable pseudogene (Kamb *et al.*, 1994), but later on it was found that the $p15^{INK4b}$ gene was also encoded by the 9p21 locus (Hannon & Beach, 1994; Jen *et al.*, 1994).

The $p15^{INK4b}$ cDNA was isolated following a series of experiments designed to identify the point at which transforming growth factor (TGF)-β, a growth inhibitory cytokine, impacted the cell cycle regulatory machinery (Hannon & Beach, 1994).

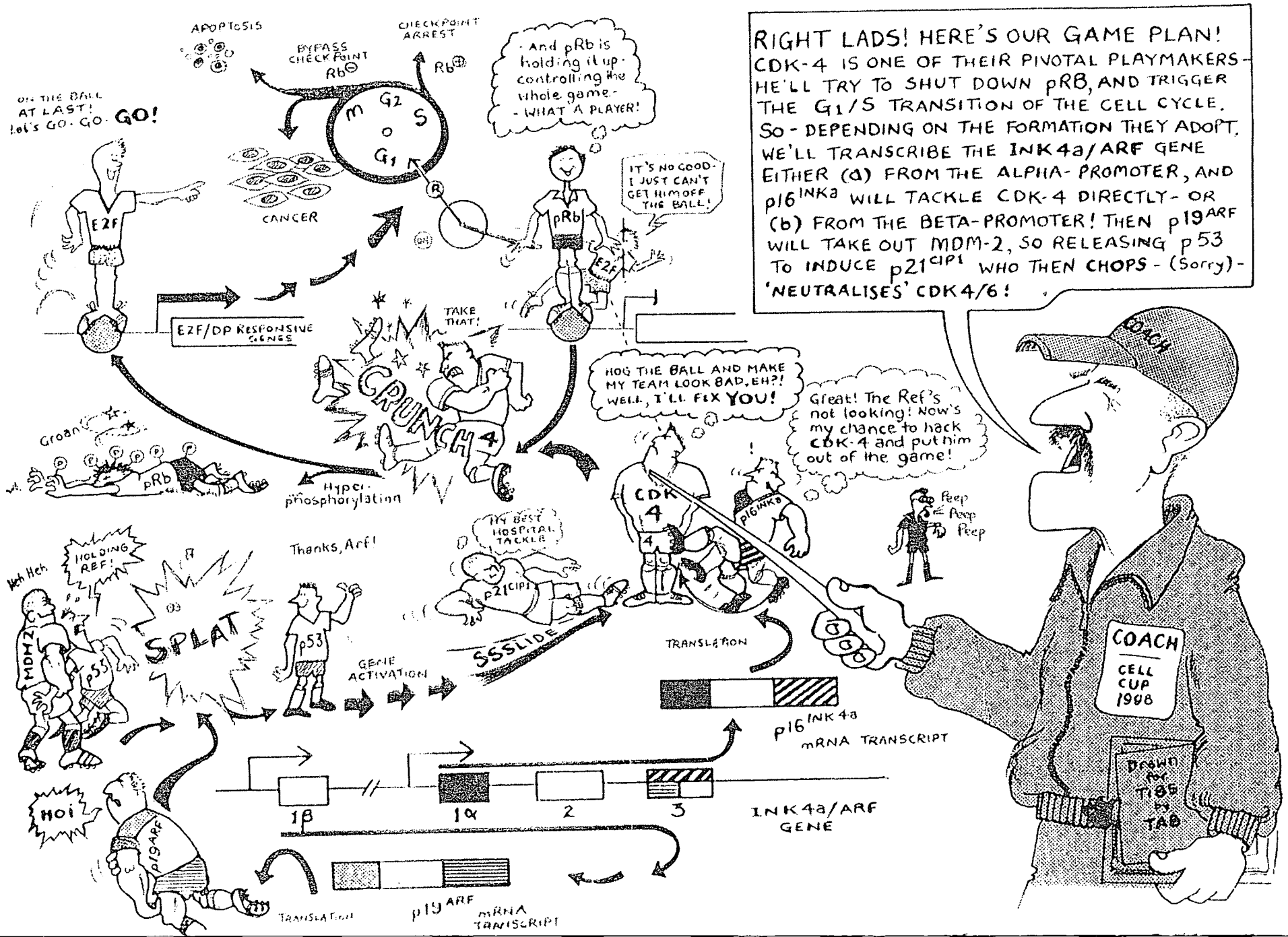


Figure 1.4. The INK4a/ARF tumour suppressor game plan. (Chin *et al.*, 1998)

When a human keratinocyte cell line was treated with TGF-β, the cells arrested in the G_1 phase. Rearrangement of the multi-protein complexes that contain the G_1-regulatory kinases, CDK4 and CDK6 also occurred. Loss of CDK4 and CDK6 activity occurred because of association with a protein about 15kDa in size. This protein was found to be the p15 protein. P15 was recognized weakly by p16 antiserum, prompting the cloning of p15 via a low-stringency hybridization approach.

When the C-terminal segments of p15 and p16 are compared, it is seen that they share a high degree of homology, whereas the N-terminal portions are considerably different. The four-ankyrin repeat structure that is characteristic of the p16 family is maintained. It's impossible to distinguish biochemically between p15 and p16 *in vitro*. Both proteins bind to and inhibit CDK4 and CDK6 with similar affinities. The position of the two genes on chromosome 9p21 is such that it suggests that these two homologs arose from gene duplication (Carnero &Hannon, 1998).

Even though p15 and p16 are indistinguishable on biochemical level, their biological roles are vastly different. When a variety of different cells are treated with TGF-β, p15's abundance is altered (Hannon & Beach, 1994). Induction of p15 occurs at both the transcriptional and post-transcriptional levels, and TGF-β responsiveness has been mapped in the p15 promoter to an SP1 element (Li *et al.*, 1995). TGF-β is a multi-functional cytokine that can act as growth factor, a differentiation factor, or a growth inhibitor (Massague & Polyak, 1995). However, no definitive model for TGF-β's growth arresting mechanism has been provided yet.

While preferential loss of the p15 gene may obviously occur as a bystander-effect of deletion of the p16 gene, it is observed in a limited, but coherent, subset of tumour types (Jen *et al.*, 1994; Zhou & Linder, 1996; Kawamata *et al.*, 1995; Zhang *et al.*, 1996; Zariwala *et al.*, 1996).

1.8 Mutations in the p16 gene and cancer

In most cells of the body, there are two functional copies of each gene, including those of the tumour suppressors. So, to disable their function, both copies of the gene have to be disrupted (Knudson, 1995). These disruptions may occur in the form of point mutations that lead to protein inactivation, frameshift mutations, nondisjunction,

deletions that lead to a loss of protein , hypermethylation (loss or reduction in expression) and chromatin condensation (Drexler, 1998).

A study done by Koh *et al.* in 1995 showed out of nine different p16 alleles derived from tumours, four were totally unable to induce cell cycle arrest, two were similar to the wild type allele and three had intermediate activities. Of this intermediate group, two could inhibit CDK4 effectively and the third could bring about intermediate levels of growth suspension but apparently had no influence on CDK4 activity.

Quite a number of p16 gene mutations occur in different tumours. A few published examples are as follows:

- Head and neck neoplasms: deletions and point mutations (Olshan *et al.*, 1997)
- Murine primary T-cell lymphomas: homozygous deletions and methylation (Malumbres *et al.*, 1997)
- Familial melanoma: point mutations (Ranade *et al.*, 1995)
- Human glioma: point mutations (Arap *et al.*, 1997)
- Oral premalignant lesions: loss of heterozygosity, point mutations (Papadimitrakopoulou *et al.*, 1997)
- Chronic myeloid leukemia: homozygous deletions (Sill *et al.*, 1995)
- Acute lymphoblastic leukemia: homozygous deletions (Fizzotti *et al.*, 1995)
- Adult T-cell leukemia: homozygous deletions (Hatta *et al.*, 1995)
- Acute lymphocytic leukemia: homozygous deletions and loss of heterozygosity (Rasool *et al.*, 1995)
- Non small-cell lung cancers: Missense mutations, methylation and homozygous deletions (Gazzeri *et al.*, 1998)

1.9 The p16 gene and Ras

It has been found that p16 also has an inhibitory effect on Ras-induced proliferation and transformation of REF-52 cells (Serrano *et al.*, 1995). REF-52 cells that were injected with a plasmid encoding activated Ha-ras (V12-Ras) immediately progressed to the S-phase and DNA synthesis occurred, but p16 effectively blocked this reaction. This inhibitory reaction of p16 was tested by inclusion of a catalytically inactive CDK4 (CDK4 - K35M) into the cells. The mutant CDK4 bound to p16 without phosphorylating Rb. As soon as this CDK4 was injected into the cells, p16 -induced repression of cell proliferation decreased significantly, suggesting that p16 functions mainly by targeting cyclin D- CDK4 kinase (Serrano *et al.*, 1995)

1.10 P16 and Development

The effect of the p16 gene on development was determined by using mice with an engineered targeted deletion of the gene (Serrano *et al.*, 1996). Both the p16 and $p19^{ARF}$ products were absent in these mice. The overall impression was that p16 has no major impact on development. The p16 gene-null mice had a slightly lighter coat colour, but it isn't clear whether the p16 gene contributed to this phenotype. The organs of the mice appeared to be normal, although there seemed to be a slight proliferation expansion of the white pulp of the spleen, and there were megakaryocytes and lymphoblasts in the red pulp (Serrano *et al.*, 1996). These features are consistent with abnormal extramedullary hematopoiesis in the spleens of young mice. This appears to be the only abnormality noticed in the development of these p16 gene-null mice and so it is believed that p16 doesn't play a major role in normal eukaryotic development.

2. Cell proliferation and cancer

The growth and development of a multicellular organism depends on numerous signals and pathways. The proliferation, differentiation and survival of cells depend on these functions. If the number of cells in a particular tissue were changed, a pathological event may occur. The reduction of cells may be presented as involution,

atrophy or hypoplasia of the tissue, whereas a cell number increase results in hyperplasia or neoplasia (Pusztai &Cooper, 1996).

2.1 Cellular increases

A neoplasm can be defined as an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues, and which continues in the same excessive manner even after the stimulus which has evoked the change has stopped (Pusztai &Cooper, 1996). Hyperplasia is defined as an increase in the number of differentiated, non-neoplastic cells in a tissue. The normal mechanisms of growth control are still functional, but the balance leans strongly to the stimulatory side (Pusztai &Cooper, 1996).

Sometimes, in clinical medicine, the terms tumour and neoplasia are used interchangeably, even though tumour mainly refers to the cell or tissue mass (Pusztai &Cooper, 1996). Neoplasia, in contrast, concerns more than just cell proliferation. It also refers to disruption of cellular differentiation which may include a disrupted or altered metabolic pathway, and abnormal relationships between the cells and their microenvironment where the cells do not stop growing, even when coming in contact with neighboring cells (Pusztai &Cooper, 1996).

Neoplasms are generally classified as benign, when the tumour is unable to infiltrate the surrounding normal tissue, or malignant, where the tumour is both invasive and metastatic (Pusztai &Cooper, 1996).

2.2 Tumour cell proliferation

The term growth fraction refers to the fact that at any given time only a fraction of the cells of the tumour are actively dividing (Mendelsohn, 1962). When the growth fraction is measured *in vitro* in conjunction with the determination of cell cycle time, it shows a higher tumour growth rate than that observed in animal models as well as in some human tumours *in vivo*. The most probable explanation is cell loss in tumours. From 40% to 80% of cells are lost during proliferation (Steel, 1968). Several mechanisms may be responsible for this loss. These include nutrient deprivation, immunological response against neoplastic cells, lethal errors in

metabolic pathways and DNA replication, programmed cell death (apoptosis) and partial differentiation of tumour cells.

2.2.1 Nutrient deprivation

Up to a certain size (1-2 mm^3), a group of neoplastic cells can survive by receiving nutrients from surrounding tissue by simple diffusion. From there on they have to generate their own capillary growth or die (Folkman, 1989). This is a very complex procedure and it may be a relatively late acquisition in the progression towards metastatic invasive tumours.

2.2.2 Immunological response against neoplastic cells

It has been found that in solid tumours, up to 50% of the cells consist of infiltrating T lymphocytes, natural killer cells and macrophages (Kelly *et al.*, 1988). In this manner, tumour growth could be controlled, but it seems as if advanced tumours do not respond to this phenomenon any more (Lotze & Finn, 1990).

2.2.3 Apoptosis

Cell death can be executed via apoptosis or necrosis. Necrosis is a passive type of cell death caused by cellular damage, whereas apoptosis is a purposeful self-destruction mechanism (Kerr *et al.*,1972). Apoptosis is very important for cellular control (Cohen, 1991) and cells that underwent this type of cell death are very easily recognized and phagocytosed, even more so than necrotic cells (Savill *et al.*,1993).

Apoptotic cells can be recognized by a certain sequence of events they undergo. These are cell shrinkage, condensation, margination and fragmentation of chromatin, and finally retention of cytoplasmic organelle structure, but loss of the positional interrelationships of organelles (Gregory, 1995). Scanning electron microscope analysis showed apoptotic cells to have a cratered surface structure. This might be caused by fusion of endoplasmic reticulum-derived vesicles with the plasma membrane. The last steps of apoptosis are overall changes in cellular morphology that leads to the formation and budding-off of so-called "apoptotic bodies" (Wyllie *et al.*, 1980; Kerr & Harmon, 1991; Wyllie, 1992; Dive & Wyllie, 1993). The apoptotic bodies are membrane bound, sometimes contain chromatin and appear to be formed by a microfilament-dependent mechanism (Cotter *et al.*, 1992). They are very susceptible to phagocytosis and might even be engulfed by cells such as fibroblasts.

This "body"-formation is not always necessary. Sometimes the whole apoptotic cell is ingested by macrophages (Savill *et al.*, 1993).

Apoptosis can be initiated by several cellular and environmental factors. There seems to be a particular stage of cell differentiation that is pre-set for initiation, but environmental factors, physiological or non-physiological, for example, a cell surface ligand, ionizing radiation or drugs, also play a role.

The size of a cell population has to maintain a balance between production and loss. If this balance were to be disturbed, a neoplastic phenotype could develop. The balance could be upset by the promotion of cell proliferation or by the inhibition of cell death (Gregory, 1995). Cells could become immortal by the suppression of genes which cause apoptosis, directly or indirectly, or by activation of genes which promote survival by growth inhibition or apoptosis (Gregory, 1995). So, apoptosis may contribute to oncogenesis through multiple mechanisms.

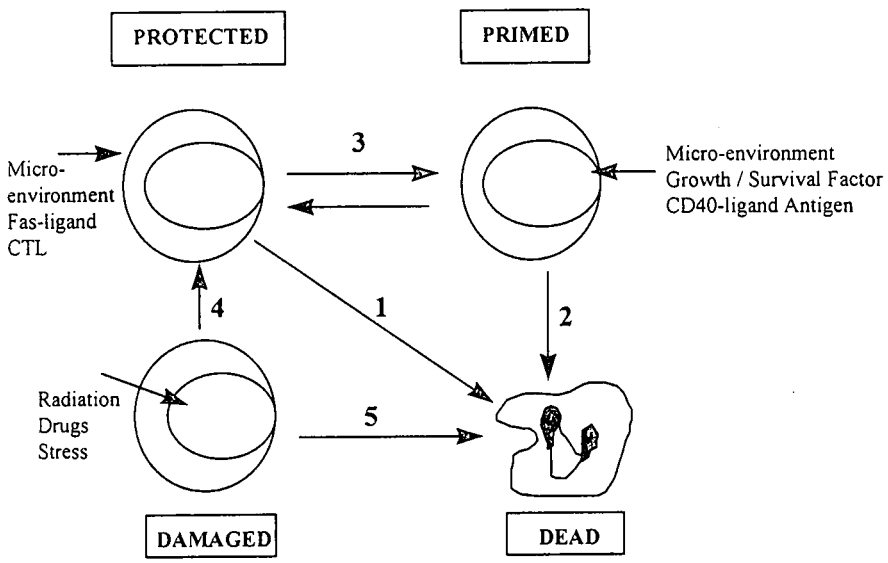


Figure 1.5. Regulation of apoptosis.

Figure 1.5 is a simplified version of the regulation of apoptosis and terms like protected or primed cells are used more for explanation than as a scientific term. The protected cells require a positive signal to trigger apoptosis (pathway 1), whereas primed cells are constitutively activated to undergo apoptosis if survival signals are absent (pathway 2). Exchange between protected and primed cells (pathway 3) may

occur as a differentiation step and/or as a result of microenvironmental influences, but it should be noted that these cells are not derived from one another. Cells which have undergone, for example, DNA damage as a result of environmental factors or drugs may choose either to repair the damage (pathway 4) or to enter apoptosis (pathway 5) (Gregory, 1995).

2.3 Multistage tumour progression

Tumour progression can be divided into three phases, namely initiation, promotion and progression (Fig. 1.6) (Pusztai &Cooper, 1996).

2.3.1 Initiation

The first step involves irreversible changes in the genome, which in turn makes it susceptible to malignant transformation. Factors such as chemical substances, physical and biological agents may initiate the process. Cells are especially susceptible during DNA synthesis, but cell division also provides an ideal opportunity for initiation.

2.3.2 Promotion

Initiated cells move on to this second step because of environmental effects until a premalignant phenotype is manifested. These cells then have an increased risk to become cancerous. Various chemicals can induce promotion by being mitogenic or by preventing programmed cell death.

2.3.3 Progression

This last step involves malignant phenotypic changes including invasiveness, metastatic competence, tendency for autonomous growth and often-increased karyotypic instability. Factors that advance progression are not well defined and may not be that different from those involved in initiation and progression.

Advances in molecular biology and genetics have highlighted the fact that a multitude of changes in DNA including gross chromosomal abnormalities and even methylation, occur in tumours. Also, the understanding of how mutations in the structure and expression of growth factors, growth factor receptors, transcription factors, cell adhesion molecules, oncogenes and tumour-suppressor genes contribute to the development of the malignant phenotype has advanced at an unprecedented rate.

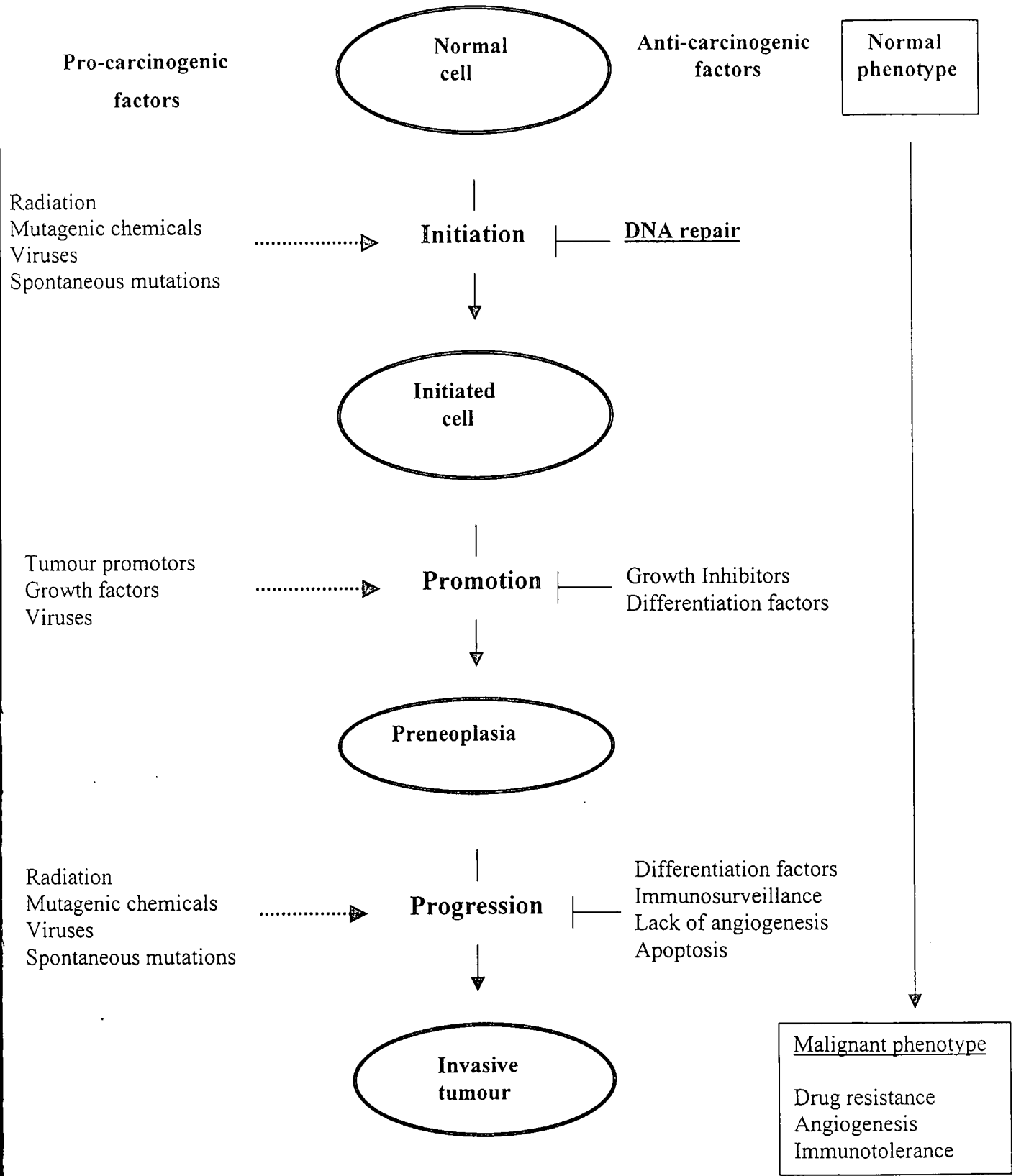


Figure 1.6. Schematic representation of multistage tumour progression.

However, only a few of the numerous changes observed in an individual tumour are thought to be rate-limiting for cancer progression (Armitage & Doll, 1954).

3. Yeast

Yeast is one of the most popular models used in the study of the cell cycle. Yeasts are unicellular fungi and are ideal for molecular studies of eukaryotic cell biology because their reproduction rates are almost as rapid as that of bacteria, they have a genome size less than 1/100 of that of mammals and they are especially useful in studies involving the cell cycle, since theirs is very similar to that of mammals.

The two most popular species of yeast are *Saccharomyces cerevisiae,* commonly used in baking and brewing and *Schizosaccharomyces pombe*, used in Japan for making the alcoholic drink Sake. *S. cerevisiae* is a budding yeast and *S. pombe* a fission yeast.

3.1 Yeast life cycles

Both the budding and the fission yeast can proliferate in either a diploid or haploid state. The state in which a portion of the life cycle is spent, be it haploid or diploid, depends on the species as well as the environment. Budding yeast, in times of plenty, will prefer to proliferate as diploid cells, with a cell cycle time of about two hours. However, when nutrients are in short supply, they go through meiosis to form haploid spores, which germinate when conditions improve to become haploid cells that can choose to proliferate, or conjugate in G_1 phase to re-form diploid cells.

In contrast, the fission yeast normally proliferates as haploid cells, and during starvation, fuse together to form diploid cells that go through meiosis and sporulation to regenerate haploid cells.

The most widely used laboratory strains of budding yeasts are mutants that can proliferate stably as haploids.

3.2 The yeast cell cycle

S. cerevisiae cells undergo biochemical as well as morphological changes while progressing through the cell cycle. These changes can be seen in bud formation.

When the bud is forming, replication of the nuclear DNA begins, the nucleus then extends to the neck of the bud, divides and the bud splits from the mother cell (Pringle & Hartwell, 1981). *S. pombe* cells, in contrast, increase in length until the cell cycle enters into mitosis, when a septum forms and two daughter cells emerge (Fantes, 1977).

Cdc (cell division cycle) conditional mutants of different yeast lines were used to study the cell cycle and so led to a better understanding of mammalian cell proliferation (Fantes, 1989).

S. cerevisiae grows by forming buds. This mode of growth makes it difficult to make a clear distinction between the different phases of the cell cycle, since the segregation of cell constituents into the bud sometimes occurs at the same time as their duplication. It is, however, possible to identify the G_1, S, G_2 and M phases of *S. cerevisiae* by studying the discrete intervals occupied by DNA replication and segregation and their associated events (Lew *et al.*, 1997).

The regulatory transition that occurs in late G_1 phase was aptly named "Start" (Pringle & Hartwell, 1981). Just before this point in the cell cycle is reached, *S. cerevisiae* cells have the option to develop in a number of ways. If the cells are in a well-fed environment, they can proceed with the cell cycle, however, if this is not the case, cells can enter a quiescent state or begin sporulation. Cells that have been exposed to mating pheromones may arrest in G_1 and initiate the mating program. As soon as the cells pass the Start point, they are set on their course to complete the cell cycle and usually not even nutrient deprivation or pheromone exposure can stop them. Thus, Start can be seen as the decision point in the cell cycle (Pringle & Hartwell, 1981).

Once the cells pass Start, a number of cell cycle events are triggered. These include initiation of DNA replication, bud emergence and duplication of the microtubule-organizing center. So, it is quite easy to identify the G_1 / S phase transition point in *S. cerevisiae*.

3.3 Cyclin-dependent kinase inhibitors of yeast

The first cyclin-dependent kinase inhibitors (CKI) were discovered in yeast (Mendenhall, 1998). The four most prominent yeast CKIs are Pho81, Sic1, Far1 and Rum1 (Table 1.1).

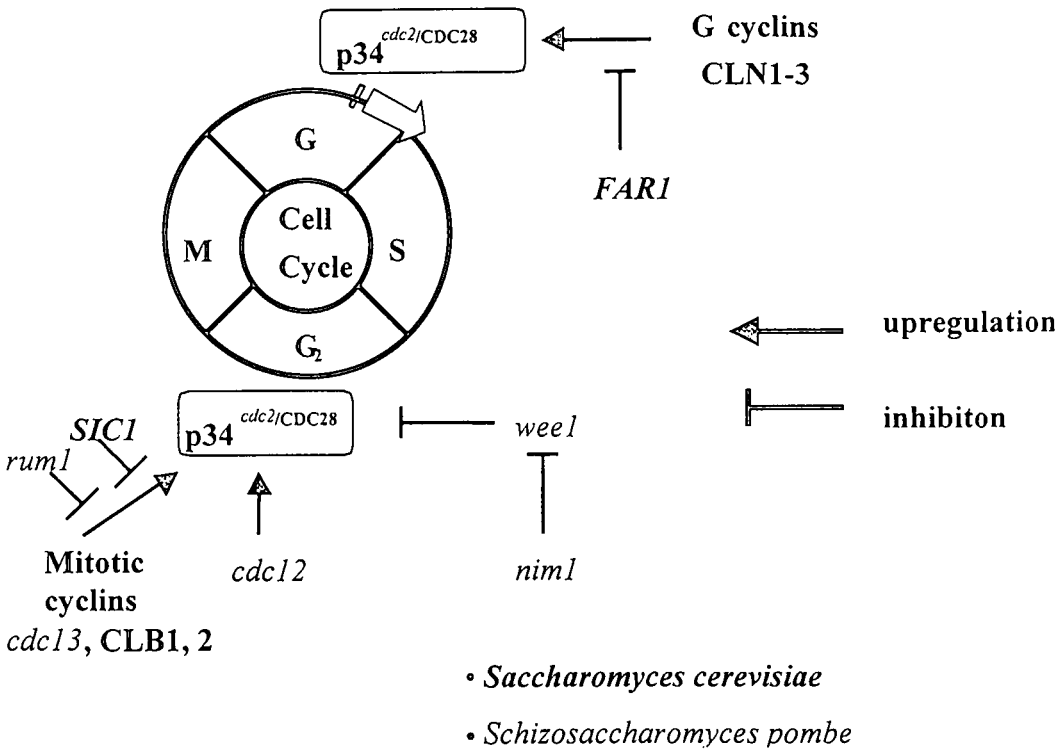


Figure 1.7. The yeast cell cycle, showing the interactions between p34, the different cyclin families and the CDK inhibitors.

Table1.1. The yeast CKIs and the cyclin-dependent kinase/cyclin pairs they inhibit.

CKI	CDK	Cyclin
Pho81	Pho85	Pho80
Far1	Cdc28	Cln1, Cln2
Sic1	Cdc28	Clb2, Clb5
Rum1	Cdc2	Cdc12, Cig2

Even though all four of these proteins have similar biological functions, each has a unique way to accomplish its task (Fig 1.7). Pho81 and Far1 have both a CKI and non-CKI function. Pho81 reacts to nutritional limitations to regulate gene transcription and Far1 links pheromone detection to cell cycle arrest (Mendenhall, 1998). This constitutes CKI function. In addition, Pho81 works directly with a transcriptional activator and a cyclin (without a CDK) to control gene transcription.

The other role that Far1 plays is to determine the cell surface site at which polarized growth will occur (Mendenhall, 1998).

Sic1 and Rum1 are involved in cell cycle regulation, especially the proper coordination of critical mitotic events. Both proteins are expressed in the G_1 phase of the cell cycle, but these proteins interact with various CDK-cyclin partners that affect events in all parts of the cell cycle (Mendenhall, 1998).

3.4 Yeast cell cycle events

Two genes, *cdc2/CDC28* (p34) and *cdc10* play a major part in the transition through the start point (Brooks *et al.*, 1989). The product of *CDC28* also plays a part in the initiation of mitosis. For each role (S phase promotion and mitosis initiation) it binds to different activation proteins. The three proteins encoded by the *CLN1*, *CLN2* and *CLN3* genes allow *CDC28* to function in S phase promotion. These genes have been isolated in budding yeast and show sequence homology with the cyclins found in mammals (Reed *et al.*, 1985). They are identifiable by their ability to periodically accumulate and be proteolytically destroyed during the cell cycle. The products of the *CLN* genes are primarily required at Start in the yeast cell cycle. The other proteins that regulate p34 at the G_2/M transition point in the cell cycle are non-cyclin-type proteins (Sorrentino, 1996).

It has not been as easy to identify the G_2 / M phase transition point. The reason for this is that many hallmarks of mitosis are absent or difficult to spot in *S. cerevisiae*. It has been concluded that there is no distinct G_2 phase in the *S. cerevisiae* cell cycle, but even so, the basic principles of the *S. cerevisiae* cell cycle do not differ much from those in other eukaryotes (Lew *et al.*, 1997).

4. The experimental approach to this study

The first part of the study entailed the development of a functional assay for the tumour suppressor gene, p16, making use of yeast as reporter system. A similar assay for mutations in the p53 gene has been developed (Flaman *et al.*, 1995). In this assay human p53 is expressed in yeast. Wild type p53 activates transcription of the *ADE2*

gene which leads to white yeast colonies, and mutant p53 containing colonies are subsequently red, because the *ADE2* gene cannot be activated.

Why develop a yeast-based assay? On balance, we see the advantages outweighing the shortcomings of such an approach.

Advantages:

1. It assays for protein function, not just polymorphisms.
2. The whole ORF is tested in one assay.
3. It is relatively quick (2-3 days), the actual work takes half a day.
4. The reagents are inexpensive: no radio-activity, sequencing gels, silver staining etc. is required.
5. A large numbers of samples can be done in parallel.
6. It is not as labour intensive as traditional screening methods, for example SSCPs (single stranded conformation polymorphism).

Disadvantages:

1. No archival material can be used, since mRNA is required.
2. The background of PCR related mutants restricts the sensitivity of the assay.
3. A knowledge of yeast is essential, and not many laboratories in the human field has the necessary infrastructure.

We chose the p16 gene as a subject since it has been found to be mutated in many types of cancer (Hirama & Koeffler, 1995). It may be involved in chronic myeloid leukemia (CML) progression (Sill *et al.*, 1995) as well as other leukemias (Olshan *et al.*, 1997; Ranade *et al.*, 1995). We would eventually like to see if advancement of the chronic phase of leukemia to the accelerated phase could be detected via p16 gene mutations before any clinical indications are seen. This may be done since p16 gene mutations are implicated in the later phase of CML, rather than in the chronic phase.

The rationale behind the assay is given schematically in Figures 1.8 to 1.10. RNA is isolated from tumour cells and subjected to RT-PCR with a set of p16 gene-specific primers, producing a PCR product of 490 bases. This is then cotransformed with a

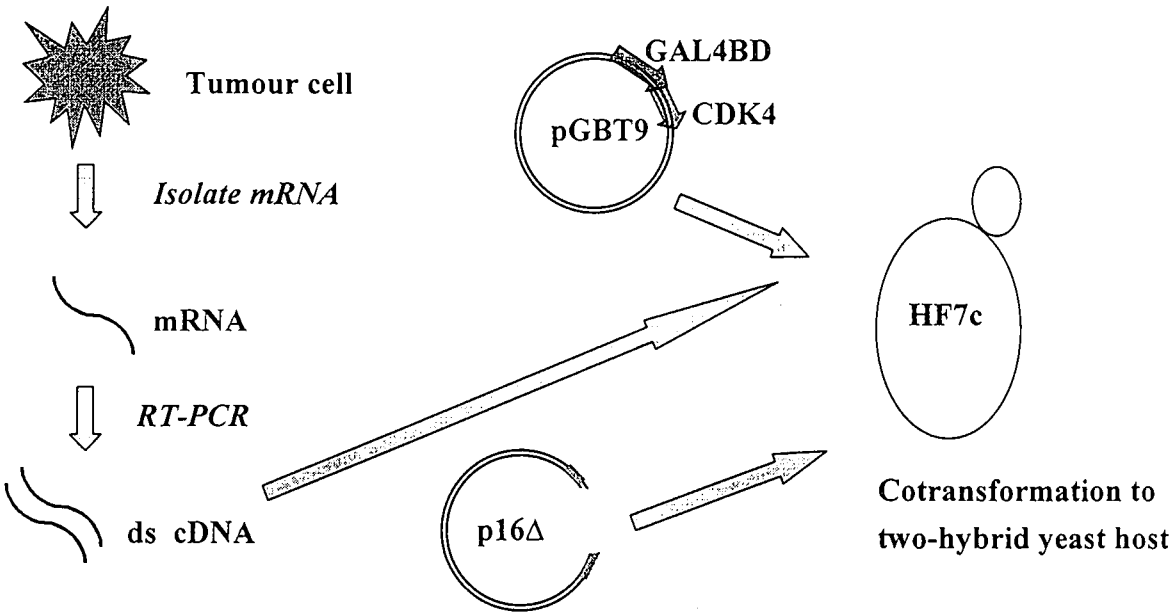


Figure 1.8 Schematic representation of the yeast transformation assay.

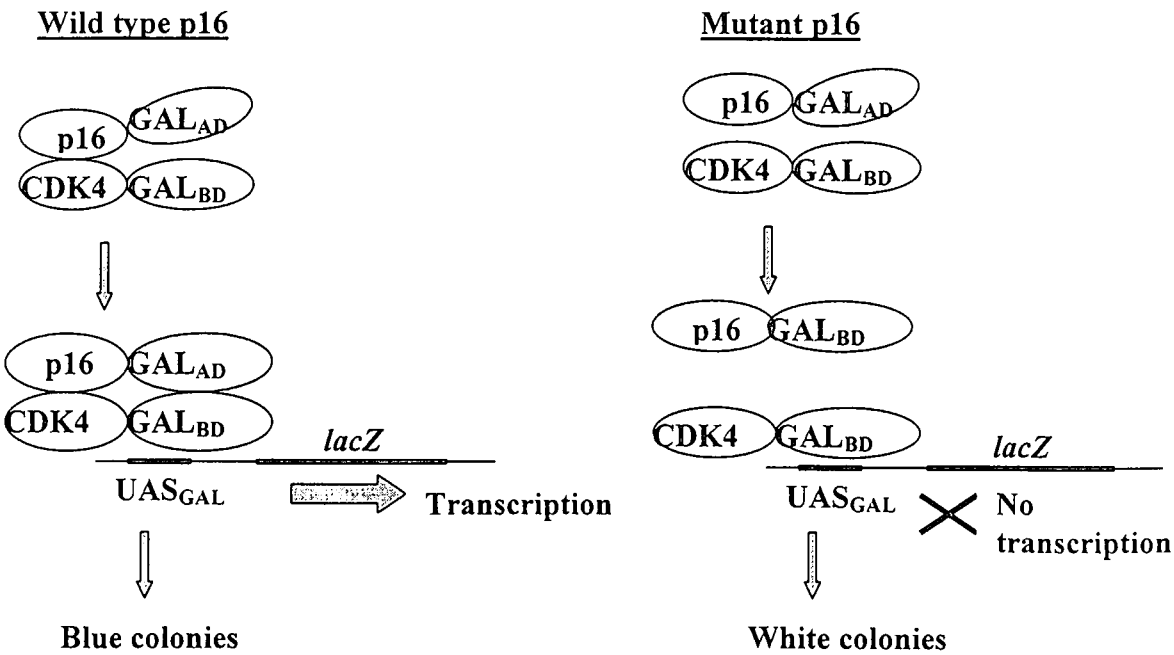


Figure 1.9 Schematic representation of protein-protein interactions that occur after yeast transformation.

gapped plasmid and a CDK4-containing plasmid into a suitable two-hybrid yeast host (Fig. 1.8).

The two-hybrid system (Fields, 1993) is a yeast-based genetic assay for detecting protein-protein interactions. It is designed around two plasmids, each containing part of the GAL4 transcription activator gene. This gene is conveniently organised into 2 domains, the DNA binding domain (BD) and the transcriptional activation domain (AD). These domains can be separated from each other without loss of individual function. Cloning sites were engineered at the C-termini of the GAL4 domain genes for insertion of open reading frames (ORFs) coding for proteins of interest. If these genes are inserted in reading frame, fusion proteins will be formed, consisting of a GAL4 partner and a protein of interest. Interaction between the two proteins would reconstitute the function of the transcriptional activator (GAL4) and lead to transcription of a reporter gene (*lacZ*) containing a GAL4 binding site in its promoter.

As wild-type p16 can bind to CDK4 *in vivo*, it should also happen in the yeast cell. This leads to transcription of the *lacZ* reporter gene, since the two parts of the GAL4 gene come in contact with one another (Fig. 1.9). The binding domain of the GAL4 gene is present on the p16 gene-containing plasmid, and the activation domain on the CDK4 gene-containing plasmid. The two GAL4 domains normally have no affinity for one another, but the p16-CDK4 binding brings them in close enough contact to ensure that they can function as transcription activator. This enables transcription of the *lacZ* gene to take place and blue yeast colonies form. A mutated form of p16 will not be able to switch *lacZ* on (Fig 1.9), resulting in white yeast colonies.

The key to the approach is the *in vivo* recombination event required to splice the PCR product into the linearised p16Δ plasmid (Fig 1.10). This is achieved by having small terminal overlaps, creating homology for the yeast homologous recombination system to insert the PCR product into the plasmid. This is a great time-saving step as conventional *in vitro* ligation, *E. coli* transformation and plasmid amplification before yeast transformation is avoided. The second part of the study is concerned with using the system on actual samples from cell lines and tumours.

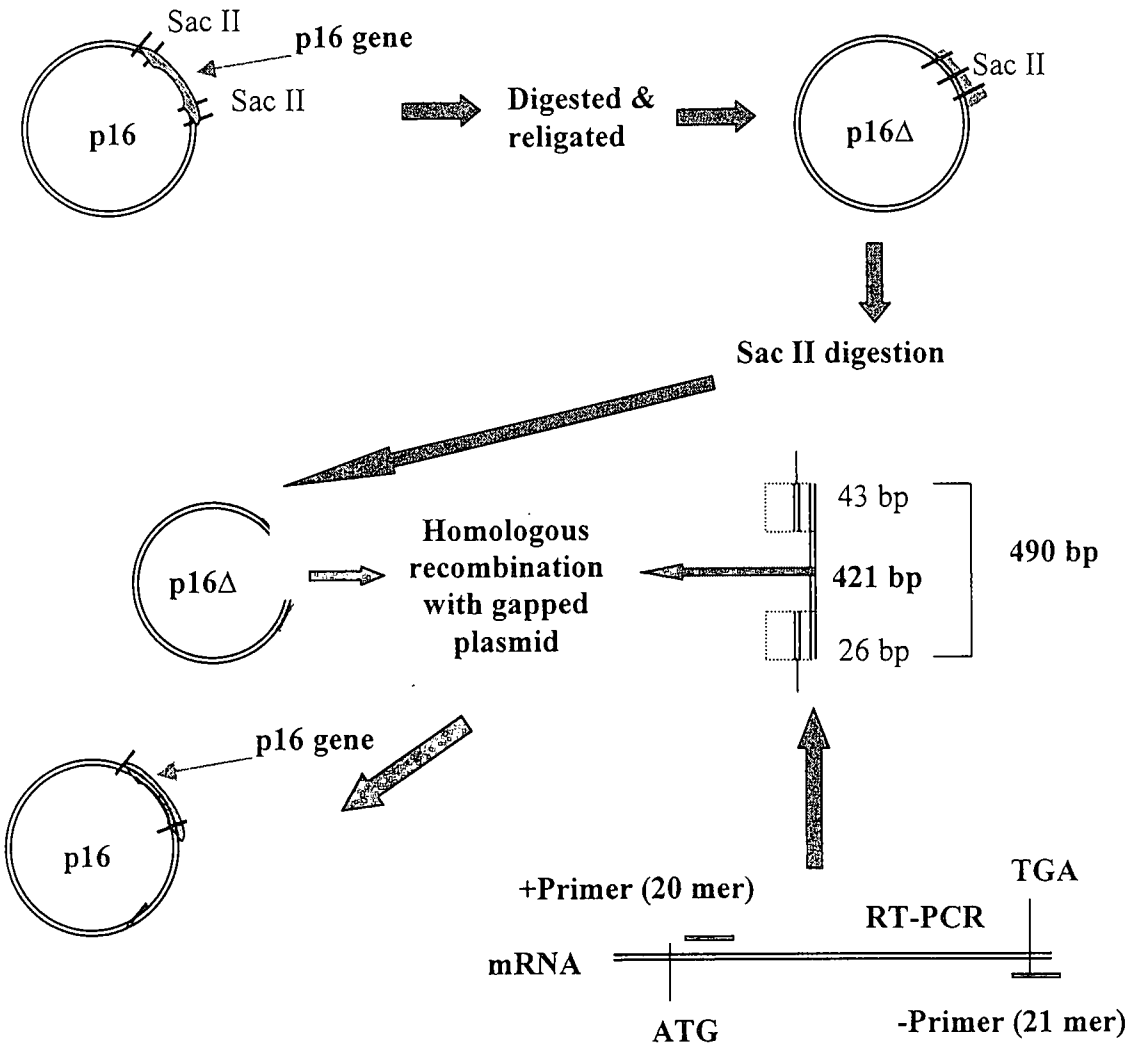


Figure 1.10 Schematic representation of the construction of the p16Δ plasmid, the RT-PCR and the homologous recombination that occurs in the yeast cells after transformation.

Chapter 2

Materials and Methods

1. Plasmids:

The CDK4 gene was ligated into the pGBT9 plasmid. This plasmid contains the GAL4 DNA binding domain sequence. The p16 gene was ligated into pGAD424, which contains the activation domain sequence for the GAL4 gene. Both plasmids were kind gifts from Dr M Serrano, Cold Spring Harbor Laboratory.

2. cDNA Library:

A human leukocyte cDNA library, constructed in pSPORTI, was bought from Gibco-BRL.

3. PCR reagents:

The primers P16P and P16M were synthesized by MWG Biotech, Germany. Both primers were designed with phosphorothioate linkages at the 3' end to protect against exonucleases (Table 2.1, Group 1).

	NAME	USE	SEQUENCE
1	P16P	Sense primer: RT-PCR	GGA GCA GCA TGG AGC CTT C-s-G
	P16M	Antisense primer: RT-PCR	GGC CCT GTA GGA CCT TCG GT-s-G
2	P1S4	Sense primer: Exon 1	GGA GAG GGG GAG AAC AGA CAA CGG
	1108	Antisense primer: Exon 1	GCG CTA CCT GAT TCC AAT TC
3	P2S2	Sense primer: Exon 2	ACC CTG GCT CTG ACC ATT CTG TTC T
	P2A2	Antisense primer: Exon 2	GTA CAA ATT CAG ATC CAT CAG TCC

Table 2.1. Different primer sets used in the study. *Group 1 represent primers used in RNA studies and group2 & 3 primers used to amplify the genomic DNA.*

Four different enzymes were tested in the PCR reaction, namely, Taq polymerase (Boehringer Mannheim), Expand HiFi polymerase (Boehringer Mannheim), AmpliTaq Gold (Perkin Elmer) and Pfu (Stratagene). The enzymes were used in the buffers supplied by the manufacturers. No additives to the $MgCl_2$ or $MgSO_4$ contents were made to ensure high stringency. A 2mM stock solution (Amersham) served as source of dNTPs for PCR.

A second PCR reaction was used on genomic DNA to test for deletion of the gene. Two different sets of primers were used (Table 2.1, Group 2 & 3) (Miller *et al.*, 1996).

4. Microbial strains:

4.1 *Saccharomyces cerevisiae:*

The host strain HF7c was used for transformation. This strain contains a GAL4-dependent HIS3 reporter gene which, due to its promoter, is not leaky and is, therefore, phenotypically auxotrophic for histidine (Feilotter *et. al.*, 1994). In addition, HF7c carries an integrated GAL4 dependent *lacZ* reporter gene and is also auxotrophic for tryptophan and leucine.

4.2 *E. coli:*

JM105 cells were used in all transformations and plasmid extractions.

5. General chemicals and media.

All chemicals were of analytical grade and media was bought from Difco. Double distilled water was used throughout.

6. Polymerase Chain Reaction (PCR):

Optimal conditions for amplification of a 500 bp fragment of the p16 gene from the pGAD424 plasmid was established using a Taguchi approach (Cobb & Clarkson, 1994). Ideal reagent combinations proved to be 10 ng DNA template, 100 pmol of each primer, 2 mM dNTPs and 1x Buffer with 1.5 mM $MgCl_2$ in a reaction volume of 50 µl. The optimal cycling conditions were: denaturation at 94 °C for 3', followed by 30 cycles of 94 °C for 1', 57.5 °C for 1', 72 °C for 2' and finally an extension cycle at 72 °C for 5'. An Omnigene Hybaid machine with a heated lid was used. A dilution series of template was used to determine the sensitivity of the reaction.

The second PCR was done to amplify the two exons of the gene, one 270 basepairs and the other 371 basepairs in size (Miller *et al.*, 1996). Reagent combinations were used as described above. This step was included to determine if deletion of the p16 gene occurred, when no PCR amplification could be achieved in some cases when mRNA was used in the reaction.

7. mRNA extraction:

Two samples of blood (5 ml each in EDTA tubes) were drawn, diluted with one volume PBS and layered onto 5 ml of Ficoll-Paque (Pharmacia). The tubes were centrifuged for 20 minutes at 1 200 rpm. The buffy coat containing the leukocytes was then collected with a pasteur pipette, mixed with one volume PBS and centrifuged for 5 minutes at 2 800 rpm. The pellet was washed with 10 ml of 1 M sodium citrate to lyse the red blood cells, then left on ice for 10 minutes and recentrifuged as above. This process was repeated until all red cells were removed. Total RNA was then isolated from the leukocytes using a kit bought from Qiagen, following the manufacturer's instructions. mRNA was isolated with a Dynal Dynabeads kit for mRNA extraction and resuspended in 20 µl of water.

8. RT-PCR:

A first strand mix was made, consisting of 18 µl extracted RNA, 6 µl 5x reverse transcriptase buffer (Superscript II, Gibco-BRL) and 1µl (100 pm) P16M primer. This mix was placed at 70 °C in a beaker containing 200 ml water and allowed to cool on the bench for 20 to 30 minutes. When the mix had cooled down sufficiently, 1 µl RNase inhibitor (Promega), 3 µl 100 mM DTT, 1 µl Superscript II and 1 µl 2 mM dNTPs were added and placed at 42 °C for 30 minutes. Five microliters of this reaction was used for a 50 µl PCR as described above.

9. Isolation of fragments from gels:

The PCR products where separated on a 2% agarose gel. The fragments were cut from the gel and cleaned by using the Gene-Clean II kit (Bio101). The concentration of the fragments were determined on a spectophotometer at 260 nm and stored at -20 °C.

10. Yeast Transformation:

Generally, the method described by A Gietz on the Internet (http://www.umanitoba.ca/ faculties/medicine/human_genetics/gietz/Trafo.html) was used.

Inoculate 2 - 5 ml of liquid YPD (*1% w/v yeast extract, 2% peptone, 2% Glucose*) with the yeast strain and incubate with shaking overnight at 30 °C.

Count the overnight culture and inoculate 50 ml of warm YPD at a cell density of $5x10^6$ ml

Incubate the culture at 30 °C on a shaker at 200 rpm until its equivalent to $2x10^7$ cells/ml. This will take 3 to 5 hours. This culture will give sufficient cells for 10 transformations.

Harvest the culture in a sterile 50 ml centrifuge tube at 5000 rpm for 5 minutes.

Pour off the medium, resuspend the cells in 25 ml of sterile water and centrifuge again.

Pour off the water, resuspend the cells in 1 ml 100 mM LiAc and transfer the suspension to a 1.5 ml microfuge tube.

Pellet the cells at top speed in a microfuge for 15 seconds and remove the LiAc.

Resuspend the cells to a final volume of 500 µl ($2x10^9$ cells/ml) with the 100 mM LiAc.

Boil a 1 ml sample of SS-DNA (10 mg/ml) for 5 minutes and quickly chill on ice.

Vortex the cell suspension and pipette 50 µl samples into labeled microfuge tubes. Pellet the cells and remove the LiAc.

The basic transformation mix consists of:

240 µl PEG (50% w/v)

36 µl 1 M LiAc

25 µl SS-DNA (2 mg/ml)

50 µl water and plasmid DNA

Carefully add these ingredients in the order list. This is very important, because the PEG should protect the cells from the detrimental effects of the LiAc.

Vortex each tube vigorously until the cell pellet has been completely mixed. Usually this takes about 1 minute. Further mixing can be done with a micropipette.

Incubate at 30 °C for 30 minutes

Heat shock in a water bath at 42°C for 20 - 25 minutes.

Centrifuge at 6 - 8000 rpm in a microfuge for 15 seconds and remove the transformation mix.

Pipette 1 ml of sterile water into each tube and resuspend the pellet by pipetting it up and down gently.

Incubate the SC minus plates for 2 - 4 days to recover transformants.

The transformants were grown on SC plates lacking the appropriate amino acid (*0.1% w/v essential amino acid mix, lacking the appropriate amino acid e.g. Leucine*) (D'Enfert *et.al.*, 1995).

11. Cloning of the p16 gene to pYES2

General recombinant methods were followed, mostly from Sambrook *et al.*, 1989. The p16 gene was cut from the pGAD424 plasmid with Xho1 and EcoR1 restriction enzymes, while the pYes2 plasmid was cleaved with the same two enzymes. Ligation was done as described by Sambrook *et al.*, 1989.

11.1 Colony hybridization with DIG

A DNA probe was constructed by incorporating DIG-labeled dNTPs (Roche) during a PCR reaction. A nylon membrane was placed on the petri dish with the *E. coli* transformants for 1 minute. Positive colonies were detected on the membrane by hybridizing the membrane overnight with the DIG-labeled probe and then adding CSPD to ensure a light reaction that can be detected on X-ray film.

The positive colonies were cultured overnight and mini-preps were done to isolate the plasmid.

A PCR reaction was done on the isolated plasmid to confirm that it contained the relevant insert.

11.2 Sequencing

The plasmids that tested positive during the PCR were sequenced to confirm the fidelity of the wild type p16. The sequencing reactions were done with the Thermo Sequenase sequencing system kit from Amersham. The same primers as in the PCR reactions were used.

The sequencing was done on a Perkin Elmer ABI Prism 377 sequencer and the data was analysed on the ABI Prism 377 data analysis software.

12. Induction of pYES2

Transformants were grown in YPD growth medium *(2% Glc, 0.67 % YNB and 0.08% amino acid mix)* and the following method was used to induce the pYES2 plasmid to transcribe the p16 gene:

Grow cells overnight in 50 ml YPD at 30 °C on a shaker.

Centrifuge cells.

Add new growth medium that contains 5% glycerol instead of 2% glucose to rid the cells of the glucose inhibition.

Grow cells for 4 hours.

Take a 1 ml sample from the culture as a non-induced control.

Divide culture in 2 equal parts and centrifuge.

To one pellet add growth medium containing glucose and to the second pellet add medium that contains 2% galactose.

Take 1 ml samples from each culture every 20 minutes for 3 hours and a last sample after overnight growth.

13. RNA extraction

All reagents were treated with 0.1% DEPC before use.

Centrifuge 1 ml of culture at 2 500 rpm for 3 minutes.

Add to the cells: 500 µl NaAc buffer *(50 mM NaAc; 10 mM EDTA; pH 5.0 with acetic acid)*

50 µl 10% SDS

660 µl Phenol (65 °C) *(melt 500 µl phenol in 500 µl NaAc buffer, pH 6.0. Keep in low light conditions at 4 °C).*

Shake vigorously for 4 minutes at 65 °C.

Centrifuge at 3 500 rpm for 5 minutes in a microfuge.

Draw off the organic matter and add 660 µl phenol to the aquase phase.

Shake vigorously for 4 minutes at 65 °C.

Centrifuge as above.

Add equal volumes Chloropane to the upper phase. *(50 % liquid phenol; 50% chloroform; 0.5% 8-hydroxychinoline. Equilibrate with ANE: 10 mM NaAc, 100 mM NaCl, 1 mM EDTA; pH 6.0)*

Vortex for 2 minutes.

Centrifuge.

Add equal volumes chloroform to upper phase.

Centrifuge.

Precipitate upper phase with 1/10 volume 3 M NaAc and 3 volumes EtOH.

Leave at -20 °C for 2 hours.

Centrifuge at top speed for 15 minutes in microfuge.

Dry the pellet completely.

Resuspend pellet in 30 µl DEPC water.

Run 3 µl on a 0.8% agarose gel to check the quality of the RNA. Two definite bands have to be visible.

14. Flow cytometry

The 1 ml cell samples taken during the induction experiment were used (Kikuchi, *et al.*, 1994).

Pellet the cells at top speed in a microfuge.

Resuspend cells in 1 ml 70% ethanol and incubate for 30 minutes at room temperature with rotation. (Cells will clump together during ethanol fixation, but will disperse after resuspension in buffer in the next step. Cells may be stored overnight in ethanol at 4 °C. Mix well before proceeding.)

Wash cells once GENTLY in 50 mM sodium citrate and resuspend in 0.5 ml of the same.

Centrifuge for 1-2 minutes at 2 000 g in a microfuge.

Add 20 µl of 10 mg/ml RNase A (40 µl/ml final) and incubate for 2 hours at 37 °C.

Wash cells once with 50 mM sodium citrate and resuspend in 500 µl propidium iodide (PI) buffer. (*50 mM sodium citrate, pH 7.0; 10 mM NaCl; 0.1% Nonidet P-40). (Stock: 5 mg/ml PI in 50 mM sodium citrate, pH 7.0. Filter sterilize before use and keep frozen in low light conditions.)*

Add 10 µl of 5 mg/ml PI stock (100 µg/ml final) and leave at room temperature for 30 minutes. Maintain samples in low light conditions.

Centrifuge samples as described above and resuspend cells in 1 ml 50 mM sodium citrate.

Add 1 µl PI stock solution (10 µl/ml final), mix and keep on ice until ready to analyse.

Prepare 1:10 dilutions of stained cells and put 300 µl in 0.5 ml pop-top tubes for use in the flow cytometer.

A Beckton Dickinson flow cytometer was used for the analysis of the cells.

5. <u>Fluorescence microscopy</u>

The cells were stained with PI using the same method as for the flow cytometry. Undiluted cells were placed on microscope slides and analyzed under a fluorescent microscope equipped with a triple band-pass filter.

Chapter 3

Results and Discussion

1. Introduction

Detection of mutations in oncogenes and tumour suppressor genes in cancer cells is a rapidly growing field with great potential for practical application. Consequently, there are many techniques being developed and reported in the literature. The vast majority of these mutation detection techniques aim to detect the mutations on the level of genomic DNA. Many of the techniques detect base changes, which could be either mutations or polymorphisms. We have decided that there is a need for a functional assay of p16 activity, since this type of assay can distinguish clearly between a neutral polymorphism and an inactivating mutation. We have further chosen yeast as a reporter system.

2. Construction of the p16Δ plasmid

A derivative of pGADp16, named p16Δ, was made by removing most of the coding region of the p16 gene. This was done by SacII (KspI) digestion and religation. Minipreps were done on 10 transformants, they were digested with SacII and checked for the correct size and absence of an insert (Results not shown). All 10 plasmids were correct and one was chosen to grow up at large scale to serve as gapped plasmid in the yeast assay.

3. Testing the primers

In a case such as this, where little leeway is allowed for primer design (as they have to be located in a very specific region), it is not always possible to have an "ideal" set of primers that satisfy all primer-design criteria. Furthermore, if a particular PCR is to be used in a quantitative approach, it has to be optimized first to ensure the highest possible sensitivity and reproducibility. Therefore, the p16 gene containing plasmid was used as a template to develop an optimal PCR regime. After employing a

Taguchi-type approach (Cobb & Clarkson, 1994), where different parameters were changed in a predetermined way, the best conditions were eventually found. The optimal PCR had the following steps: Denaturation at 94 °C for 3', followed by 30 cycles of 94 °C for 1', 57.5 °C for 1', 72 °C for 2' and finally an extension cycle at 72 °C for 5'. This program was used in all subsequent work. Despite the effort that went into optimizing the PCR, it proved to be inconsistent. After thorough investigation of all possible parameters, two critical factors emerged. Firstly, it was found that the block of the PCR machine did not have a uniform temperature distribution. "Good" and "bad" wells were identified and labeled accordingly (Fig.3.1). If only "good" wells were used, the results were consistent and reproducible. Secondly, the PCR only worked consistently if thin-walled tubes were used. Only one block of a particular PCR machine could be made to work, and was used for all subsequent experiments.

4. Sensitivity of the assay

As a test of the sensitivity of the PCR, the pGADp16 plasmid as well as a human leukocyte cDNA library were used as templates. The plasmid could be diluted to 0.4 pg per reaction and the library to 12.5 pg per reaction (Fig. 3.2 & 3.3), before the PCR became negative. This indicates that p16 mRNA is present in human leukocytes. For the plasmid, 0.4 pg is equivalent to about 800 copies of the gene, indicating that the sensitivity of this assay could be improved. One way of doing it, is by employing a nested PCR approach utilising two sets of primers. For this particular application, however, it is not advisable to do too many rounds of amplification, as the error-rate increases with increasing number of cycles. It seemed as if the PCR done with the plasmid DNA had a more definite cut-off than the PCR done with the library DNA. This might be due to continued heating differences in the "good" wells of the PCR block. It should also be remembered that this PCR cannot be used for quantitative analysis.

5. Yeast transformation

Since the yeast transformation represents the actual assaying step of the planned technique, it needs to be as efficient as possible. Therefore, much effort was put into

finding the optimum conditions for transforming the HF7c strain with the particular plasmids used in the two-hybrid assay. The CDK4 gene containing plasmid presented

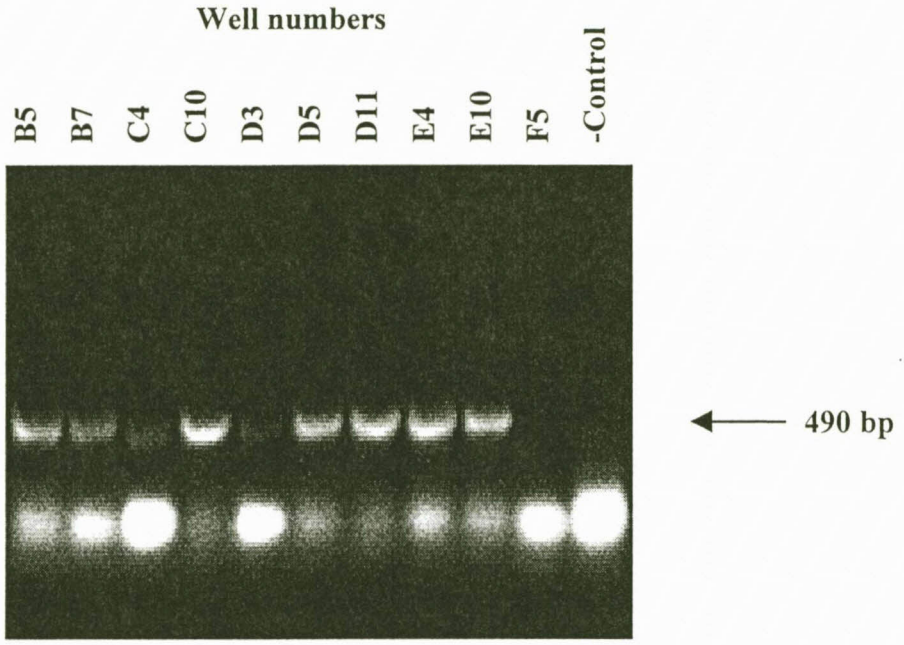


Figure 3.1. A PCR reaction was constructed and divided equally into eleven tubes. The tubes were placed into eleven different wells of the PCR machine (Hybaid OmniGene with hotlid) to determine the best wells for amplification. *Lane numbers represent well numbers in PCR block.*

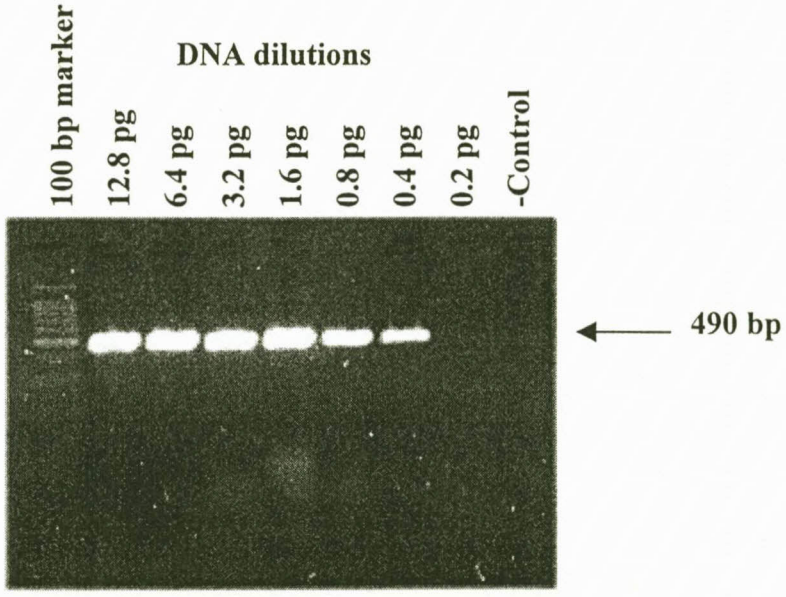


Figure 3.2. A dilution series of the pGADp16 plasmid, starting with 12.8 pg and a 2x dilution of each sample to determine the sensitivity of the PCR reaction.

no problems and was used to establish the optimal transformation protocol. In contrast, despite many different attempts, changes of protocol, repurification of plasmid and going back to frozen master cultures of the host strain, no transformation with the p16 gene containing plasmid on its own was ever accomplished. When the transformation mixture contained both plasmids and selection was on -Trp-Leu plates, the presence of both plasmids could be demonstrated by PCR and recovery in *E. coli*. This proved an interesting development and warranted further investigation.

We also tested the effect of combining the different elements of the assay in various ways. No difference was found between first transforming the host with the CDK4 plasmid and subsequently bring in the gapped plasmid and PCR product or transforming directly with all three DNAs simultaneously (Results not shown).

Blue yeast colonies proved that there was contact between the relevant proteins and that this technique could be used to detect mutations in the p16 gene. However, some white colonies were detected among the blue ones and this we attributed to errors occurring during the PCR.

6. Testing different enzymes

The same PCR conditions as described above were used to amplify the p16 gene, using different polymerase enzymes. This was done to determine which enzyme showed the lowest error rate during the PCR. The error rate of a PCR reaction is determined by the amount of "wrong" nucleotides incorporated in the amplified DNA. Four enzymes, Taq Polymerase, Expand HiFi, Pfu and AmpliTaq Gold were chosen because of their different properties: Taq polymerase was chosen as an ordinary, not too expensive enzyme, Expand HiFi to test an enzyme mixture (Pwo and Taq polymerases), Pfu as a single high-fidelity proofreading enzyme and Amplitaq Gold as representing the new class of enzymes that are initially inactive, becoming active during the course of the PCR. The products of the different enzymes were run on an agarose gel and used in a yeast transformation. The results are summarised in Table 3.1 and the surprising finding was that there is not much difference in fidelity among the enzymes under our conditions. One of the reasons may be that we have used only 30 cycles in the PCR reaction. Increasing the number of cycles to 35 led to non-specific products appearing on gels (Results not shown) and 30 is therefore regarded

as the limit for this specific PCR. Since Expand HiFi gave a slightly higher product yield at a slightly lower error-rate than Taq polymerase, we have decided to

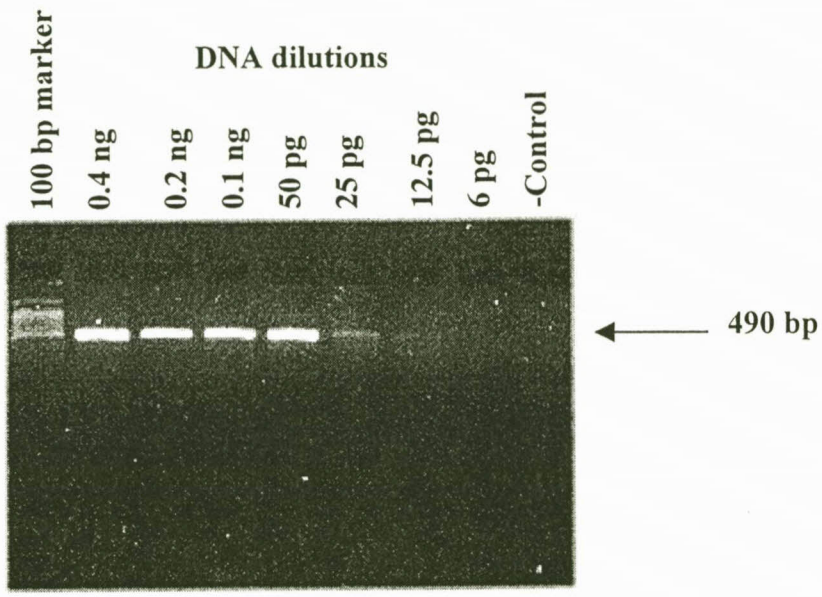


Figure 3.3. A dilution series of the leukocyte cDNA library was constructed to determine the presence of the p16 transcript *in vivo.*

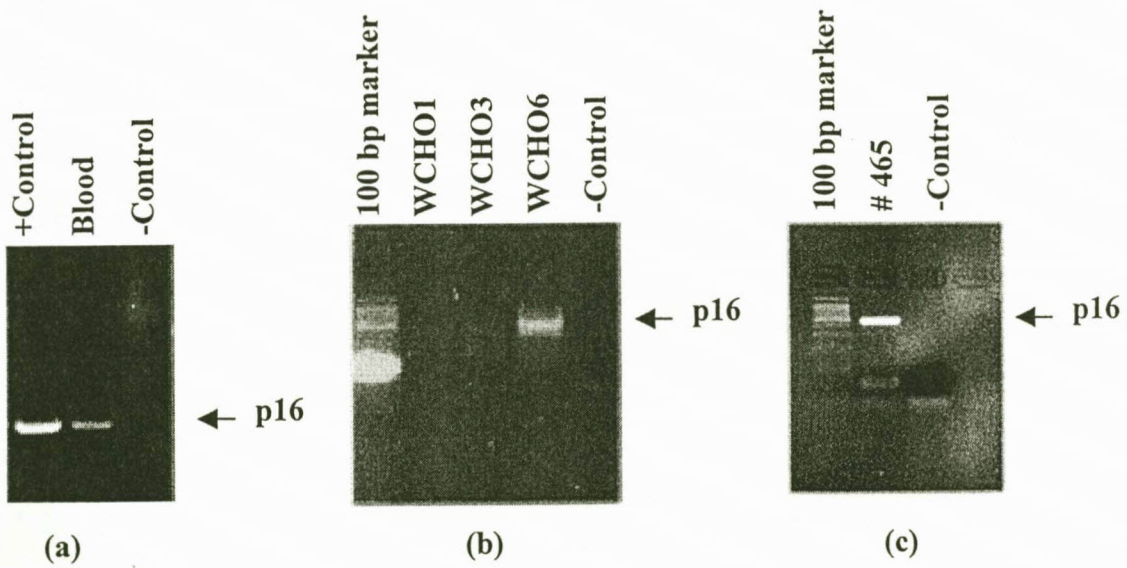


Figure 3. 4. Amplification of the p16 transcript from **(a)** normal blood, **(b)** esophageal cell lines and **(c)** blood from a leukaemia patient. *Lane numbers indicate patient file numbers and cell line names.*

standardize on this enzyme formulation for subsequent work. It is also cheaper than Pfu and Amplitaq Gold.

Table 3.1 Comparing the error-rate of four different DNA polymerases.

Enzyme	Taq polymerase	Amplitaq Gold	Expand HiFi	Pfu
Percentage of blue colonies	96 ± 2.5	96 ± 2	97 ± 2	97 ± 2

7. Amplification of the p16 ORF from biological samples

When amplification of the p16 reading frame was tried by using total RNA isolated from fresh leukocytes in the PCR reaction, no product was obtained, indicating that it would be necessary to first enrich for mRNA before doing the RT-PCR. When this was done, a positive result was obtained (Fig.3.4a). Volumes of whole blood down to 300 microliter were successful. When these products were used in a yeast transformation, only wild-type levels of blue colonies were found (Results not shown).

RNA was extracted from three esophageal carcinoma cell lines (a kind gift of Alet Zerwick of the Department of Oncotherapy, National Hospital). The mRNA was subjected to RT-PCR to amplify the p16 gene. (Fig. 3.4b). Only one of the three samples showed a positive amplification of the reading frame. Genomic DNA of the same cell lines were subjected to two separate PCRs to amplify the two exons of the gene. This was done to determine if WCHO1 and 3 perhaps had homozygous deletions of the p16 gene, which is a frequent occurrence in tumours (Hirama & Koeffler, 1995). From the results of the p16 genomic PCR it can be seen that only WCHO3 (despite repeated attempts) showed consistent amplification of the p16 exons, and not WCHO6 as expected (Fig. 3.5). The absence of products in the case of WCHO1 could be explained by homozygous deletion of the p16 genomic region, while hypermethylation of the p16 gene promoter may be the cause of the absence of an RT-PCR product in the case of WCHO3. An explanation for the results of WCHO6 has to await further investigation. A way should be devised to distinguish between RNA degradation, failed PCR and absence of the p16 gene and we are in the

process of evaluating a number of internal controls. Actin and c-abl have been tried so far, but their optimal conditions are far from that of the p16 PCR, leading to non-specific PCR products. A reliable internal control, amplified from a constitutively expressed gene, with compatible PCR parameters, still has to be found.

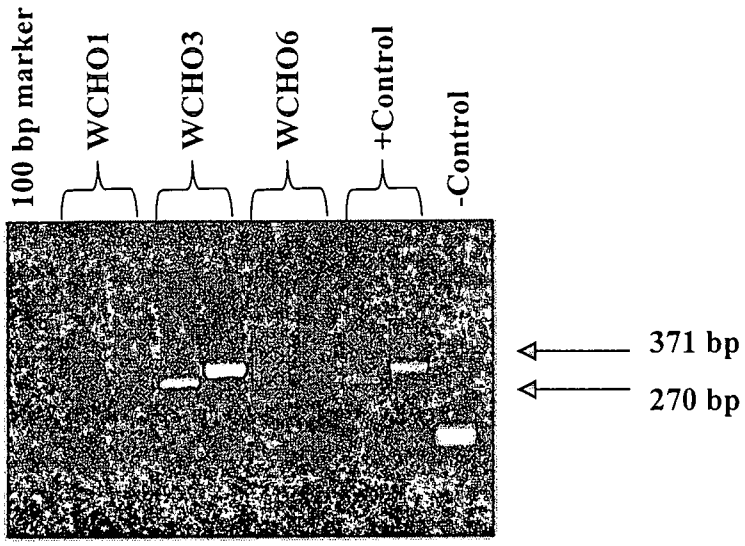


Figure 3.5. The PCR products of the three esophageal genomic DNAs. *Lane numbers are cell line names.*

8. The p16 gene and its influence on yeast:

Since transformation of the p16 gene containing plasmid led to cell death, the gene was cloned into a regulatable expression plasmid, pYES2, to determine the exact effect the gene product had on yeast. This plasmid was named pCB1.

The plasmid could be brought to express the p16 gene by growing the yeast cells in galactose containing medium. These cells were then compared with cells grown in glucose medium in which the p16 gene could not be switched on. Cell samples were taken at different time intervals and analysed by flow cytometry and fluorescense microscopy.

The flow cytometric analysis is presented in graphical form, with the x-axis representing the fluorescense intensity (DNA content) and the y-axis the number of cells. Fig 3.6a shows the yeast sample grown in glycerol. Three peaks were observed namely n, 2n and 2n+. The n peak represents haploid cells, 2n shows cells after

replication and 2n+ represents cells that have started budding before being separated from their mother cell (chains of cells). This last peak is usually seen in cell populations during active growth. The distribution seen in this graph is typical of the slow growth expected from an overnight culture using glycerol as carbon source.

In Fig 3.6b the yeast had been grown for four hours in glucose medium. This ensured that the p16 gene would not be expressed. As can be seen from the graph, the 2n and 2n+ peaks showed an increase and the n peak a decrease. This indicates that new growth has occurred.

Fig 3.6c shows the data from yeast grown in galactose for 16 hours, containing the pYES2 plasmid without the p16 gene, a control to ensure that the medium and plasmid itself have no effect on the growth of the yeast. Here growth is also detected, with a relatively big increase in the 2n+ peak, a sign of accelerated growth.

Fig. 3.6d. represents yeast grown in galactose for 16 hours, but in which the p16 gene is actively expressed. When compared to the control (Fig. 3.6a.), almost no difference can be seen. This leads to the conclusion that the human p16 gene does inhibit the growth of yeast cells, thus making it impossible to detect transformants expressing p16.

The results obtained from the fluorescent microscopy supported those obtained from flow cytometry. When the cells that were grown in galactose medium (active p16 gene) were analysed, one could see that the nuclei of the cells appeared compact and small. In contrast, the cells that didn't express the p16 gene had nuclei that seemed "looser" and more budding cells were visible (Fig 3.7)

The simplest explanation for this observation is that p16 binds to the yeast equivalent of CDK4, namely *Cdc28*. This is supported by the finding that if a host is used that contains an integrated copy of CDK4, very small colonies are found after transforming it with pGADp16 (Results not shown). We postulate that p16 can bind to both CDK4 and *Cdc28* and if CDK4 is present in excess, enough free *Cdc28* would be around to drive progression of the cell cycle. In the case of the integrated CDK4 gene, just enough free *Cdc28* remains to allow very slow growth, leading to small colonies.

9. Conclusion

This project has succeeded in its primary aim, namely demonstrating that human p16 gene mutations can be investigated in yeast. Although Reymond & Brent (1995) also used yeast as a testbed for p16 function, their approach was much more cumbersome, relying on conventional cloning techniques to get p16 into the correct plasmid. Using the gapped repair system as pioneered by Flaman *et al.*(1995), made a major difference. The technique now becomes potentially useful for routine screening of many samples.

A few issues still have to be cleared up however, before we can consider using this assay as a routine test. First of all, PCR errors have to be minimised to ensure that the p16 mutations are *in vivo* mutations and not due to the PCR, which might lead to a wrong result. At the moment we consider all samples with 5% or less mutant colonies as wild type. We have not yet come across a sample that has a high percentage of mutants and only time will tell precisely where to draw the line between wild type and mutants.

A second problem we encountered was that in some cases we were not able to distinguish between loss of the gene, hypermethylation of the gene or PCR inhibitors to explain the absence of a PCR product. A possible solution for this problem would be to incorporate some internal controls to enable us to distinguish between an absent gene or an inhibited PCR reaction.

Once the yeast transformation has been done and colonies appear, it takes at least two days for the colonies to start changing colour, adding up to about 5 days before the results can be scored. This might cause a problem, since such delays have to be kept to a minimum in routine tests.

A major question that arises is whether mutations of the p16 gene observed in yeast genuinely reflect mutations in humans. Reymond and Brent (1995) used known p16 mutants in their study and showed that the mutations observed in yeast reflect the mutations in the tumours accurately. A parallel project to this one is currently underway to mutate p16 via mutagenic PCR. Sequencing these mutants and comparing them with the database available on p16 mutations should ensure that mutations of the p16 gene observed in the yeast are not due to the technique or something peculiar to yeast, but are genuine *in vivo* mutations. At the moment there about 10 mutants waiting to be sequenced. A major advantage of this technique is

that individual mutations can be recovered and sequenced. This is a big improvement on other methods where mixtures of normal and mutant DNA have to be used.

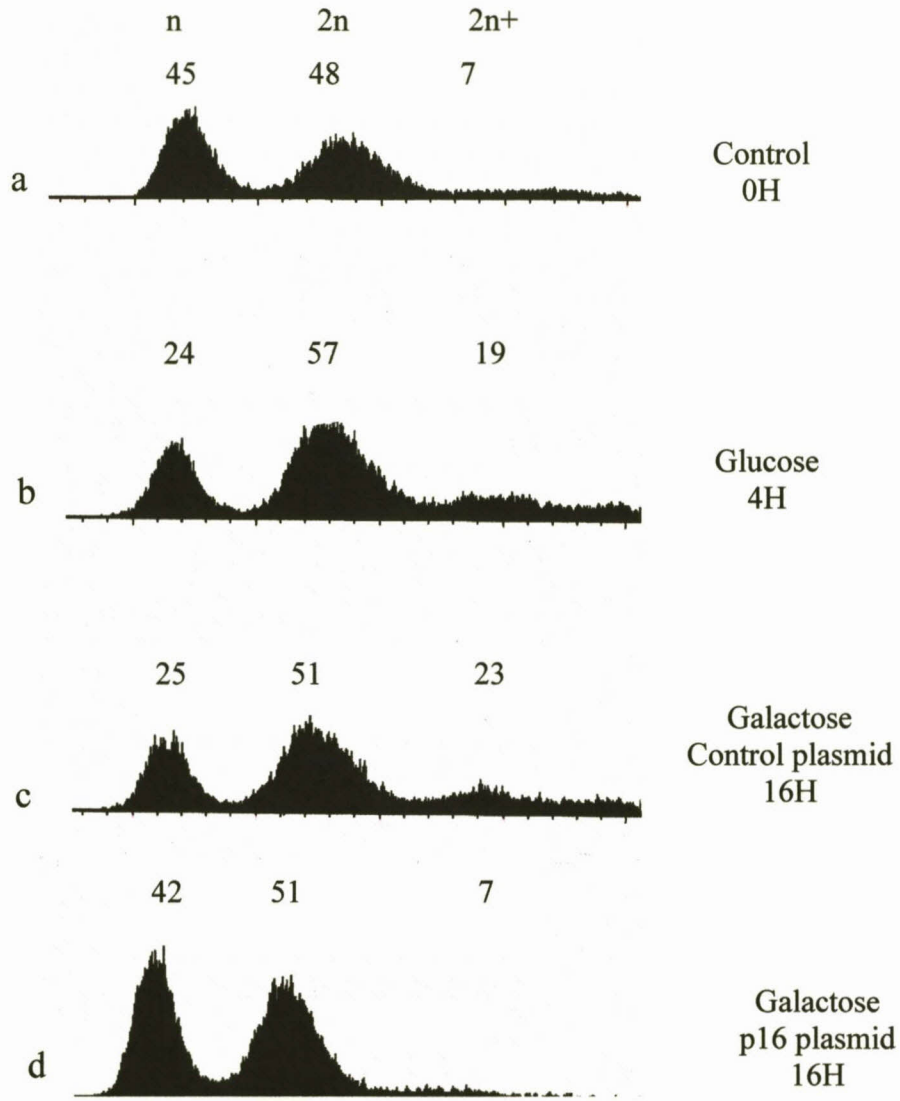
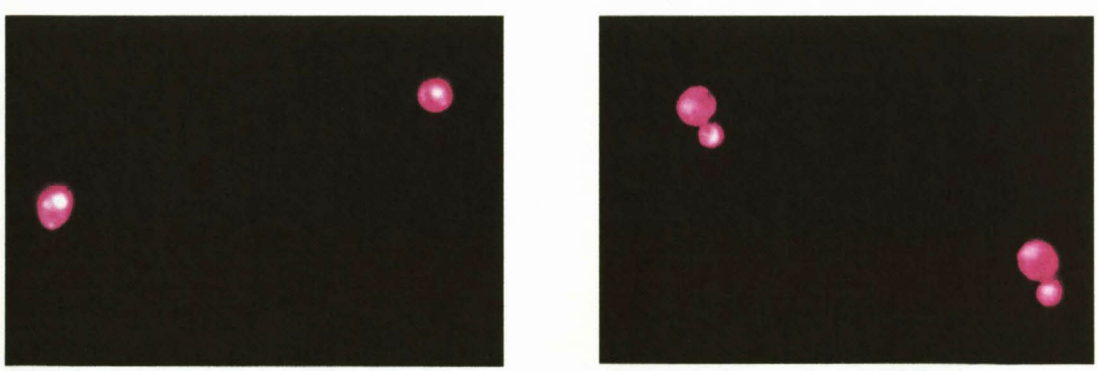


Fig 3.6 Flow cytometric analysis of induced expression of p16 in yeast. The percentage of cells in a particular growth phase is indicated above each peak.



(a) **(b)**

Figure 3.7 Fluorescent microscopy results. The yeast cells expressing the p16 gene **(a)** and those not expressing the gene **(b)**.

Abstract

Cyclin-dependent kinases (CDKs) are crucial regulators of the cell cycle. CDKs themselves are subject to control by both cyclins and CDK inhibitors. Among the inhibitors, p16 is very prominent, since it has been found to be mutated or lost in a variety of tumours. We are interested in mutations involved in the progression of leukemia from the chronic to the acute phase. The p16 gene has been implicated in this progression, therefore we needed an assay for p16 status that could be applied to screen patients in chronic phase regularly.

Traditional mutation screening makes use of physical methods such as Single Stranded Conformational Polymorphism (SSCP) analysis. These methods are generally labour intensive and are not always informative. If tests for the actual function for the gene products could be devised, it could be used to screen tumour samples for the status of these genes.

We have decided to develop a yeast-based test that would directly assay for activity rather than just nucleotide changes. The assay is based on the yeast two-hybrid system, where protein-protein contact is reflected in colony colour. We have designed a primer set to amplify the p16 reading frame by RT-PCR from small amounts of leukocyte mRNA. This cDNA is then cotransformed with a gapped plasmid containing terminal p16 overlaps, allowing homologous recombination to splice the reading frame into the plasmid. The host strain also contains a CDK4-expressing plasmid and if the amplified p16 can still bind to CDK4, the colonies would turn blue.

We have successfully constructed and tested the system and found it to be very sensitive, being able to assay p16 from as little as 300 microliters of whole blood.

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