Identification of novel protein interactors of the SV40 large T antigen using the yeast two-hybrid system

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December 2001

Thesis submitted for the degree of Doctor of Philosophy
in the University of London

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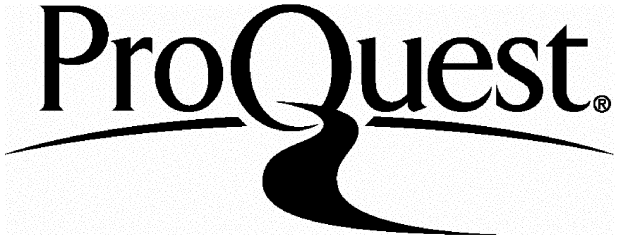

Acknowledgments

My first thanks go to Dr. Parmjit Jat for his help and supervision of this project, and to Professor Mike Waterfield for giving me the opportunity to work at the Ludwig Institute for Cancer Research. My very special thanks go to Dr. Erica Golemis at the Fox Chase Cancer Center in Philadelphia, for giving me the unique opportunity to visit the United States and work at her laboratory, and for all the immensely useful discussions and technical advice. I also would like to thank Dr. Anne Ridley for her support and encouragement, and for all the advice and discussions on this project. Thanks to Dr. Derek Davies at the ICRF for providing the flow cytometric analysis, Dr. Athena Nikitopoulou for the sequencing, Dr. Ilya Serebriiskii for advice on the two-hybrid, Dr. Roy Katso for help with kinase assays, and Dr. Noriko Shikama, Dr. Peter Sorger and Dr. Mark Kirschner for providing reagents. Thanks also to Dr. Yann Leverrier, Dr. Beata Wojciak-Stothard, Katie Adams and Alan Entwistle for their help with immunofluorescence. I would especially like to thank all the members of the transformation laboratory, both past and present, for their friendship and their support through all the good times and the bad times.

More than anyone else, I would like to thank my boyfriend Harry for his help and advice during this Ph.D., for the constructive reading of this manuscript, but mostly, for all his love, support and patience, and for keeping me sane when times were crazy. I would be lost without you. Σ'αγαπω.

This work was supported by a studentship from the Medical Research Council.

Abstract

The SV40 large T antigen is a viral oncogene able to immortalize mammalian cells in culture, and to occasionally transform them to tumourigenicity. It can also cause genomic instability by inducing chromosome aberrations and aneuploidy. T antigen is a multifunctional protein which interacts with a number of cellular proteins. Studies have previously shown that the critical activities required for these functions map to the N-terminus of the protein. The N-terminus carries the pRb binding activity and the J domain, but data suggests that there may be additional proteins binding in this region.

To isolate such proteins, the yeast two-hybrid approach was chosen, and the N-terminus of T antigen fused to the LexA DNA binding domain was used as a bait. In order to optimize the screen, different versions of the system were setup, by cloning T antigen into an N-terminal fusion vector, two C-terminal fusion vectors and an inducible C-terminal fusion vector. A HeLa library and two mouse embryonic libraries carrying cDNAs fused either to the B42 or the GAL4 activation domains were screened. A number of proteins were identified as potential interactors with the N-terminus of T antigen in yeast.

One of these proteins was the Bub1 mitotic checkpoint protein, a member of the family of proteins that monitor the assembly of the mitotic spindle. The interaction of Bub1 with T antigen was investigated in mammalian cells. T antigen was found to co-immunoprecipitate with endogenous Bub1 from extracts of rat embryo fibroblasts conditionally immortalized with T antigen, as well as from NIH 3T3 cells ectopically expressing T antigen. The interaction of T antigen with Bub3, another checkpoint protein of the same family and a binding partner of Bub1, was also demonstrated. In addition, T antigen was found to co-localize with Bub1 in the nuclei of early prophase cells. To investigate the functional significance of this interaction, FACS analysis was used to show that expression of T antigen in cells treated with nocodazole (a microtubule disrupting agent) makes them more refractory to the spindle assembly checkpoint. Moreover, kinase assays indicated that T antigen expression affects the kinase activity of Bub1. The T antigen/Bub1 interaction suggests a novel role for T antigen and provides a new insight into its ability to cause aneuploidy.

Finally, a preliminary investigation of two other proteins isolated from the yeast two-hybrid screens, Hsp40 and β-tubulin, indicated that these proteins may also co-localize with T antigen in mammalian cells.

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Abbreviations

aa	amino acid
Ad	adenovirus
ADH1	alcohol dehydrogenase gene
Amp	ampicillin
APC	anaphase promoting complex
APC	Adenomatous Polyposis Coli
APS	ammonium persulphate
ARF	alternative reading frame
ars	autonomously replicating segment
ATM	*Ataxia Telangiectasia Mutated*
ATP	adenoside triphosphate
BH	Bcl-homology domain
bp	base pair
BSA	bovine serum albumin
BTG1	B-cell translocation gene 1
Bub	*Budding uninhibited by benomyl*
C-	carboxy
CBP	CREB-binding protein
CD	conserved domain
Cdk	cyclin-dependent kinase
cDNA	complementary deoxyribonucleic acid
C.elegans	*Caenorhabditis elegans*
CENP-E	centromere protein-E
Ci	Curie
CR1	conserved region 1
CR2	conserved region 2
DCS	donor calf serum
DNA	deoxyribonucleic acid
DNA MeTase	DNA methyltransferase
ddNTP	dideoxynucleoside triphosphate
DMSO	dimethylsulphoxide
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
E1A	early region 1A
ECL	enhanced chemilluminescence
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetra-acetic acid

FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
Gal	galactose
Glu	glucose
GST	glutathione S-transferase
H1	histone 1
HA	hemagglutinin
HAT	histone acetyltransferase
HDAC1	histone deacetylase 1
HeLa	human cervical carcinoma cells
His	histidine
HPV16	human papillomavirus 16
IGF-BP3	insulin-like growth factor binding protein 3
IP	immunoprecipitation
Kan	kanamycin
kb	kilo base
kDa	kilo dalton
Leu	leucine
LiOAc	lithium acetate
Lys	lysine
Mad	*Mitotic arrest defective*
MDM2	Murine Double Minute Clone 2
MEFs	mouse embryo fibroblasts
MOPS	3-[*N*-morpholino]propanesulfonic acid
mRNA	messenger RNA
myc	myelocytomatosis
N-	amino
NLS	nuclear localization signal
NRS	normal rabbit serum
O.D.	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCNA	proliferating-cell nuclear antigen
PCR	polymerase chain reaction
Pds1	precocious division of sister chromatids
PEG	polyethylene glycol
PI	propidium iodide
PIG3	p53-induced gene 3
PKA	protein kinase A

PMSF	phenylmethylsulphonylfluoride
PP2A	protein phosphatase 2A
psi	pounds per square inch
Py	polyomavirus
REFs	rat embryo fibroblasts
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
S.cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulphate
Ser	serine
SOD	superoxide dismutase
SV40	simian virus 40
TAE	Tris-acetate/EDTA
T-Ag	large tumour antigen
t-Ag	small tumour antigen
TE	Tris-EDTA
TEMED	N,N,N,N-tetramethylethylenediamine
Thr	threonine
Tris	Tris (hydroxymethyl) amino-methane
Trp	tryptophan
U	unit
UAS	upstream activator sequences
Ura	uracil
UTR	untranslated region
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
X-Gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

CHAPTER ONE

Introduction

When primary cells are placed in culture, they can only proliferate for a limited time after which they stop dividing. This phenomenon, known as cellular senescence, is the subject of extensive research as it is suggested to be the basis of organismal aging *in vivo*. Insights into the regulation of normal cellular aging may also be found from the study of immortalization. This, in contrast to senescence, is a state of unlimited proliferation that cells can acquire, among other ways, through the introduction of viral oncogenes, such as the simian virus 40 (SV40) large tumour antigen (T-Ag).

This thesis describes the research that I carried out in order to identify novel cellular targets of T-Ag, to further elucidate how this viral protein induces growth deregulation in mammalian cells. This introduction is divided into five main sections. In the first two sections I discuss the states of cellular senescence and immortalization. The third section is a brief overview of cell cycle regulation in normal cells. I then go on to describe the various functions and properties of T-Ag and the mechanisms it uses to override cell cycle controls to induce immortalization. Finally, in the last section I describe the yeast two-hybrid system, which is the approach that I used to identify novel T-Ag - interacting proteins.

1.1 CELLULAR SENESCENCE

Aging research is today considered one of the most exciting and promising scientific fields. The quest to understand aging, however, began more than a hundred years ago (historical perspective reviewed in Hayflick, 1997). In 1881 the German biologist August Weismann, although presenting no experimental evidence, suggested that death in higher animals occurs because cells have a finite capacity for cell division and therefore tissues cannot forever renew themselves. Since then, scientific advances have enormously enhanced our understanding of the aging process. An overview of these findings will be presented in this section.

1.1.1 The finite lifespan of normal cells *in vitro*

Normal mammalian cells cultured *in vitro* cannot proliferate indefinitely. They undergo a limited number of divisions before entering a senescent phase, which is termed cellular or replicative senescence. This phenomenon of cellular senescence was first demonstrated in 1961, when Leonard Hayflick and Paul Moorhead showed that normal human fibroblasts grown in culture have a finite mitotic lifespan (Hayflick and Moorhead, 1961). It has since been shown in many laboratories that a variety of other human cell types, as well as fibroblasts of other species undergo senescence, and is now considered a universal phenomenon of cells in culture.

Senescent cells in culture do not die. They are viable cells that can be maintained *in vitro* for long periods and they remain metabolically active as shown by their ability to synthesize mRNA and protein (Lumpkin et al., 1986). However, even though they retain some responsiveness to mitogens and can be induced to synthesize DNA, these cells cannot be induced to

undergo mitosis, re-enter the cell cycle and divide (Tavassoli and Shall, 1988 ; Cristofalo et al., 1989).

1.1.2 Correlation to *in vivo* aging

Is cellular senescence correlated to *in vivo* aging? It is hard to determine this correlation, especially since there is no clear definition of what constitutes biological aging. However, attempts have been made to understand the process of organismal aging by studying aging at the cellular level.

It has been suggested that the finite proliferative potential exhibited by cells cultured *in vitro* is simply an artifact of cell culture, and therefore not correlated to the aging of the organism as a whole. This hypothesis is based on the suggestion that *in vitro*, it is impossible to mimic the natural environment of the cells within the organism. Cells do not retain their three-dimensional environment and their original cell-cell interaction, which are critical for functional cooperation among cells. It is also suggested that the explantation of cells from the organism, their serial subcultivation and enzymatic treatment, and the cell culture conditions, are severely stressful factors. This stress causes the accumulation of damage and results in the decline of growth rate and the subsequent replicative death of the cells (Rubin, 1997). Supporting this idea are two recent studies which have demonstrated that under very specific culture conditions rat Schwann and oligodendrocyte precursor cells can proliferate indefinitely in culture (Tang et al., 2001 ; Mathon et al., 2001).

However, even though physiological conditions cannot be perfectly duplicated in cell culture, there is strong evidence to support the theory that cellular senescence is indeed the manifestation of organismal aging at the cellular level (Hayflick, 1965 ; Smith and Pereira-Smith, 1996 ; Campisi, 1996).

Indefinite cell renewal does not take place in tissues *in vivo*, and this phenomenon is reflected in the *in vitro* cell cultures.

Various observations support this hypothesis. Firstly, experiments have been carried out in different laboratories and using various cell types, to rule out the possibility that cellular senescence is a phenotype observed only due to the artificial environment of cell culture. These experiments were based on the serial transplantations of normal cells into new young animals every time the animal approached old age. So it was suggested that even in the presence of a proper *in vivo* environment, the cells did not proliferate indefinitely (Hayflick, 1997).

Secondly, an inverse correlation has been found between the division potential of cells in culture and the age of the donor from which these cells originated. Cells taken from young donors undergo more rounds of divisions in culture than cells taken from older donors (Hayflick, 1965 ; Bierman, 1978). In addition, it has been shown that cells taken from patients with Werner's syndrome - a rare hereditary premature aging syndrome - have a shorter *in vitro* lifespan than cells taken from normal individuals of the same age (Brown, 1990).

Thirdly, the maximum lifespan of the donor species seems to be directly proportional to the maximum replicative potential of its cells in culture (Rohme, 1981). For example, rodent fibroblast cultures will undergo 20-30 population doublings before entering senescence, whereas human fibroblasts undergo as many as 60-70 population doublings (Bayreuther et al., 1988).

Finally, a biomarker has been identified which is common between cells in culture and tissues *in vivo*. A ß-galactosidase histochemically detected at pH 6, was found to be present both in senescent human fibroblasts and keratinocytes in culture as well as in dermal fibroblasts and epidermal keratinocytes from samples of aging skin (Dimri et al., 1995). This marker increases in intensity and abundance in samples taken from older donors

compared to those from young donors. This provides strong evidence that senescent cells are indeed accumulating with *in vivo* aging, and are therefore not an artifact of cell culture.

It should be noted however, that until there is a clear definition of what constitutes biological aging, the absolute correspondance between cellular senescence and organismal aging still remains to be proved.

1.1.3 Characteristics of the senescent state

Senescence versus quiescence

The senescent state is distinct from another type of growth arrest called quiescence. Quiescent cells are growth arrested in the G0 phase of the cell cycle, whereas senescent cells arrest at late G1 phase and at the G1/S phase boundary (Sherwood et al., 1988), and sometimes at the G2 phase of the cell cycle (Gonos et al., 1996). In addition, quiescence is a reversible state. Quiescent cells growth arrest due to growth factor deprivation or by contact inhibition, and, in contrast to senescent cells, will rapidly re-enter the cell cycle upon the addition of fresh growth factors or by replating.

Senescence versus terminal differentiation

Cellular senescence and terminal differentiation have been suggested to be analogous states, mainly because in both of these states cells undergo irreversible growth arrest and their phenotype is altered (Bayreuther et al., 1988 ; Seshadri and Campisi, 1990). In addition, the two states share a common molecular profile. Key regulatory cell cycle proteins such as the cdk inhibitors p16, p21 and p27 as well as pRb and p130 which are involved in cellular senescence, have been shown to be associated with the induction of terminal differentiation (Harvat et al.1998).However, even though senescence and terminal differentiation share common features, evidence suggests that

they are independent cellular pathways. In the case of the epidermis, for example, cellular senescence is exhibited when skin stem cells reach the end of their proliferative capacity, which in turn reduces cell renewal and contributes to skin aging. On the other hand, terminal differentiation is a much more active form of growth arrest exhibited by keratinocytes with the purpose of performing a specialized function, i.e. to move from the basement membrane upwards, differentiate into cornified squames and form the protective skin layer (Gandarillas, 2000).

Molecular changes during senescence

In addition to their irreversible growth arrest senescent cells have various other characteristics. Studies have shown that extensive molecular differences exist between young and senescent cells cultured *in vitro*, both at the mRNA and at the protein level (Lumpkin et al., 1986 ; Seshadri and Campisi, 1990 ; Goldstein, 1990 ; Smith and Pereira-Smith, 1996 ; Hubbard and Ozer, 1995). These include mRNAs and proteins of the cell cycle, proteins of the extracellular matrix, mitochondrial proteins, as well as differences in chromosomal DNA structure.

Changes in the expression and activity of important cell cycle proteins is one of the main characteristics of the senescent phenotype. The expression of the cyclin-dependent kinase (Cdk) inhibitors p16 and p21 increases significantly in senescent cells (Alcorta et al., 1996 ; Tahara et al., 1995). Overexpression of both of these proteins in normal early passage cells results in a premature senescence phenotype (McConnell et al., 1998). Similarly, expression of p16 in malignant cells reverts their immortal phenotype (Uhrbom et al., 1997), and disruption of the p21 gene enables normal cells to bypass senescence (Brown et al., 1997). In addition, the tumour suppressor protein p53 which induces the expression of p21, is found to be more transcriptionally active in senescent cells (Atadja et al., 1995).

Other changes identified in senescent cells include the increased expression of extracellular matrix proteins like fibronectin (Kumazaki, 1992), collagenase and stromelysin (Millis, 1992), as well as mitochondrial changes like the accumulation of mitochondrial deletions (Cortopassi, 1992), and the changes in expression of mitochondrial proteins, e.g. the decreased expression of the mitochondrial prohibitin proteins (Coates et al., 2001) and the overexpression of the Mn-superoxide dismutase (SOD) and the cytochrome *c* oxidase (Kumar, 1993).

These are only some of the changes that have been observed in cellular senescence. Many other genes have been found to be differentially expressed between young and senescent cells. It should be noted, however, that not all of these changes are observed in every senescent cell. A comparison of three different cell types - dermal fibroblasts, retinal pigment epithelial cells and vascular endothelial cells - using DNA microarray analysis to compare the profiles of early- and late-passage cells, has revealed that the pattern of gene expression at senescence varies considerably from one cell type to another (Shelton et al., 1999).

1.1.4 Theories of aging

The elucidation of the causes of aging is a biological problem that has generated extensive research. In fact, it has been estimated that over 300 theories of aging have been proposed so far (Brown, 2000). These theories generally fall into two broad categories, which suggest that senescence is either a stochastic or a programmed event.

Stochastic theories

The stochastic theories suggest that cellular senescence occurs as a result of the random accumulation of cellular damage (Orgel, 1973). Based on

these theories, when cells divide *in vitro* they accumulate various forms of damage like mutations, karyotypic alterations and loss of DNA methylation. This damage leads to genomic instability and to the altered expression of key growth regulatory molecules, which results in the loss of proliferative potential i.e., cellular senescence (Sherwood et al., 1988).

Accumulation of cellular damage is also the mechanism proposed by the "oxidative stress" theory of aging. According to this hypothesis, respiration and other normal biological processes within cells, generate a variety of toxic by-products, which include some highly reactive oxygen species. Anti-oxidant cellular defenses are not efficient enough to completely eliminate these species from the cell, and so some are left to attack both DNA and protein molecules. It is proposed that their accumulation over time results in the irreversible cellular damage that causes senescence (Harman, 1981 ; Stadtman, 1992 ; Ames, 1993).

This hypothesis is supported by observations from various species including *C.elegans*, *Drosophila* and mice (Finkel and Holbrook, 2000). Mutations in the *C.elegans age-1* gene which increase both the mean and the maximum lifespan of the organism (Klass, 1983 ; Friedman and Johnson, 1988a ; Friedman and Johnson, 1988b), were shown to confer resistance to oxidative stress by elevated expression of SOD and catalase compared to wildtype nematodes (Larsen, 1993 ; Vanfleteren, 1993). Similarly, experiments in *Drosophila* have shown that the simultaneous overexpression of SOD and catalase in transgenic flies results in lower levels of oxidative damage and an extended lifespan (Orr and Sohal, 1994). In mammals, supporting evidence for the oxidative stress theory comes mainly from experiments in mice, where disruption of the mouse $p66^{shc}$ gene was shown to enhance the resistance of mice to damaging oxidative agents and to increase their lifespan by 30% (Migliaccio et al., 1999).

Finally, two other theories are based on the accumulation of cellular damage. One is the "mitochondrial theory", which suggests that aging is caused by the accumulation of defective mitochondria with time (Harman, 1981) that harbor mitochondrial DNA alterations (Cortopassi, 1992). The other is the "caloric intake" theory, which proposes that the rate of aging is in direct relation with the metabolic rate, supported by caloric restriction

experiments, where limiting food intake has shown to prolong lifespan in many species (Masoro, 2000). Again, oxidative stress may be the basis of both of these theories. Mitochondria are the sites where oxidative phosphorylation takes place, a process that generates many oxygen species and, therefore, the accumulation of mitochondrial DNA alterations may be the result of continuous exposure to oxygen free radical damage (Wallace, 1999 ; Raha and Robinson, 2000). Likewise, caloric restriction extends lifespan by lowering the metabolism and therefore, by limiting oxidative stress (Sohal and Weindruch, 1996).

However, it has recently been proposed that caloric restriction may also regulate lifespan by directly affecting gene expression, using a mechanism independent of oxidative stress. Experiments in yeast have shown that the extension of lifespan in response to caloric restriction is dependent on the presence of the Sir2 protein, a NAD-dependent histone deacetylase which regulates gene transcription by silencing, therefore providing a link between metabolism, gene expression and aging (Imai et al., 2000 ; Lin et al., 2000).

Genetic program

In contrast to the stochastic theories, the genetic theories suggest that cellular senescence is a programmed event governed by the genome. This is mainly supported by the up- or down-regulation of various genes in senescent cells compared to young cells, as described earlier.

It has been suggested that a biological clock mechanism exists in cells. This clock does not count chronological time, instead it counts the number of cell divisions and determines the entry into senescence when a specific number of divisions has taken place. Components of this clock would be proteins whose expression, phosphorylation or other modification, changes as cells accumulate divisions. In addition to p16 and p21, which accumulate in cells approaching senescence (see 1.1.3), other proteins involved in

senescence and implicated in the biological clock are $p27^{Kip1}$, $p19^{ARF}$, p24 and $p33^{ING1}$.

$p27^{Kip1}$ is a Cdk inhibitor which progressively accumulates in oligodendrocyte precursor cells as they proliferate, and is expressed at high levels in post-mitotic terminally differentiated oligodendrocytes, and is therefore, part of an intrinsic timer that arrests the cell cycle (Durand et al., 1997 ; Durand et al., 1998 ; Burton et al., 1999). In addition, p27-null mice have increased number of cells in many organs suggesting that in addition to the oligodendrocyte cell lineage, p27 is involved in the control of proliferation in many other cell types (Fero et al., 1996). $p19^{ARF}$ is the protein encoded by the alternative reading frame of the p16 locus, and even though it is not a cdk inhibitor like p16, it can induce cell cycle arrest in a p53-dependent manner. Fibroblasts derived from $p19^{ARF}$-null mice proliferate continuously and do not undergo senescence (Kamijo et al., 1997). p24 and $p33^{ING1}$ are two other proteins which accumulate in senescent rodent and human fibroblasts respectively, and are suggested to be involved in the measurement of the replicative lifespan of cells (Mazars and Jat, 1997 ; Garkavtsev and Riabowol, 1997).

In addition to these proteins, the biological counting mechanism of human cells is now thought to be primarily regulated by telomeres. Telomeres are the structures at the end of the chromosomes consisting of repetitive TTAGGG sequences (Moyzis et al., 1988). These short sequences ensure the complete replication of the chromosomes and protect them from degradation, fusion, and recombination. Telomeres are synthesized by the enzyme telomerase (Greider and Blackburn 1985). However, adult somatic cells do not express telomerase, and due to the incomplete replication of DNA at the ends (the *"end replication problem"*), the chromosomes lose approximately 50 bp of telomeric DNA after each cell division (de Lange, 1994). It has been shown that the telomeres of human cells decrease after passaging *in vitro*

(Harley et al., 1990), and that ectopic expression of telomerase results in an extension of life span (Bodnar et al., 1998 ; Vaziri and Benchimol, 1998). It is therefore proposed that in human cells this progressive shortening of the telomeres is the biological clock, and that senescence is triggered when the chromosomes have reached a crirtical size.

It is possible that a common pathway exists in humans, that links the stochastic and the genetic theories, and more specifically, the oxidative damage theory with the telomere clock theory. Interestingly, it has been shown that in human fibroblasts the rate of telomere attrition increases in the presence of oxidative damage (Petersen et al., 1998). Furthermore, fibroblasts taken from patients with premature aging syndromes, which have been shown to have very short telomeres (Kruk et al., 1995), also contain decreased levels of antioxidant enzymes (Yan et al., 1999). This data links aging with both telomeres and DNA damage, and suggests that senescence is regulated by a genetic clock counting telomere length, which may run faster or slower depending on "stochastic environmental factors", i.e. the presence of damaging oxidative species.

Finally, it should be noted, that expression of oncogenic *ras* in primary human or rodent cells has been shown to provoke cells to senesce prematurely, proposing that senescence may also be triggered in response to a certain stimulus, and not solely dependent upon the completion of a certain number of divisions (Serrano et al., 1997 ; Zhu et al., 1998).

In conclusion, it is clear that aging is one of the most difficult and complex biological problems. As Nobel Laureate Francois Jacob once wrote: *" It is truly amazing that a complex organism, formed through an extraordinarily intricate process of morphogenesis, should be unable to perform the much simpler task of merely maintaining what already exists."* (Arking,1998).

1.2 IMMORTALIZATION

Further insights into the mechanisms of senescence could be found by the study of the opposite phenomenon, which is immortalization. Cells that have overcome senescence and have acquired an infinite proliferative potential, are said to have become immortal.

Immortal cells can either arise spontaneously, or be induced by radiation, chemical carcinogens, or the introduction of viral and cellular oncogenes.

1.2.1 Rodent versus human immortalization

Often, when rodent embryo fibroblasts are continuously passaged, some cells will spontaneously become immortal (Curatolo et al., 1984). The exact nature of the lesions enabling these cells to escape senescence is not yet clearly defined. However, alterations of the p53 negative growth regulator (Rittling and Denhardt, 1992), as well as increased transcription of the c-*myc* cellular oncogene (Tavassoli and Shall, 1988), have been observed in some spontaneously immortal cell lines.

Furthermore, rodent fibroblasts are efficiently immortalized by the introduction of cellular or viral oncogenes. Cellular immortalizing oncogenes include *myc* (Kelekar and Cole, 1987), *fos* (Jenuwein and Muller, 1987), and *jun* (Schutte et al., 1989), and some mutant forms of p53 (Jenkins et al., 1984 ; Jenkins et al., 1985). Viral oncogenes capable of immortalizing rodent cells include the early region 1A (E1A) gene of adenovirus (Ad) (Ruley, 1983), the E7 gene of human papillomavirus type 16 (HPV16) (Vousden et al., 1988) and the large tumour antigen (T-Ag) of polyomavirus (Py) and simian virus 40 (SV40 ; Jat and Sharp, 1986).

In contrast, spontaneous immortalization of human fibroblasts is extremely rare, and immortal cells, even after the introduction of oncogenes, are obtained at a very low frequency. This difference between the two species, can be explained by a two-stage model of senescence for human cells (Wright et al., 1989). According to this model, when normal human fibroblasts are grown in culture, they undergo a limited number of divisions before entering cellular senescence, called Mortality stage 1 (M1). These cells can be pushed to cycle further past M1 by the introduction of viral oncogenes, however, their lifespan is extended only by a few population doublings, after which almost all cells undergo crisis and cease dividing, what is called Mortality stage 2 (M2). Therefore, immortalization of human cells requires the bypass of two mortality stages. Spontaneous immortalization is extremely rare since the probability for both stages to be overcome in the same cell is very low. Rodent cells can spontaneously immortalize and are also readily immortalized by oncogenes, meaning that they either lack M2 or they have a greater capacity to overcome it (Gonos et al., 1992).

It should also be noted at this point, that there is a significant difference between rodent and human cells regarding the biological counting mechanism. As described previously, the mitotic clock in human cells is thought to be the progressive telomere shortening of chromosomes (Harley et al., 1990). In normal human somatic cells telomerase activity is absent, whereas in the majority of immortal cell lines telomerase is reactivated and telomere length is stabilized (Counter et al., 1992). In contrast, rodent cells have very long telomeres, and telomerase activity is present in normal somatic cells (Prowse and Greider, 1995). Furthermore, telomerase-null mouse cells can be immortalized and transformed with the same efficiency as telomerase positive cells (Blasco et al., 1997). Therefore, telomerase is not required for the immortalization and transformation of rodent cells, and senescence is most

likely to be regulated by a telomere-independent clock (Wright and Shay, 2000).

Taken together, the evidence shows that while research in rodents is an indispensable tool for human research, due to the differences between the rodent and the human system, findings in one species should never be unequivocally accepted for the other.

1.2.2 The relation between senescence, immortality and cancer

Immortalization versus tumourigenesis

It is important to note that immortalized cells are not tumourigenic. Even though they have a reduced requirement for mitogens, they are still dependent on them and they cannot overgrow a confluent monolayer or cause tumours if introduced into mice, i.e. they lack the properties of fully transformed cells. The transformation of immortalized cells into malignant cells, requires the introduction of a second oncogene (Land et al., 1983), or a spontaneous mutational event (Land et al., 1986).

Even though immortalization alone is not sufficient for tumourigenesis, it is, however, a prerequisite for its development (Newbold et al., 1982 ; Newbold and Overell, 1983). The progression of cells to the malignant state is a multistep process, and it is unlikely that a tumour could develop without having overcome the finite proliferative lifespan (Reddel, 2000).

Common pathways

It is important to note that growth regulatory proteins and mechanisms implicated in cellular senescence and immortalization, are also linked to tumourigenesis. As described in section 1.1, the cell cycle proteins p53 and p16 are involved in the regulation of the cellular senescence, as is the attrition of telomeric ends on chromosomes. Similarly, the inactivation of p53 and p16

(Rittling and Denhardt, 1992 ; Rogan et al., 1995 ; Munro et al., 1999), as well as the elongation of telomeres (Counter et al., 1992), are changes associated with immortality.

It is therefore of great interest that, inactivation of p53 and p16 are two of the most common alterations in human tumours (Levine et al., 1994 ; Serrano, 1997), and telomerase activity is found in the majority of human cancers (Kim et al., 1994). The fact that the same genetic alterations that confer the immortal phenotype are also characteristic traits of cancer, strongly suggests that senescence is a tumour suppressor mechanism that safeguards against neoplasia (McCormick and Campisi, 1991 ; Smith and Pereira-Smith, 1996 ; Parkinson et al., 2000). In addition, studies showing that the senescent phenotype can be triggered prematurely in response to an oncogenic stimulus or a proliferative stress, further reinforce the role of replicative senescence as a mechanism for restricting cancer progression (Serrano et al., 1997 ; Lee et al., 1999a).

The states of senescence, immortality and cancer are therefore closely linked, and the study of one could provide insights into the mechanisms of the other.

1.3 CELL CYCLE OVERVIEW

In order to understand how T-Ag deregulates the cell cycle to induce immortalization, this section will give an overview of cell cycle regulation in normal cells in the absence of T-Ag (summarized in Figure 1.1), and will focus mainly on the proteins targeted by T-Ag (see 1.4.3).

1.3.1 Cyclins, Cdks and Cdk Inhibitors

The eukaryotic cell cycle is divided into four phases: G1, the phase during which the cell prepares for DNA synthesis; S, the DNA synthesis phase; G2, the preparatory phase for mitosis; and M, the mitosis.

Progression through the cell cycle phases is regulated by the successive assembly and activation of cyclin/cdk complexes. The cyclin is the regulatory subunit of the complex, and the cdk is the catalytic subunit which catalyses the phosphorylation of specific substrates important for cell cycle progression. Whereas the levels of the cyclin component are regulated by synthesis and degradation, the cdk subunit is activated by the binding of the cyclin subunit and by phosphorylation/de-phosphorylation on specific residues, and is inhibited by the binding of specific cdk inhibitors (Nigg, 1995 ; Sherr and Roberts, 1999). There are two families of cdk inhibitors: the INK4 family which consists of p15, p16, and p18, which inhibit cyclin D/cdk4 and cdk6 complexes ; and the Cip/Kip family consisting of p21, p27 and p57 which inhibit cyclin D, E and A/cdk complexes (Sherr and Roberts, 1999).

Different cyclin/cdk complexes are required at different phases of the cell cycle (Kamb, 1995 ; Gao and Zelenka, 1997 ; Johnson and Walker, 1999). For example, cyclin D/cdk 4 and cyclin D/cdk 6 complexes form during early G1, cyclin E/cdk 2 at the G1/S transition, cyclin A/cdk 2 during S phase, cyclin A/cdk 1 during G2, and cyclin B/cdk 1 at the G2/M transition.

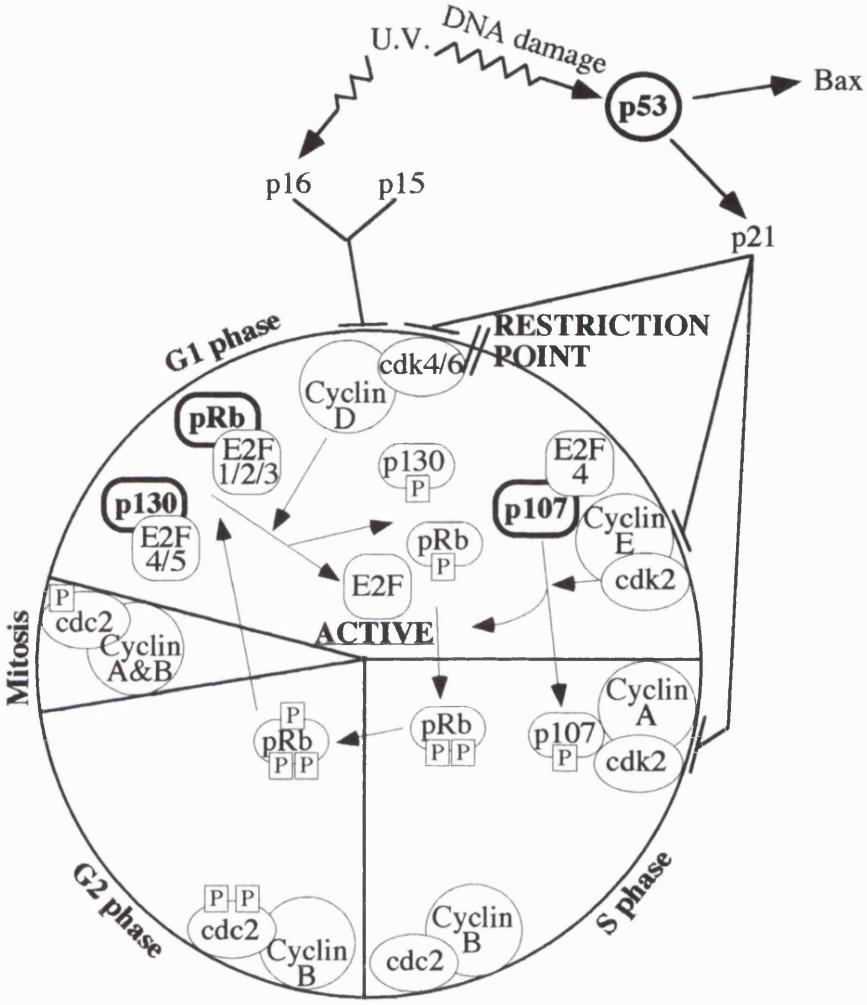


Figure 1.1 Summary of cell cycle regulation. The proteins with which large T antigen interacts are indicated in bold. Taken from Darmon and Jat, 2001.

1.3.2 The pRb family of proteins

When a cell has divided and has returned to G1, it has to decide whether to enter the S phase and ultimately undergo another round of division, or whether to exit the cell cycle and become quiescent (i.e., enter G0). The cell decides this by assessing the availability of extracellular mitogenic signals two thirds into the G1 phase at a point called the "R" (restriction) point (Pardee, 1989). Once a cell has passed the R point, it is then fully committed to completing the rest of the cell cycle, and it is no longer dependent on mitogens to do so. After the R point, progression through the cell cycle can only be halted in the event of severe cellular damage (Planas-Silva and Weinberg, 1997).

Studies to investigate the nature of the R point transition, have shown that this point in G1 coincides with the phosphorylation of the retinoblastoma (pRb) susceptibility protein (Weinberg, 1995). In early G1, pRb is in an underphosphorylated state which is inhibitory for the G1/S transition. As the cell progresses through G1, pRb gets increasingly phosphorylated, resulting in a hyperphosphorylated form which lifts the G1/S blockade. pRb remains hyperphosphorylated throughout the remainder of the cell cycle, until it enters again the following G1 phase (Chen et al., 1989 ; DeCaprio et al., 1992). The phosphorylation of pRb is dependent on the activity of the cyclin D-dependent kinases cdk 4 and cdk 6 (Kato et al., 1993), and can be inhibited by the binding of the cdk inhibitors p16 and p21 (Serrano et al., 1993 ; Harper et al., 1993).

Underphosphorylated pRb is found in a complex with the E2F transcription factor (Hiebert et al., 1992 ; Flemington et al., 1993). The hyperphosphorylation of pRb allows its dissociation from E2F, which is then released and is free to activate the transcription of its target genes (Suzuki-Takahashi et al., 1995 ; Dyson, 1998). E2F targets include DNA synthesis

genes (DNA polymerase α, thymidine kinase, PCNA) as well as cell cycle genes (cyclin A, cyclin E, cdc2) (Lavia and Jansen-Durr, 1999), which proves that E2F is important for driving the cell towards S phase.

pRb is a member of a family of three proteins which share homology in their protein-binding "pocket" domains (also called the "pocket" family of proteins). The other two proteins are p107 and p130 (Ewen et al., 1991 ; Mayol et al., 1993 ; Mulligan and Jacks, 1998). p107 and p130 also associate with E2F, but have been found to regulate the transcription of different E2F target genes than pRb (Hurford et al., 1997).

The E2F transcription factor is a heterodimeric factor consisting of an E2F subunit and a DP subunit. Six E2F proteins and three DP proteins have been identified so far in mammalian cells, each preferentially binding different members of the pRb family at different points in the cell cycle (Figure 1.1) (Dyson, 1998 ; Lavia and Jansen-Durr, 1999). The pRb family of proteins inhibit the transcriptional activity of the E2F proteins via two methods. Firstly, by directly binding and blocking the E2F activation domains (Flemington et al., 1993), and secondly, by recruiting the histone deacetylase HDAC1, a protein which removes acetyl groups from histone and therefore, condenses the chromatin and reduces the accessibility for transcription factors (Ferreira et al., 1998 ; Luo et al., 1998).

1.3.3 p53

After passage through R, the next important step in the cell cycle is the G1/S transition. At this point, the cell has to ensure that there is no DNA damage before initiating DNA replication. The cdk inhibitor p16 can induce G1 arrest in response to DNA damage (Shapiro et al., 1998), however, the main protein responsible for the G1/S checkpoint is p53 (Levine, 1997). Depending on the extent of the DNA damage, p53 will either drive the cell

into temporary cell cycle arrest (until the damage is repaired), or into apoptosis (if the damage is so severe it cannot be repaired).

Under normal conditions, p53 has a very short half-life and is therefore found at very low levels in the cell. This is due to its association with the p53 inhibitor MDM2, which constantly promotes the degradation of p53 by the proteasome (Haupt et al., 1997 ; Momand et al., 2000). When there is DNA damage however, p53 levels accumulate rapidly, mainly by decreased degradation and to a lesser extent by increased translation (Kubbutat and Vousden, 1998). The increase in the amount of p53 is dependent on the extent of the damage and determines cell fate, that is, low levels of p53 induced by modest DNA damage lead to temporary growth arrest, whereas higher levels of p53 induced by severe damage lead to the apoptotic pathway (Chen et al., 1996).

The mechanism by which p53 induces both cell cycle arrest and apoptosis, is through the transactivation of its target genes. p53 transactivates a variety of genes that contain p53-responsive elements (the cdk inhibitor p21, the nucleotide excision repair protein GADD45, the p53 inhibitor MDM2, the growth-inhibitory protein IGF-BP3, the apoptotic protein Bax, the apoptotic protein PIG3, and cyclin G ; Wiman, 1997).

p53 induces G1 arrest through the transactivation of p21 (El-Deiry et al., 1994). As described earlier, p21 inhibits the cyclin D-dependent kinases cdk4 and cdk6 and thus prevents the phosphorylation of pRb. Underphosphorylated pRb remains bound to E2F and the E2F-responsive genes required for progression into S phase are not induced, therefore the cell arrests in G1. In addition, p21 binds and inhibits PCNA, a component of the DNA replication machinery. The association with p21 blocks the ability of PCNA to activate DNA polymerase δ and therefore inhibits DNA replication (Waga et al., 1994).

p53 induces the apoptotic pathway mainly through the transactivation of Bax (Miyashita and Reed, 1995). Bax promotes apoptosis by heterodimerizing with Bcl-2, an anti-apoptotic protein thought to promote cell survival by blocking the activation of the cysteine proteases called caspases (Adams and Cory, 1998).

Finally, in addition to the direct DNA damage by ionizing radiation and chemicals, p53 is also activated in response to stress signals such as hypoxia, ribonucleotide depletion and heat shock, and is also implicated later in the cell cycle, at the G2/M checkpoint and the mitotic spindle checkpoint (Levine, 1997 ; Oren, 1999). It is clear from all these functions, that p53 is crucial for protection against the proliferation of "damaged" cells, and this may explain why it is the most commonly mutated gene in all human cancers (Levine et al., 1994).

1.3.4 Mitotic checkpoints

Finally, after progressing through S and into G2, the next phase where the cell has to ensure that the cell cycle is proceeding normally is mitosis. Mitotic checkpoints consist of the DNA damage checkpoint and the spindle checkpoint.

The first checkpoint is the cell's mechanism for preventing mitosis in the event of DNA damage. This damage could result from mistakes introduced by the polymerases during replication, by incomplete DNA replication, by ionizing radiation, by free radicals, or by incomplete DNA repair (Lengauer et al., 1998). Key proteins of the DNA damage checkpoint in G2/M include the ataxia telangiectasia mutated protein (ATM) which is involved in the recognition and repair of damaged DNA, as well as the Chk1 kinase, which in the presence of DNA damage inhibits the activation of the

cyclin B/cdc2 complex and therefore blocks the entry into mitosis (Rhind and Russell, 1998).

The spindle checkpoint is a surveillance mechanism, which monitors the proper assembly of the mitotic spindle and is essential for ensuring the accurate segregation of chromosomes, thus preventing any gain or loss of genetic material (aneuploidy). When a spindle defect, like failure of chromosomes to attach to the spindle is detected, the checkpoint mechanism generates a signal that blocks cell cycle progression until the defect is repaired (Amon, 1999). This signal is transmitted through the proteins of the mitotic checkpoint machinery. Genetic screens in budding yeast have identified some of the components of this machinery, which is conserved in higher organisms including humans. These include the *Bub* (*Bub1* , *Bub2* , *Bub3*) and *Mad* (*Mad1* , *Mad2* , *Mad3*) family of genes (Hoyt et al., 1991 ; Li and Murray, 1991). Spindle checkpoints are crucial for maintaining genome integrity, since aneuploidy is the leading cause of genetic instability in cells, and is observed in almost all types of cancer (Lengauer et al., 1998 ; Pihan and Doxsey, 1999). The spindle checkpoint will be further discussed in chapter 5.

1.4 THE SV40 LARGE T ANTIGEN

The SV40 T-Ag is one of the viral oncogenes capable of immortalizing mammalian cells. An enormous amount of research on T-Ag has been carried out since the early 1960's, which makes it impossible to cover all the information acquired on this protein in this thesis. This section will cover some aspects of this research, by first introducing the SV40 virus, and then describing T-Ag and discussing its immortalizing function and its association with various cellular proteins. The final section will focus on the functions of the N-terminal domain of the protein, which is the specific region of T-Ag studied in this project.

1.4.1 The SV40 small DNA tumour virus

SV40 is a member of the papovavirus family of small double-stranded DNA tumour viruses (Tooze, 1981). Its genome is 5243 bp, divided into an early and a late region (Figure 1.2). The early region encodes three proteins called large T, small t and tiny t antigens, from differentially spliced mRNAs of a common translational start site (Griffin, 1981). The late region encodes the virion capsid proteins VP1, VP2 and VP3. These are generated by alternative initiation codons and not by differential splicing like the early proteins.

The permissive host of SV40 is the African green monkey. This means that when SV40 infects monkey cells the lytic cycle of the virus takes place, which results in the production of new viral particles and eventually cell lysis. During lytic infection, the virus is adsorbed in the cell and penetrates the nucleus, the viral genome is uncoated and the early genes are expressed (which are required for the initiation of replication, and for using the host replication machinery), and viral DNA replication is carried out. After viral

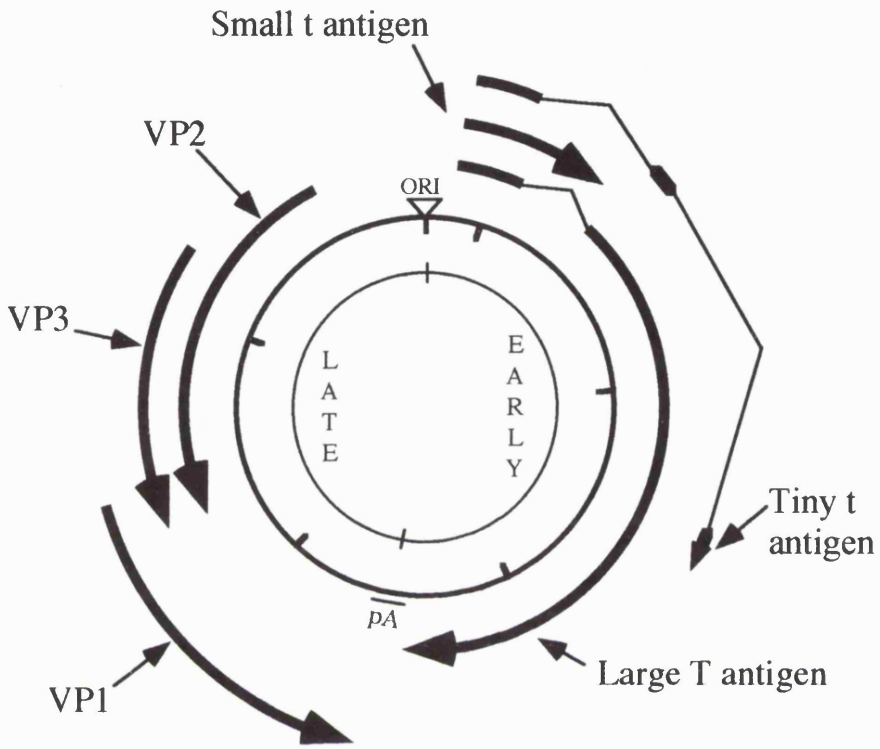


Figure 1.2 Structure of the SV40 genome. The early and late regions and their corresponding proteins are shown. ORI is the origin of replication and pA is the polyadenylation signal sequence. Taken from Darmon and Jat, 2001.

replication, the late genes are expressed to provide the structural components for the new viruses, which are then assembled and exit the cell by lysis.

In contrast, rodents are non-permissive hosts for SV40, so the infection in rodent cells is abortive, and the lysogenic cycle of the virus takes place instead. In this case, SV40 cannot replicate and produce new virions, however the viral DNA may integrate into the host genome and the early genes can be expressed. The expression of the T-Ag early gene in non-permissive cells has been shown to stimulate cellular growth (see below).

1.4.2 The large T antigen

T-Ag is a multidomain protein of 708 amino acids. It mainly localizes to the nucleus by a nuclear localization signal (NLS) in the N-terminus, although a very small fraction is cytoplasmic and some bound to the plasma membrane (Kalderon et al., 1984). T-Ag has various biochemical functions and can associate with a number of cellular proteins (Figure 1.3 ; Fanning and Knippers, 1992).

Functions of T-Ag

T-Ag is a multifunctional protein. It has DNA and RNA helicase activity (Stahl et al., 1986 ; Scheffner et al., 1989), topoisomerase activity (Marton et al., 1993), ATPase activity (Tjian and Robbins, 1979 ; Clark et al., 1981), it binds RNA covalently (Carroll et al., 1988), and it can bind DNA both specifically and non-specifically (Carroll et al., 1974 ; Prives et al., 1982). T-Ag also has the ability to activate and repress transcription from a number of viral and cellular promoters (Hansen et al., 1981 ; Mitchell et al., 1987 ; Feuchter and Mager, 1992 ; Gilinger and Alwine, 1993 ; Gruda et al., 1993 ; Rice and Cole, 1993 ; Sompayrac, 1997 ; Sompayrac et al., 1998).

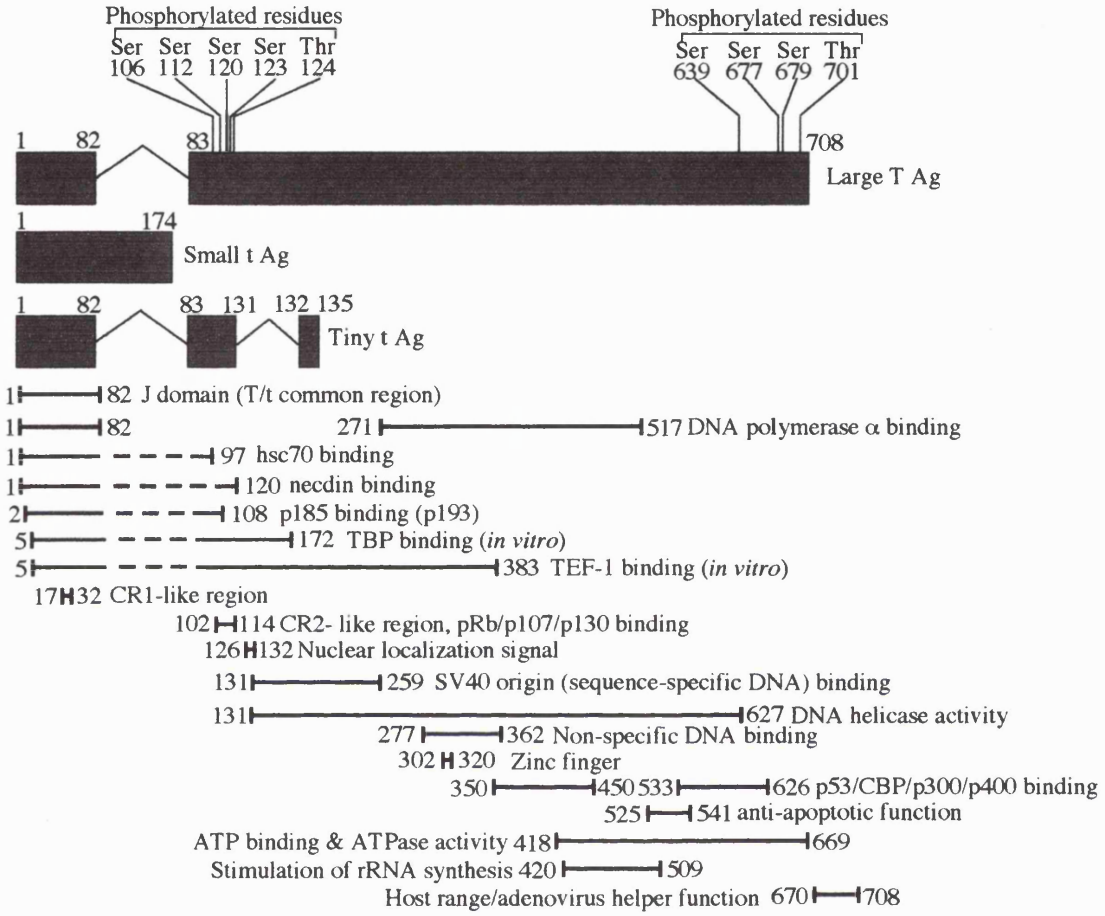


Figure 1.3 Schematic representation of the T-Ag functional domains. Adapted from Darmon and Jat, 2001.

Association of T-Ag with cellular proteins

T-Ag has the ability to bind a number of host cellular proteins. These include p53 (Lane and Crawford, 1979 ; Linzer and Levine, 1979), pRb (DeCaprio et al., 1988), p107 (Dyson et al., 1989), p130 (Hannon et al., 1993), DNA polymerase α (Smale and Tjian, 1986 ; Gannon and Lane, 1987 ; Dornreiter et al., 1990), Hsc70 (Sawai and Butel, 1989 ; Sawai et al., 1994), the CREB-binding protein (CBP) and p300 (Avantaggiati et al., 1996 ; Eckner et al., 1996 ; Lill et al., 1997a). From these, the proteins important for immortalization will be discussed further in the next sections.

In addition, the association of T-Ag with other cellular proteins is not yet fully characterized, but could prove to be very important in the immortalization and transformation process. These include necdin, a neuron-specific growth suppressor protein (Taniura et al., 1998) and p185, a protein of unknown function (Kohrman and Imperiale, 1992), although recent data suggests it could be a novel apoptosis-promoting protein (Tsai et al., 2000). Preliminary evidence also suggests that T-Ag could associate with the SUG1 proteasome component (Grand et al., 1999).

T-Ag and immortalization

T-Ag has the ability to immortalize primary rodent cells (Petit et al., 1983 ; Jat and Sharp, 1986) and primary human cells at a low frequency (Shay et al., 1993), and to transform established cell lines to tumourigenicity (Brown et al., 1986).

It is interesting to think of these properties of T-Ag as a function of its evolution. As a viral protein, T-Ag is highly unlikely to have evolved to infect and immortalize non-permissive cells, because this is of no advantage to the virus with respect to its propagation (Fanning and Knippers, 1992). On the other hand, in permissive cells the function of T-Ag is to drive resting cells back into the cell cycle and stimulate DNA synthesis, in order to have access

to the replication machinery that it requires and that quiescent cells cannot provide. Therefore, immortalization can be viewed as a "by-product" of T-Ag's ability to induce DNA synthesis and stimulate cell growth for its own advantage.

T-Ag immortalization studies have focused on rodent cells, since infection with SV40 results in a 100% of these cells becoming immortalized, whereas human cells are harder to immortalize (Jha et al., 1998). The immortal phenotype of these cells is dependent on the presence of T-Ag, since its inactivation results in growth arrest (Jat and Sharp, 1989 ; Gonos et al., 1996).

Only some of the activities of T-Ag are required for its ability to immortalize and transform rodent cells. In order to identify which are the critical functions, a number of T-Ag mutants have been generated and used in various assays. The outcome of these studies is somewhat confusing, since conflicting results are sometimes obtained depending on the assays and the cells used. Assays normally used include the ability to induce DNA synthesis in quiescent cells, the immortalization of primary cells, the transformation of primary cells in cooperation with a second oncogene, the transformation of established cell lines, and the ability to induce tumours in model animals. A number of different cells are also used, including rat embryo fibroblasts (REFs), mouse embryo fibroblasts (MEFs), rat REF52 fibroblasts and mouse C3H10T1/2 fibroblasts. REFs and MEFs are primary cells, while REF52 and C3H10T1/2 are established cell lines. Established cell lines already carry alterations that enable them to proliferate continuously, therefore, immortalization assays should be carried out using primary cells (Conzen and Cole, 1995 ; Darmon and Jat, 2001). It is important to note however, that even when using primary cells, during the course of the immortalization assay, primary REFs and MEFs have the ability to spontaneously immortalize (see section 1.2.1).

Focusing on immortalization, assays on REFs have shown that the N-terminus of T-Ag is sufficient for inducing the immortal phenotype (amino acids 1 to 137 for primary REFs and amino acids 1 to 311 for secondary REFs) (Colby and Shenk, 1982 ; Asselin and Bastin, 1985). In MEFs, immortalization is dependent on the activity of three regions, the extreme N-terminal region (aa 1 to 82), the N-terminal pRb-binding domain (aa 102 to 114), and the p53-binding domains (aa 350 to 450 and 533 to 560) (Conzen and Cole, 1995). Taken together, the importance of the N-terminal domain in immortalization is evident, and interestingly, even in transformation assays the N-terminus plays a critical role both in rat and mouse cells (Sompayrac and Danna, 1988, 1991, 1992 ; Zhu et al., 1992). The N-terminal domain function will be discussed separately in the final section.

The C-terminal domain of T-Ag

Although the N-terminus carries critical functions, the C-terminal domain is required for efficient immortalization. The most important C-terminal interaction of T-Ag is the binding of p53 (Lane and Crawford, 1979 ; Linzer and Levine, 1979). Two domains of T-Ag are required for this interaction, one between amino acids 350 to 450 and one between 533 to 626 (Zhu et al., 1991 ; Kierstead and Tevethia, 1993). The interaction with T-Ag stabilizes p53, and significantly increases its half-life, which in normal cells is very short (Oren et al., 1981). At the same time, the binding of T-Ag to p53 is thought to inhibit the p53 transactivation function by preventing it from binding to DNA (Segawa et al., 1993), although recent evidence suggests that T-Ag can inhibit p53 function even with intact DNA binding (Sheppard et al., 1999). Thus, the stabilization of p53 leads to increased levels of p53, which is however transcriptionally inactive (Mietz et al., 1992). T-Ag therefore, abrogates the p53-dependent G1 checkpoint, and cells cannot arrest in response to DNA damage. In addition, p53-dependent apoptosis is

also thought to be inhibited by the binding of T-Ag (McCarthy et al., 1994). Blocking apoptosis is important in inducing immortalization, since apoptosis is one of the cell's defense mechanisms against deregulated growth.

In addition to binding p53 for the purpose of inactivating its function, it is proposed that the metabolic stabilization of p53 by T-Ag is an active process, which correlates with the efficiency of transformation. Presumably, the enhanced levels of the stabilized protein cause p53 to act as a cooperating oncogene in SV40 transformation (Tiemann and Deppert, 1994). This seems to be the case, because if no function of p53 was required by SV40, then inactivation of p53 function could also be achieved by p53 degradation. However, T-Ag has been found to also interact with the p53 inhibitor mdm2 which targets p53 for proteolysis, thereby preventing its degradation and promoting its stabilization (Henning et al., 1997).

T-Ag is also thought to bind pCBP and p300 within its C-terminus (Avantaggiati et al., 1996 ; Eckner et al., 1996 ; Lill et al., 1997a). CBP and p300 are transcriptional co-factors involved in a number of cellular processes, and are mainly involved in transcription regulation, through the bridging of transcription factors with the basal transcriptional machinery (Giles et al., 1998). p400, a protein structurally related to p300, has also been shown to bind T-Ag (Lill et al., 1997a). The CBP/p300 proteins are found in a stable complex with p53 in normal cells, suggesting that they are p53 transcriptional regulators (Gu et al., 1997 ; Lill et al., 1997b). The binding of T-Ag to CBP/p300 requires the p53-binding domain of T-Ag and therefore, it seems that T-Ag binds CBP/p300 through p53. So, in addition to directly sequestering p53, T-Ag also deregulates p53 function by disrupting the association with its transcriptional co-factors.

In addition to the p53 and p300/CBP binding, the C-terminus of T-Ag carries an anti-apoptotic function mapped to amino acids 525 to 541. This is a region of high homology with a domain of the adenovirus type 5 E1B 19K

protein required to overcome E1A-induced apoptosis, a domain homologous to the Bcl-2 BH1 domain (Conzen et al., 1997). The N-terminus is also thought to have an anti-apoptotic function, as recent data shows that within the N-terminus T-Ag binds a novel protein called p193 (Tsai et al., 2000). This protein also has homology with BH3, another Bcl-2 homology domain. Due to the similarity of binding site and molecular mass, it is possible that p193 is the p185 T-Ag-bound protein identified by Kohrman and Imperiale in 1992. In conclusion, it seems that T-Ag inactivates apoptosis in two ways, by overcoming the p53-induced apoptotic pathway as discussed previously, and, independently of p53 by binding and inactivating Bcl-2 family members.

Finally, it has been shown that the C-terminus beyond amino acid 627 carries functions that are not required for either immortalization or transformation (Tevethia et al., 1988).

1.4.3 The N-terminal domain of T antigen

The N-terminus of T-Ag carries the J domain and the binding sites for the Hsc70 protein and the pRb family of proteins (Figure 1.3). These will all be discussed together in the following section, as they are thought to be part of the same pathway, and are the most critical functions for immortalization associated with the N-terminus.

In addition, the N-terminus contains a region of homology (between amino acids 17-32) with the conserved region CR1 of the Ad E1A viral oncogene (Figge et al., 1988). In E1A this region is required for the binding with the p300 protein (Yaciuk et al., 1991). T-Ag also interacts with p300, although not at the CR1 region like E1A, but in the C-terminus as previously discussed.

T-Ag also binds DNA polymerase α between amino acids 1 and 82 (Smale and Tjian, 1986 ; Gannon and Lane, 1987 ; Dornreiter et al., 1990),

although the existence of a second binding region between amino acids 271 and 517 makes it unlikely that DNA polymerase α binding is a critical function for the N-terminus.

The NLS is also present in the N-terminus within amino acids 126 to 132, but mutants in that region have shown that the NLS is also not required for immortalization and transformation by T-Ag (Kalderon et al., 1984 ; Fischer-Fantuzzi and Vesco, 1987 ; Woods et al., 1994).

Finally, the immortalization and transformation role of p185/p193 and necdin, the two other proteins also binding in the N-terminus, has not yet been established.

The J domain and binding of Hsc70, and the pRb family of proteins

The first 82 N-terminal amino acids of the T-Ag and the small t antigen (t-Ag) are identical, so this domain is known as the T/t common region. Even though the importance of this domain in immortalization and transformation has been known for many years, no critical function residing in this region had been found.

Database comparisons of the T/t sequence revealed that this domain is similar to the J domain of the DnaJ family of molecular chaperones, and carries the conserved HPD tripeptide found in all the DnaJ proteins (Kelley and Landry, 1994). The DnaJ molecular chaperones are involved in protein folding, assembly and transport. They act by binding to DnaK proteins and stimulating their ATPase activity, which facilitates the folding and assembly of their protein substrates.

It has been shown that the J domain of T-Ag can functionally substitute the J domain of the *E.coli* DnaJ chaperone (Kelley and Georgopoulos, 1997). In addition, the N-terminus of T-Ag has the ability to stimulate the ATPase activity of two DnaK proteins and to release unfolded polypeptide substrates from DnaK, which are both activities of the DnaJ

proteins (Srinivasan et al., 1997). Taken together, these results demonstrate that in addition to sequence similarity, the N-terminus of T-Ag can actually function as a J domain.

Interestingly, T-Ag has been shown to bind the Hsc70 protein within its N-terminus (Sawai and Butel, 1989 ; Sawai et al., 1994). Hsc70 is a mammalian DnaK homologue, and its interaction with T-Ag is dependent upon the presence of an intact J domain (Campbell et al., 1997). The T-Ag mutant *dl*1135, which lacks immortalizing and transformation activity (deletion of aa 17 to 27), does not have a functional J domain and cannot associate with Hsc70. It has therefore been proposed, that the T-Ag-Hsc70 complex acts as a DnaJ-DnaK complex. T-Ag recruits Hsc70 to the protein complexes it is bound with, in order to inactivate them (Stubdal et al., 1997 ; Zalvide et al., 1998).

The most important complex T-Ag targets at the N-terminus is the pRb-E2F complex. T-Ag binds the pRb family of proteins (pRb, p107 and p130) at the CR2-like region (Figure 1.3), another region of homology with E1A (DeCaprio et al., 1988 ; Moran, 1988). T-Ag preferentially binds the underphosphorylated form of pRb, which, as described in section 1.3.2, is the inhibitory form for cell cycle progression (Ludlow et al., 1989). Thus, pRb is bound by T-Ag and the E2F transcription factor is free to induce the expression of its downstream targets and allow the cell to progress through G1/S.

It is suggested, that out of the three pocket proteins, the targeting of p107 and p130 by T-Ag could be more critical than its interaction with pRb. Srinivasan et al. (1997) used two T-Ag mutants (3213 and K1) which are defective in the CR2-like region, and assayed them for their ability to transform cells. These mutants do not interact with pRb, however, were able to transform C3H10T1/2 cells. Interestingly, these T-Ag mutants retain the ability to interact with p107 and p130. However, this is only in the context of

a full-length T-Ag and not an N-terminal truncated T-Ag, suggesting that p107 and p130 could also be binding in the C-terminus.

The proposed model for the function of the T-Ag J domain, is that it recruits Hsc70 to the pRb/p107/p130-E2F complexes and promotes their disruption (Stubdal et al., 1997 ; Zalvide et al., 1998), so that in the absence of a functional J domain, the T-Ag-pRb/p107/p130 interaction is not productive. This model is challenged by data which shows that in REFs, the T-Ag-p130 interaction is productive even in the absence of a functional J domain. Using a J domain mutant T-Ag (the *dl*1135 mutant), increased transcription of the B-*myb* gene was observed, which is an E2F dependent gene transcriptionally downregulated by p130 (Powell et al., 1999). Therefore, the interaction of the J domain-deficient T-Ag with p130 was productive, strongly suggesting that the J domain could be involved in the targeting of proteins other than the pocket proteins.

T-Ag might disrupt pRb function in other ways too. It was recently shown that prohibitin and a prohibitin-related protein called BAP37 are specifically recognized by a T-Ag antibody (Darmon and Jat, 2000). Even though this does not demonstrate a T-Ag-prohibitin interaction, this finding is interesting since prohibitin is a protein implicated in senescence and immortalization (Liu et al., 1994 ; Jupe et al., 1995 ; Coates et al., 1997 ; Coates et al., 2001). It has also been shown that prohibitin is a regulator of E2F activity through its association with the pRb family of proteins (Wang et al., 1999). This association is suggested to stabilize the pRb-E2F complex. Further research will determine whether T-Ag can also disrupt the prohibitin/BAP37-pRb complex in order to deregulate cell growth.

Finally, it was also shown that acetylation and methylation are two modifications linked to T-Ag through its association with pRb. The mechanism by which the pRb family proteins repress transcription is now suggested to be the modification of chromatin structure. That is, they inhibit

the transcriptional activity of E2F by recruiting the histone deacetylase HDAC1, a protein which removes acetyl groups from histone. Deacetylation results in the condensation of the chromatin, therefore reducing the accessibility for transcription factors (Ferreira et al., 1998 ; Luo et al., 1998), and actively repressing the E2F promoter (Brehm et al., 1998 ; Magnaghi-Jaulin et al., 1998).

It must be noted here that T-Ag is linked to acetylation also through its association with the CBP/p300 proteins. In addition to regulating transcription through association with the transcription machinery, the CBP/p300 proteins also have a role as histone acetyltransferases (HATs), which by acetylating histone, relax the DNA and promote transcription (Bannister and Kouzarides, 1996 ; Giles et al., 1998).

Methylation is also linked to T-Ag. DNA methyltransferase (DNA MeTase) is the enzyme responsible for methylating CpG dinucleotides in the genome, an activity important for repressing gene transcription (gene silencing ; Ng and Bird, 1999). T-Ag has been found to upregulate DNA MeTase at the post-transcriptional level by adjusting mRNA stability, an activity found to be necessary for the transformation of Balb/c 3T3 cells. A T-Ag mutant which does not bind pRb did not exhibit this upregulation, suggesting that induction of DNA MeTase is in someway regulated by the pRb pathway (Slack et al., 1999). Interestingly, a common pathway seems to emerge, by recent reports which show that DNA MeTase silences transcription through the recruitment of HDACs (Jones et al., 1998 ; Nan et al., 1998).

The N-terminus of T-Ag and genomic instability

The expression of T-Ag in human cells can cause genomic instability by inducing chromosomal aberrations and aneuploidy (Ray et al., 1990 ; Stewart and Bacchetti, 1991). While this function could be explained by

disruption of p53 activity, interestingly, studies have shown that the N-terminal 147 amino acids of the protein, which do not contain p53 binding sequences, can induce genome destabilization as effectively as the wildtype protein (Woods et al., 1994). In addition, T-Ag mutants that do not bind pRb, or mutants that lack amino acids 1 to 127, still induce chromosomal changes suggesting that an important genome destabilizing activity lies between amino acids 128 and 147 of the N-terminus.

The ability of T-Ag to cause such karyotypic instability in SV40 infected human cells, has been found to correlate with its ability to deregulate normal mitotic checkpoints. In contrast to control cells, T-Ag-expressing cells re-entered mitosis faster after exposure to ionizing radiation, and also proceeded to enter a new round of DNA synthesis, even when mitotic spindle assembly was compromised (Chang et al., 1997). These results are further supported by similar findings for the HPV16 E6 and E7 viral oncogenes, which have also been found to disrupt mitotic spindle checkpoints (Thompson et al., 1997 ; Thomas and Laimins, 1998).

The T/t common region and the small t antigen

As already described (section 1.4.1 and Figure 1.2), part of the N-terminus of T-Ag is homologous to the other two early proteins expressed by SV40, the small t antigen (t-Ag) and the tiny t antigen. Little is known about the function of the tiny t antigen, however, evidence suggests that it has a supportive role in SV40 infection and transformation (Zerrahn et al., 1993).

t-Ag is not essential for the immortalization and transformation activity of SV40 (Conzen and Cole, 1995), and, unlike T-Ag, it is not required for the induction of chromosome aberrations and genome destabilization (Stewart and Bacchetti, 1991), suggesting that it may have a role only in permissive cells. Despite not being essential however, t-Ag can increase the efficiency of the immortalization and transformation activity of SV40, especially when the

concentration of T-Ag is low (Bikel et al., 1987). This is mainly attributed to the ability of t-Ag to cooperate with T-Ag in the metabolic stabilization of p53 (Tiemann et al., 1995).

t-Ag is also thought to be involved in the regulation of phosphorylation of T-Ag, however, conflicting data exists depending on the system studied. T-Ag is a phosphoprotein, with two clusters of phosphorylated residues, one at the N-terminus and one at the C-terminus (Figure 1.3). The level of phosphorylation of T-Ag varies, and it can affect the function of T-Ag both positively and negatively. For example, phosphorylation of T-Ag on Thr-124 is required for SV40 replication both *in vitro* and *in vivo*, whereas phosphorylation on Ser-120 and Ser-123 inhibits viral DNA replication *in vitro* (Fanning, 1994).

t-Ag is thought to regulate the activity of T-Ag, through its association with the protein phosphatase 2A (PP2A). The dephosphorylation of Ser-120 and Ser-123 on T-Ag activates DNA replication, and in a cell-free system this dephosphorylation is carried out by PP2A (Virshup et al., 1989). It has been shown that t-Ag can bind PP2A in rodent cells (Pallas et al., 1990), inhibiting its dephosphorylating activity towards T-Ag (Scheidtmann et al., 1991), and that this inhibition is important for efficient viral transformation (Mungre et al., 1994). This inhibition of dephosphorylation is in contrast to t-Ag's function in monkey cells, where it has been shown to stimulate SV40 DNA replication (Cicala et al., 1994), and to enhance the transactivation of the SV40 early and late gene promoters (Bikel and Loeken, 1992).

Further research is clearly required in order to elucidate the exact functions of t-Ag, although it is entirely possible that, in contrast to T-Ag, t-Ag has a role only in permissive monkey cells.

Conclusion

The main activities of T-Ag which allow it to deregulate cellular growth and induce immortalization, have been found to be the J domain, the binding of the pRb family of proteins, the p53 binding, the CBP/p300 binding and the anti-apoptotic domain. The cooperation of all these domains allow T-Ag to induce a highly efficient immortalization program. However, at least in REFs, it has been found that the N-terminus of T-Ag is sufficient for immortalization.

At the time when this project began, the function of the J domain and its importance for pRb binding was not yet established. Furthermore, there was later evidence that suggested, that even in the absence of a functional J domain, T-Ag still retains a productive interaction with the pRb proteins. We therefore proposed, that there is an additional activity residing in the N-terminus of T-Ag, possibly a novel protein interaction, also targeted by the J domain. We decided to investigate this possibility using the yeast two-hybrid system, a system that had not been used previously to identify T-Ag interactors.

1.5 THE YEAST TWO-HYBRID SYSTEM

A very powerful approach for studying and identifying interacting proteins *in vivo* is the yeast two-hybrid system, developed in 1989 by Fields and Song. The general principle of the yeast two-hybrid system is discussed in this section, while the specific setup of the system used for the T-Ag screens is described in chapter 3.

1.5.1 Description of the system

The principle of the yeast two-hybrid system is based on the regulation of eukaryotic gene transcription. The system makes use of hybrid genes to detect protein-protein interactions via the activation of reporter gene expression. This is achieved by functionally restoring a eukaryotic transcriptional activator (Fields and Song, 1989).

More specifically, in eukaryotic gene transcription many genes are expressed at low levels unless induced by transcriptional activators. Most transcriptional activator proteins have two separable functions residing on two independent functional domains, namely a DNA-binding domain and an activation domain. The DNA-binding domain binds to specific sequences on the DNA , called upstream activator sequences (UAS). The activation domain increases the rate of transcription of the adjacent gene through association with proteins of the transcriptional complex which binds to the promoter TATA box, and with the RNA polymerase (Figure 1.4).

In the yeast two-hybrid system (Figure 1.5), a transcriptional activator molecule is used whose DNA-binding domain and activation domain have been separated. Chimeric transcriptional activators can also be used whose DNA-binding domain and activation domain come from two different molecules, for example using the DNA-binding domain of the LexA protein

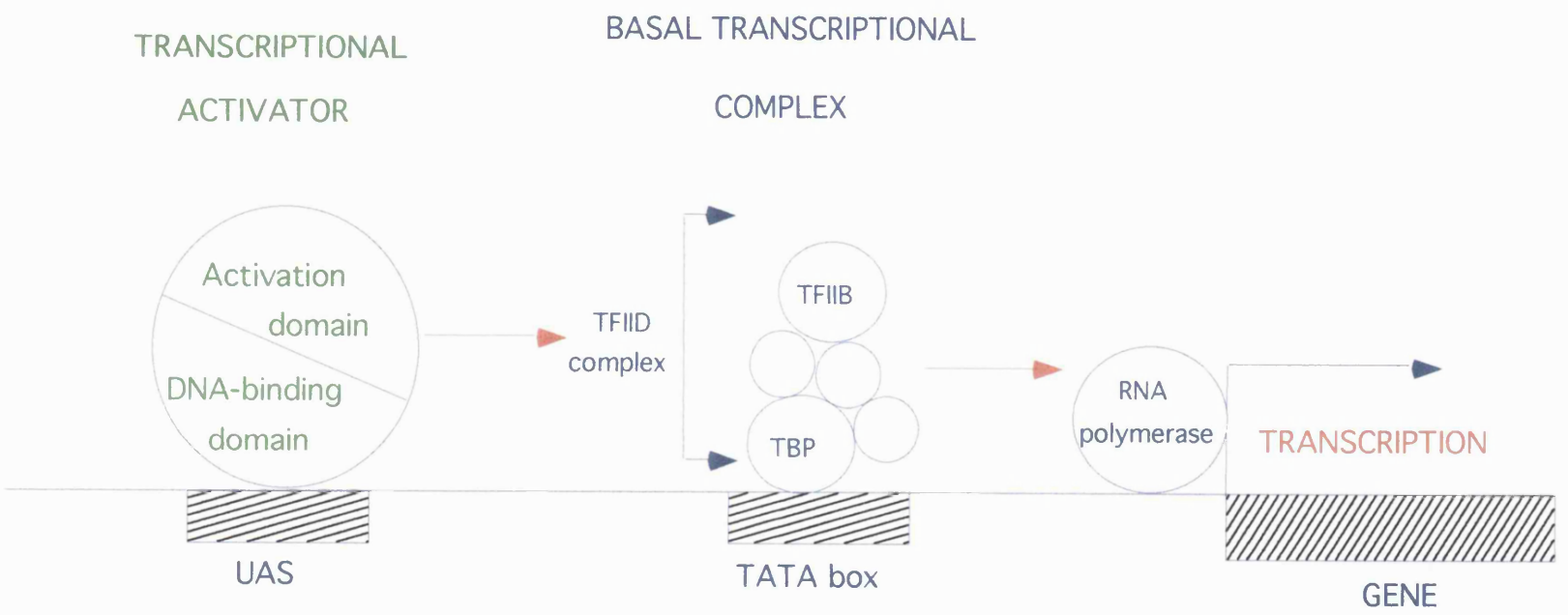


Figure 1.4 Simplified schematic of eukaryotic gene transcription.

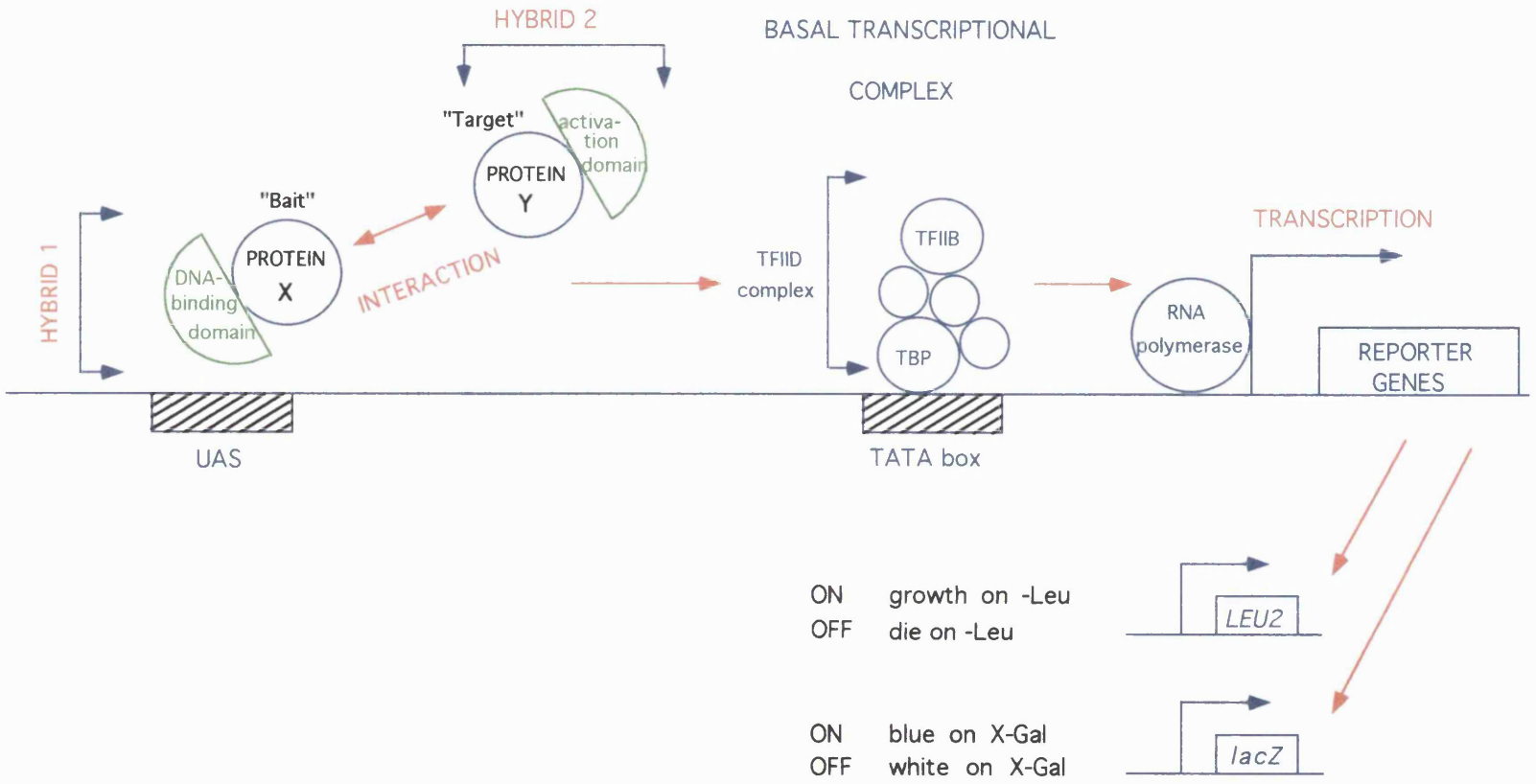


Figure 1.5 Schematic representation of the yeast two-hybrid system.

and the activation domain of B42 (Ma and Ptashne, 1987). Typically, the protein of interest X (or else called the "bait") is expressed as a fusion protein with the DNA-binding domain of the transcriptional activator (Hybrid 1). Another protein Y (or else called the "target") is fused to the activation domain of the transcriptional activator (Hybrid 2). The two hybrids are then transformed into the yeast *Saccharomyces cerevisiae* and tested for association. Any two interacting proteins will bring the whole complex together and reconstitute the transcriptional activator protein. This will then result in the transcription of reporter genes which provide a detectable phenotype, for example transcription of the *LEU2* gene will provide growth on media lacking leucine and transcription of the *lacZ* gene will give blue colonies on 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal) media.

1.5.2 Advantages and applications

Investigating protein interactions *in vivo* has the advantage that the binding detection is a lot more sensitive than *in vitro*. This is because the proteins are overexpressed by strong promoters and by high copy vectors, but mainly because the interaction of the proteins is stabilized by the association of the activation domain with the proteins of the transcriptional complex. This way, even weak interactions can be detected which would not be identified otherwise.

The yeast two-hybrid system has a number of useful applications (Luban and Goff, 1995 ; Colas and Brent, 1998). It can be used to study known protein-protein interactions, as well as to identify novel ones (Chien et al., 1991). A number of novel interactions have been identified, including interactions between cell cycle regulators which has significantly contributed to the understanding of the cell cycle (reviewed in Hannon, 1997). For example, the p21/cdk 2 and the p16/cdk 4 interactions were identified by the

yeast two-hybrid system (Harper et al., 1993 ; Serrano et al., 1993). In addition, known interactions can be studied to verify the association of proteins that have been shown to interact using other methods, or to define the particular domains of the interaction, as it has been done for the T-Ag-p53 interaction (Li and Fields, 1993). Also, the yeast two-hybrid system provides a very useful approach for the design of drugs and therapeutic molecules which will bind and inhibit proteins of bacterial and viral origin.

Moreover, the system has been successfully modified for studying various other interactions. A one-hybrid system has been developed for the identification of DNA-binding proteins that can initiate transcriptional activation (Luo et al., 1996). Reverse two-hybrid and one-hybrid systems are also used to detect the dissociation of protein-protein and DNA-protein interactions, and a three-hybrid system has been developed to identify RNA-protein interactions (Vidal et al., 1996 ; Drees, 1999).

However, there are a few limitations to the system (Fields and Sternglanz, 1994). One limitation is that the proteins are targeted to the nucleus. Therefore any proteins normally found in the cytoplasm, may not fold properly or interact with their partners in the nucleus. In addition, some proteins may need to be modified by non-yeast proteins, e.g. tyrosine kinases. Another limitation is the ability of some baits to activate the transcription of the reporter genes in the absence of the activation domain hybrid, either because the proteins contain domains that are similar to the activation domain, or because the proteins normally function as transcriptional activators. Also, it is possible that the expression of some baits is toxic or lethal to yeast. Finally, a number of false positive clones may result from a two-hybrid screen, so tests are required to verify the specificity of the interactions identified.

Nevertheless, the system is constantly being modified to improve its efficiency, and remains a popular method for protein identification.

1.6 AIMS OF THE PROJECT

When this project began, research on the SV40 large T antigen had already been carried out for more than three decades. The vast amount of information acquired by this research, has now given us a much better understanding of the functions of this viral oncogene. It is now known that the N-terminus of the large T antigen carries critical functions for immortalization, and major cellular proteins have already been found to bind in this domain. However, research indicates that an additional activity is involved and other proteins may be binding in this region. The isolation of such proteins would further elucidate the function of the large T antigen and, more significantly, would identify proteins important for growth regulation. Thus, the aims of the project were as follows:

1. To set-up and optimize the yeast two-hybrid system.
2. To identify novel proteins which interact with the N-terminal domain of the SV40 large T antigen using the yeast two-hybrid system.
3. To demonstrate the interaction of these proteins with the large T antigen in mammalian cells.
4. To carry out a functional study in order to investigate the effect of the large T antigen on the function of these proteins.
5. To explore the biological significance of these interactions, and to investigate the role of the isolated proteins in normal cell growth, as well as in tumourigenesis.

CHAPTER TWO

Materials and Methods

2.1 MATERIALS

2.1.1 Standard reagents

Analar grade standard reagents were supplied by Merck, Fisons and Sigma.

2.1.2 Enzymes

Restriction enzymes were supplied by New England Biolabs and Boehringer Mannheim. Vent polymerase was from New England Biolabs. *Taq* polymerase and T4 DNA ligase were from Promega. The AmpliTaq DNA polymerase, FS was from Applied Biosystems.

2.1.3 Miscalleneous

Protease inhibitors, antibiotics and salmon sperm DNA were supplied by Sigma. Nitrocellulose Hybond-C membrane filters were from Amersham. The 1kb and 100bp DNA ladders were from Life Technologies. The λDNA HindIII digest was from NBL Gene Sciences. Prestained broad range and rainbow protein markers were from Bio-Rad. X-ray films were either Fuji-RX or Amersham Hyperfilm. Gene Pulser electroporation cuvettes were from Bio-Rad. Protein A-sepharose CL-4B beads were from Pharmacia Biotech. 10x PBS and agarose were from Life Technologies, and 30% acrylamide (0.8%Bis) was from Merck.

2.1.4 Solutions, buffers and gels

All solutions were made in distilled water, unless otherwise stated.

General:

- 10x TAE: 0.4M Tris-acetate, 20mM EDTA
- 1x TE: 10mM Tris-HCl, 1mM EDTA, pH 7.5 or 8.0
- 1x PBS: 137mM NaCl, 1.5mM KH_2PO_4, 8mM Na_2HPO_4, 2mM KCl

Extracts:

- 6x Lysis buffer: 0.35M Tris pH 6.8, 10.28% (w/v) SDS, 36% (v/v) glycerol, 5% (v/v) ß-mercaptoethanol, 0.2mg/ml bromophenol blue
- NP-40 Lysis buffer: 150mM NaCl, 1% (v/v) NP-40, 50mM Tris-HCl pH 8.0, 5mM EDTA
- Laemli sample buffer: 80mM Tris-HCl pH 6.8, 2% (w/v) SDS, 15% (v/v) glycerol, 100mM DTT, 2mM EDTA, 0.2mg/ml bromophenol blue

Protein gels:

- 10x SDS-PAGE Running buffer: 0.25M Tris base, 1.92M glycine, 1% (w/v) SDS
- Separating gel: 7.5 or 10 or 12% (w/v) acrylamide, 0.37M Tris-HCl pH 8.8, 0.1% (w/v) SDS, 0.03% (w/v) APS, 0.06% (v/v) Temed
- Stacking gel: 5% (w/v) acrylamide, 0.12M Tris-HCl pH 6.8, 0.1% (w/v) SDS, 0.1% (w/v) APS, 0.25% (v/v) Temed

Westerns:

- 1x Transfer buffer: 25mM Tris base, 190mM glycine, 20% (v/v) methanol
- Blotto: 5% (w/v) non-fat dry milk, 0.5x PBS, 0.05% (v/v) Tween 20
- Wash solution: 1x PBS with 0.05% (v/v) Tween 20

Kinase assays:

- Hepes-modified Beach lysis buffer: 50mM Hepes/NaOH pH 7.4, 150mM NaCl, 20mM EDTA, 0.5% (v/v) Triton X-100, 10mM β-glycerophosphate, 2mM DTT, 10mM NaF, 2mM Na_3VO_4

- Kinase assay buffer: 50mM Hepes/NaOH pH 7.4, 10mM $MgCl_2$, 10mM $MnCl_2$, 1mM DTT, 10mM β-glycerophosphate, 0.1 μM PKA inhibitor
- Substrate mix: kinase assay buffer with 200μM ATP, 5μCi/reaction ^{32}P-ATP (and 500ng H1/reaction if not autophosphorylation assay)

Yeast:

- 10x BU salts (per litre): 70g $Na_2HPO_4.7H_2O$, 30g NaH_2PO_4
- Glycerol solution: 65% (v/v) glycerol, 0.1M $MgSO_4$, 25mM Tris-HCl pH 8.0
- 2.5x Laemli stacking buffer: 0.3M Tris-HCl pH 6.8, 0.25% (w/v) SDS
- 2x Laemli sample buffer: 10% (v/v) ß-mercaptoethanol, 6% (w/v) SDS, 20% (v/v) glycerol, 0.025x Laemli stacking buffer, 0.2mg/ml bromophenol blue
- TE/LiOAc: 1x TE pH 7.5, 0.1M LiOAc pH 7.5
- TE/LiOAc/PEG: 40% (w/v) PEG 4000, 1x TE pH 7.5, 0.1M LiOAc pH 7.5
- PCR incubation solution: 1.2M sorbitol, 100mM $NaPO_4$ pH 7.4, 2.5 mg/ml zymolase

2.1.5 Plasmids

The yeast two-hybrid cloning vectors pEG202, pMW103, pNLexA and pGilda, the LacZ reporter plasmid pJK103, the repression assay plasmids pJK101 and pRFHM1, and the positive and negative control plasmids pSH17-4, LexA-RPB7 and B42-RPB4 were gifts from Dr. Erica Golemis (Fox Chase Cancer Center, Philadelphia). The pZipNeoSVLT vector was made by Dr. Parmjit S. Jat.

2.1.6 Libraries

The HeLa B42 library was a gift from Dr. Erica Golemis ($9.6x10^6$ independent clones, average size of inserts 1-2 Kb). The mouse day 14.5 embryo GAL4 library was a gift from Dr. Noriko Shikama (University of Glasgow, Glasgow ; $1.92x10^6$ independent clones, average size of inserts 0.3-2 Kb). The mouse day 19 embryo B42 library was from OriGene

Technologies,Inc. ($1.0x10^7$ independent clones, average size of inserts 0.2-2.5 Kb).

2.1.7 Commercial kits

The Quantum Prep kit from Bio-Rad was used for bacterial plasmid DNA mini-preps, and the tip-100 kit from Qiagen was used for DNA midi-preps. The Nucleon kit from Scotlab was used for yeast total DNA mini-preps. The Geneclean II kit from Bio101 was used for the glassmilk purification of PCR fragments. The "ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq DNA polymerase, FS" from Applied Biosystems was used for automated sequencing. The ECL kit from Amersham was used for antibody detection.

2.1.8 Cells

Bacterial strains: *Escherichia coli* (*E.coli*) strains JS4 and DH5α

Yeast strains: *Saccharomyces cerevisiae* strains:

- EGY48 *(MATα, trp1,his3, ura3, leu2 :: 6LexAop-LEU2)* (high sensitivity)
- EGY191 (*MATα, trp1,his3, ura3, leu2 :: 2LexAop-LEU2)* (low sensitivity)
- RFY206 *(MATa, trp1Δ::hisG his3Δ200 ura3-52 lys2Δ201 leu2-3)* (mating)

Mammalian cell lines:

- Rat fibroblasts: SV4 cells (wild-type T-Ag), tsa8 and tsa14 cells (*tsA*58 mutant T-Ag)
- NIH 3T3 mouse fibroblasts: KS cells (control cells carrying pBluescript, Stratagene) and SE cells (wild-type T-Ag)

2.1.9 Media

Bacterial media:

- L-broth (per litre): 10g Bacto Tryptone, 5g Yeast Extract, 10g NaCl.
- L-agar (per litre): as L-broth plus 15g Bacto Agar.

- Super-broth (per litre): 32g Bacto Tryptone, 20g yeast extract, 10g MOPS sodium salt.

Bacto Tryptone, Yeast Extract and Bacto Agar were supplied from Oxoid.

Yeast media:

YPD Medium, YPD Agar Medium, Minimal SD Base Gal/Raf and Minimal SD Agar Base Gal/Raf were supplied from Clontech. Dropout glucose media (DOB) and amino acid supplements were supplied from Bio 101.

X-Gal plates: DOB or SD Gal/Raf with amino acids supplements, 2% (w/v) agar, 1 NaOH pellet, 1x BU salts, 0.08 mg/ml X-Gal

Mammalian media:

SV4, tsa8 and tsa14 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% (v/v) fetal calf serum (FCS), 2mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. NIH 3T3 cells were maintained in 10% (v/v) donor calf serum (DCS), 2mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. All media and components were obtained from Life Technologies.

2.1.10 Antibodies

The anti-T-Ag mouse monoclonal antibodies PAb416, PAb100 and PAb423 were made by Dr. Parmjit Jat. The anti-Bub1 and anti-Bub3 rabbit polyclonal antibodies were a gift from Dr. Peter Sorger (MIT, Cambridge Massachusetts). The anti-securin rabbit polyclonal antibody was a gift from Dr. Mark Kirschner (Harvard Medical School, Boston). The anti-LexA antibody was a gift from Dr. Erica Golemis. The anti-p21 antibody and the anti-cyclin B1 (GNS1) antibody were from Santa Cruz. The anti-actin antibody and the anti-β-tubulin antibody were from Sigma. The anti-Hsp40 antibody was from Stressgen Biotechnologies. The secondary antibodies for immunoblotting were from Amersham, and for immunofluoresence were from Jackson ImmunoResearch and Southern Biotech.

2.1.11 Oligonucleotide primers

The primers used for cloning, sequencing, and colony PCR amplifications are shown in Table 2.1. All primers were supplied by Genosys.

Primer	Sequence (5′ → 3′)	Use	Origin of sequence	Annealing Temp
1. PJ-PS1N	CATGGATCCGTATGGATAAAGTTTTAAACAGA	T-Ag subcloning (F)	T-Ag	65°C
2. PJ-136	ATCCTCGAGTCACTTGGGGTCTTCTACCTTTCT	T-Ag subcloning (R)	T-Ag	65°C
3. LEX1	CGTCAGCAGAGCTTCACCATTG	T-Ag sequencing (F)	pEG202 vector	55°C
4. LEX2	CGTTTTAAAACCTAAGAGTCAC	T-Ag sequencing (R)	pEG202 vector	55°C
5. NLEXA(F)	CATGAATTCCATGGATAAAGTTTTAAACAGA	T-Ag subcloning (F)	T-Ag	65°C
6. NLEXA(R)	ATCGGATCCACTTGGGGTCTTCTACCTTTCT	T-Ag subcloning (R)	T-Ag	65°C
7. JG4-5 SEQ	CTGAGTGGAGATGCCTCC	library cDNAs sequencing (F)	pJG4-5 vector	60°C
8. BCO1	CCAGCCTCTTGCTGAGTGGAGATG	colony PCRs (F)	pJG4-5 vector	56°C
9. BCO2	GACAAGCCGACAACCTTGATTGGAG	colony PCRs (R)	pJG4-5 vector	56°C

Table 2.1 Sequences and annealing temperatures of oligonucleotide primers. F and R denote forward and reverse primers. 1 and 2 are the primers for cloning into pEG202, pMW103 and pGilda. 3 and 4 are the primers for sequencing the pEG202, pMW103 and pGilda inserts. 5 and 6 are the primers for cloning into pNLexA and sequencing inserts. 7 is the primer for sequencing pJG4-5 inserts. 8 and 9 are the primers for bacterial colony PCRs.

2.2 METHODS

All glassware was sterilized by autoclaving (121°C for 20 minutes). Solutions for nucleic acid work were sterilised, either by autoclaving, or by filter sterilisation (through 0.22μm pore size 'Acrodisc' filters, Gelman Sciences). Media for bacterial and yeast manipulations were sterilized by autoclaving (121°C for 20 minutes and 15 minutes respectively). All plates used for yeast manipulations were further sterilized after setting, by exposure to ultraviolet (UV) irradiation for 10-20 minutes in a tissue culture hood.

2.2.1 Bacterial manipulations

2.2.1.1 Generation of competent cells

$CaCl_2$ competent cells

A 10ml overnight culture of *E.coli* JS4 was used to inoculate 1 litre of L-broth. The culture was grown until it reached an $O.D_{550}$ of 0.5, and then chilled on ice for 30 minutes. The cells were harvested by centrifugation at 3,000rpm for 10 minutes at 4°C, and the pellet was resuspended in 250ml of 100mM $CaCl_2$. The cells were centrifuged again as before, and the pellet was resuspended in 125ml of 100mM $CaCl_2$. Finally, the cells were centrifuged and resuspended in 5ml of 85% (v/v) 100mM $CaCl_2$ / 15% (v/v) glycerol solution. The cells were aliquoted into 50μl aliquots, snap frozen in liquid nitrogen and stored at -70°C until use.

Electrocompetent cells

The JS4 cells were manipulated as above, except that the successive washes were done in: 1 litre of cold water, 500ml of cold water and 20ml of 10% (v/v) glycerol. JS4 cells were resuspended in 1ml of 10% (v/v) glycerol.

2.2.1.2 Bacterial transformation and selection of transformants

Transformation by $CaCl_2$

The $CaCl_2$ method was used for transforming ligation reactions into JS4 *E.coli* cells. A 50µl aliquot of frozen competent cells was thawed on ice, and mixed gently with 950µl of ice cold 100mM $CaCl_2$. 1/4 to 1/2 of the ligation reaction (section 2.2.4.5) was added to 100µl of cells and mixed gently. After incubation on ice for 20 minutes, the cells were heat shocked at 42°C for 90 seconds, 1ml of L-broth was added and the cells were incubated at 37°C for 30 minutes. 100µl of the transformed cells were plated on L-agar plates, supplemented with 100µg/ml ampicillin or carbenicillin.

Transformation by electroporation

The electroporation method was used for transforming yeast total DNA into JS4 or DH5α *E.coli*. A 50µl aliquot of competent cells was thawed, and mixed with 1/50 to 1/10 of total yeast DNA, at the bottom of a 0.2cm prechilled electroporation cuvette. The Gene Pulser apparatus was set at 25µF and 2.5kV, and the Pulse Controller apparatus was set to 200 Ω. The cells were pulsed once, and 1ml of L-broth was immediately added to the cuvette. The cells were then transferred into a microcentrifuge tube and incubated at 37°C for 30 minutes. 100µl of the cells were plated on L-agar plates supplemented with 100µg/ml ampicillin or carbenicillin.

2.2.1.3 Colony PCR amplification

Colony PCRs were carried out to identify bacterial transformants carrying the library pJG4-5 plasmid. A small part of a fresh colony was removed using a sterile tip. The tip was then washed directly into the PCR reaction. The reaction consisted of: 1x buffer, 30pmoles of each of the library specific primers BCO1 and BCO2 (see Table 2.1), 1.6mM $MgCl_2$, 0.25mM dNTPs, and 2.5 units of *Taq* polymerase, in a final volume of 30µl. Each

reaction was covered with 20µl of paraffin oil to prevent evaporation during thermal cycling. An initial denaturation step was carried out at 94°C for 10 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealling at 56°C for 1 minute, and extension at 72°C for 2 minutes.

2.2.1.4 Storage of cells

A colony from a fresh plate was used to inoculate a 5ml overnight culture. 300µl of the culture were then mixed with 100µl of 80% glycerol, snap frozen on dry ice and stored at -70°C.

2.2.2 Yeast manipulations

For brevity, the yeast media described in this section will be denoted as:
- rich glucose medium: YPD
- glucose complete medium: Glu/CM
- 2% galactose / 1% raffinose complete medium: Gal/CM
- selective medium lacking histidine, uracil, tryptophan and leucine (and any combinations of these): -His-Ura-Trp-Leu

2.2.2.1 Standard transformation

The basic lithium acetate (LiOAc) transformation protocol was followed from Golemis et al. (1997). A single yeast colony was used to inoculate a 5ml liquid culture of the appropriate complete or selective medium. The culture was grown overnight in a 30°C shaking incubator. The following morning the culture was diluted to an $O.D_{600}$ of 0.15 with fresh medium, to a final volume such that 10ml of culture were grown for each transformation, and put back in the 30°C shaking incubator. When the culture had reached an $O.D_{600}$ of 0.5 to 0.7 after approximately 4 to 6 hours, the cells were transferred into 50ml conical tubes and harvested by

centrifugation at 3,000rpm for 10 minutes at room temperature. The cell pellet was resuspended in an equal volume of sterile water and re-centrifuged. The yeast was then resuspended in TE/LiOAc solution (500µl of TE/LiOAc for every initial 100ml of culture).

The following were mixed in microcentrifuge tubes: 50µl of yeast, 2 to 5 µg of the DNA to be transformed, 50µg of salmon sperm DNA (making sure that the combined volume of DNA transformed and salmon sperm DNA was less than 20µl), and 350µl of TE/LiOAc/PEG solution. The tubes were incubated at 30°C for 60 minutes, and then placed in a 42°C heat block for 10 to 15 minutes. The tubes were then removed to room temperature, and 200µl of each transformation were plated on the appropriate selective media, using 5-10 sterile 4mm glass beads for uniform spreading of the cells. The plates were incubated at 30°C for two days to select for transformants. Between 3 and 6 colonies were picked from each transformation, and streaked onto a master plate. The master plate was grown overnight at 30°C and then kept at 4°C as a working stock.

2.2.2.2 Bait characterization assays

The lithium acetate method described above, was used to transform the EGY48 yeast strain with the following combinations of plasmids:

1. Bait + pJK103
2. pSH17-4 + pJK103
3. LexA-RPB7 + pJK103
4. Bait + pJK101
5. pRFHM1 + pJK101
6. pJK101

"Bait" denotes the LexA-T-Ag fusion plasmids (Appendices I, II and III). Transformation no.1 was repeated for all the different T-Ag baits assayed (see chapter 3). pJK103 is the *lacZ* reporter plasmid (Appendix VI), pSH17-4 is the positive control plasmid for activation, LexA-RPB7 is the negative control for activation, pJK101 is the repression plasmid, and pRFHM1 is the positive control for repression. The bait, pSH17-4, LexA-RPB7 and pRFHM1 plasmids carry the *HIS3* marker, while the pJK103 and pJK101 plasmids carry the *URA3* marker.

EGY48 was first transformed with the plasmid combinations 1 to 3, and plated on Glu/CM-His-Ura plates to select for transformants. After two days, 6 colonies were picked from each transformation and streaked on a master Glu/CM-His-Ura plate.

LEU2/*lacZ* reporter assays

The LEU2 and the *lacZ* reporter assays were carried out by picking part of these streaks, and streaking them successively onto 4 selective plates in this order:

1. Gal/CM-His-Ura/X-Gal
2. Glu/CM-His-Ura/X-Gal
3. Gal/CM-His-Ura-Leu
4. Glu/CM-His-Ura-Leu

The streaking was done strictly in this order, so that most of the cells would be transferred onto the X-Gal plates, on which the cells do not grow well (and therefore fewer cell divisions would be required to have enough cells on the plate in order to see the blue/white phenotype), and very few cells would be transferred onto the -Leu plates (and therefore no cross-feeding could occur between cells).

The 4 plates were put in a 30°C incubator. The colour phenotype was determined after 12 to 24 hours, and the streaks were scored as white, light

blue, blue, and dark blue. The growth phenotype was determined after 2 to 4 days, and the streaks were scored as slow, medium, and fast growers.

Expression assay

To determine the expression of the baits, cultures were inoculated from the same streaks of the Glu/CM-His-Ura master plate, as for the LEU2 and *lacZ* assays. The cultures were setup in Gal/CM-His-Ura liquid media, to test the expression under the conditions that would be used for the actual screen, i.e. in galactose media. The cultures were grown overnight in a 30°C shaking incubator. The following morning the cultures were diluted using fresh media to an $O.D_{600}$ of approximately 0.15. After 4 to 6 hours, when the cultures had reached an $O.D_{600}$ of 0.5-0.7, 1.5ml were removed from each culture and transferred into microcentrifuge tubes. The tubes were centrifuged and the supernatant was aspirated. Immediately, 50µl of the 2x Laemli sample buffer were added to the cell pellet, the tubes were well vortexed, placed in dry ice and transferred to -70°C.

Prior to loading the protein gel, the extracts were removed from the -70°C, and boiled at 100°C for 5 minutes. Between 20-50µl of each sample (depending on total cell number used in extract) were loaded onto 12% protein gels. The SDS-PAGE and western blotting were carried out as for mammalian cells (see 2.2.5.3 and 2.2.5.4).

Repression assay

After the LEU2 and *lacZ* assays, EGY48 was transformed with the plasmid combinations 4, 5 and 6. The transformations 4 and 5 were plated onto Glu/CM-His-Ura plates, and transformation 6 was plated onto a Glu/CM-Ura plate. Six colonies from each transformation were streaked onto master plates, and from these, onto a Glu/CM-Ura/X-Gal and a Gal/CM-Ura/X-Gal plate to assay for repression (only Ura was omitted from these plates to

allow all three types of transformants to be assayed together). The colour phenotype of the streaks was determined after 12 to 24 hours incubation at 30°C.

2.2.2.3 Library screening by direct transformation (used in mouse screen)

Library transformation

The library screens were carried out on 16 large square 24cm x 24cm plates in total (15 plates transformed with the library, and 1 plate with the empty library vector pJG4-5 as control). A couple of days prior to the library transformation, the 16 plates were poured, allowed to set and immediately placed uncovered under a tissue culture hood and exposed to UV irradiation for 10-20 minutes. The plates were then left at room temperature to dry until the day of the screening.

A 10ml culture was inoculated in Glu/CM-His-Ura liquid medium, using a characterized EGY48 or EGY191 colony, freshly transformed with the T-Ag bait and the pJK103 plasmid as described in 2.2.2.1. The culture was grown overnight in a 30°C shaking incubator. The following morning the culture was diluted to an $O.D_{600}$ of 0.15 in a final volume of 160ml (10ml per transformation required) and incubated at 30°C until the $O.D_{600}$ was approximately 0.5. The 160ml culture was aliquoted into 4 conical 50ml tubes, and centrifuged at 3,000rpm for 10 minutes at room temperature. The cell pellets were resuspended each in 4ml of sterile water, and then pulled together in the same tube. After centrifugation at 3,000rpm for 5 minutes at room temperature, the cell pellet was resuspended in 800µl of TE/LiOAc solution.

Into each of 15 sterile microcentrifuge tubes, the following were added: 50µl of the resuspended yeast, 50µg of salmon sperm DNA, 1µg of the pJG4-5 library DNA, and 300µl of TE/LiOAc/PEG solution. In 1 control tube, the same were added except that 1µg of empty pJG4-5 vector DNA was

used instead of the library DNA. All the tubes were mixed well by inversion and incubated at 30°C for 30 minutes. 40µl of DMSO were then added, mixed by inversion, and heat shocked at 42°C for 10 minutes. The content of each tube was plated onto a 24cm x 24cm Glu/CM-His-Ura-Trp plate, using 20-30 sterile glass beads and agitated vigorously for uniform spreading of the cells. To estimate the number of transformants obtained, 40µl of the suspension were removed from one of the 15 tubes before plating, to make a series of 10^{-1} dilutions in sterile water. The dilutions were plated onto 10cm Glu/CM-His-Ura-Trp plates. All the plates were incubated at 30°C until colonies appeared (2-3 days).

The plates were then transferred to 4°C for a few hours in order for the agar to harden. 10ml of sterile water and 20-30 sterile glass beads were added to each plate. The plates were agitated vigorously to resuspend the yeast cells. 5ml of yeast slurry were collected from each plate and pooled together in two 50ml conical tubes. The yeast cells from the control transformation were transferred into a separate tube. Cells were centrifuged at 3,000rpm for 5 minutes at room temperature. The library-transformed yeast cells were resuspended in 40ml of sterile water and pooled together in one tube, mixed well by vortexing, and centrifuged again. The cells were resuspended in 1 pellet volume of glycerol solution, mixed well, split into 1ml aliquots, and stored in -70°C.

Selection of interactors

The following day one aliquot of the library transformants and one aliquot of the empty vector transformants were thawed, and diluted in Gal/CM-His-Ura-Trp liquid media to an $O.D_{600}$ of 0.5 (representing approximately a concentration of $1x10^7$ cells/ml). The cultures were put in a 30°C shaking incubator for 4 to 6 hours to induce the expression of the library. The yeast cells were then plated on Gal/CM-His-Ura-Trp-Leu 10cm

plates to select for interactors. Between 3-10 cells were plated for every primary transformant to fully represent the library. For example, if $3x10^5$ total transformants were obtained from the library transformation, between $9x10^5$-$3x10^6$ cells were plated and screened on Gal/CM-His-Ura-Trp-Leu. A maximum of $1x10^6$ cells were plated per 10cm dish to prevent cross-feeding between densely plated cells. The same number of cells were also plated for the empty vector transformants on Gal/CM-His-Ura-Trp-Leu. All the plates were incubated at 30°C for 4 days. In addition, a series of dilutions for both the library and the empty vector transformants, were plated on Glu/CM-His-Ura-Trp 10cm plates, to confirm the total number of cells plated. These plates were incubated at 30°C for 2 days.

Every day for the following 4 days, the -Leu plates were observed and any arising colony was marked. On day 4 all the colonies were picked and streaked onto a Glu/CM-His-Ura-Trp master plate which was incubated at 30°C. To test for Gal-dependence of the LEU^+ and laZ^+ phenotype, the colonies were then streaked from the master plate onto each of the following 4 selective plates:

1. Gal/CM-His-Ura-Trp/X-Gal
2. Glu/CM-HIs-Ura-Trp/X-Gal
3. Gal/CM-His-Ura-Trp-Leu
4. Glu/CM-His-Ura-Trp-Leu

The 4 plates were put in a 30°C incubator. The colour phenotype was determined after 24-48 hours, and the streaks were scored as white, light blue, blue, and dark blue. The growth phenotype was determined after 2 to 4 days, and the streaks were scored as slow, medium, and fast growers. The Gal-dependent colonies (that is, colonies that were light blue, blue, or dark blue on galactose but white on glucose, and that grew on -Leu in galactose but not in glucose) were isolated.

2.2.2.4 Library screening by interaction mating (used in HeLa screen)

Mating

This method is based on the mating of two haploid yeast strains, one carrying the library, and the other carrying the bait and the *lacZ* reporter. The RFY206 yeast strain was first transformed with a pJG4-5 HeLa library or with the empty pJG4-5 vector as a control, using the library transformation method descibed in 2.2.2.3. The EGY48 or the EGY191 yeast strain was transformed with the bait and the pJK103 *lacZ* reporter as described in 2.2.2.1.

A characterized colony of EGY48 or EGY191 was used to inoculate a 30ml Glu/CM-His-Ura liquid culture. The culture was grown overnight in a 30°C shaking incubator, until it reached an $O.D_{600}$ of approximately 1.5 (mid to late log phase approximately $3x10^7$ cells/ml). The cells were then harvested by centrifugation at 3,000rpm for 10 minutes at room temperature, and the pellet was resuspended in 1ml of sterile water. 200µl of the resuspended cells (~ $2x10^8$ cells) were transferred into a new microcentrifuge tube to be used for the mating with the library, and 200µl were transferred into another tube to be used for the control mating.

The mating was setup so that there was a two-fold excess of bait strain over the library and the control strains (prior to mating, an aliquot of the frozen library strain and an aliquot of the control had been thawed, and serial dilutions were plated on Glu/CM-Trp media to determine their plating efficiency). Therefore, a total of $1x10^8$ library cells or control cells were added to 200µl of bait cells, and the two strains were mixed and centrifuged to remove the medium. The combined cell pellets were resuspended in 200µl of YPD liquid medium, plated on 10cm YPD plates, and incubated at 30°C to allow the strains to mate. Note that to account for possible errors in plating efficiencies, additional matings were setup at this stage, using different concentrations of the bait and library strains. The mating with the highest efficiency was then selected for the screening.

After 15 hours at 30°C, 1ml of sterile water and 5-10 sterile glass beads were added to the YPD plates to detach the cells. After agitation, the cells were transferred into two microcentrifuge tubes, centrifuged, and washed once with sterile water. The cells were then resuspended in 1 pellet volume of glycerol solution (~ 500µl), and divided in 100µl aliquots, which were stored at -70°C.

Selection of interactors

The cells that grew on the YPD plate consisted of a mixture of haploid (unmated) and diploid (mated) cells. In order to estimate how many cells to screen for interactors, the mating efficiency, i.e. the percentage of mated cells out of the total number of cells on the plate, had to be determined. An aliquot of the library and the control mating were therefore removed from -70°C and thawed. Serial dilutions of the aliquots were plated on both YPD and Glu/CM-His-Ura-Trp 10cm plates. After incubation at 30°C for 2 days, the total number of cells plated (YPD plates), and the number of mated cells (Glu/CM-His-Ura-Trp plates) were calculated.

Based on the mating efficiency, the appropriate number of cells were screened. For example, if the mating efficiency was 10%, and $1x10^5$ of diploid cells were to be screened, $1x10^6$ cells were plated. An aliquot of the mated library and an aliquot of the control mating were grown in Gal/CM-His-Ura-Trp for five hours to induce library expression. The cells were then plated on 10cm Gal/CM-His-Ura-Trp-Leu plates and incubated at 30°C for 4 days to select for interactors. After this stage, the screening process was identical to the direct library screening method (2.2.2.3 selection of interactors).

2.2.2.5 DNA preparation

Yeast total DNA was extracted for the isolation of the library pJG4-5 plasmid. So in order to favour the loss of the non-library plasmids, the

overnight cultures were setup in Glu/CM-Trp liquid media (*TRP1* is the marker for pJG4-5). DNA extraction was carried out using the Nucleon kit (Scotlab) and according to the manufacturer's instructions, with the only exception that in the final step, the yeast DNA was resuspended in 100µl of sterile water. To aid resuspension, the DNA was placed in a 50-60°C water bath. DNA was stored in -20°C.

2.2.2.6 Colony PCR amplification

A fresh colony, or a cell pellet from an overnight culture were used for PCRs. A small part of the colony or the cell pellet was removed using a sterile tip, and washed in 10µl of zymolase incubation solution. The solution was incubated at 37°C for 5 minutes and 5µl were added in the PCR reaction. The reaction mixture and the PCR cycles were as for bacterial colony PCRs (see 2.2.1.3).

2.2.2.7 Specificity tests

The library pJG4-5 plasmids carrying the cDNA inserts of putative interactors, were re-introduced into a characterized EGY48 strain freshly transformed with the T-Ag bait and the pJK103 plasmid, using the standard lithium acetate transformation (section 2.2.2.1). In addition, the plasmids were re-introduced into an EGY48 yeast strain carrying the LexA-RPB7 control bait to test for non-specific interactions. Three independent transformants from each transformation were picked and streaked onto a Glu/CM-His-Ura-Trp master plate. From the master plate, the transformants were restreaked onto the 4 selective plates and scored as previously described (section 2.2.2.3).

2.2.2.8 Plasmid evictions

Plasmid evictions were carried out for yeast clones carrying the PC67 library vector by growing them on non-selective media, that is media containing tryptophan, which is the marker for the pPC67 vector used. This way, yeast cells evict the vector since it is no longer required for growth. The clones isolated from the screen, were restreaked from the Glu/CM-His-Ura-Trp master plate onto a Glu/CM-His-Ura plate and incubated at 30°C. The colonies were then continuously restreaked onto new Glu/CM-His-Ura plates, until no colonies could grow on Glu/CM-His-Ura-Trp media, i.e. until all colonies had evicted the pPC67 vector.

2.2.2.9 Storage of cells

A colony from a fresh plate was used to inoculate a 10ml overnight culture. Glycerol solution was added to aliquots of the culture to a final concentration of 15% (v/v). The aliquots were snap frozen on dry ice, and then stored at -70°C.

2.2.3 Mammalian manipulations

2.2.3.1 Cell culture

The SV4, tsa8, tsa14 and NIH 3T3 cells were maintained in Dulbecco's modified Eagle medium supplemented as described in 2.1.9, in a humidified 5% CO2 atmosphere. The SV4, tsa8 and tsa14 cells were cultured either at the permissive temperature of 33°C or the non-permissive temperature of 39.5°C as required. The NIH 3T3 cells were cultured at 37°C.

2.2.3.2 Preparation of cell lysates

For western blotting

Cell lysates were prepared from 15cm dishes of sub-confluent cells. The medium was aspirated off the dish, and the cells were washed twice with 15ml of 1x PBS. The cells were then scraped off the dish and transferred into microcentrifuge tubes. The tubes were centrifuged at 1,000rpm for 1 minute at room temperature. The cell pellet was resuspended in 160µl of 1x PBS. 40µl of 6x lysis buffer were added, and the lysates were boiled for 5 minutes at 100°C. The lysates were then stored at -20°C until use.

For immunoprecipitations

Cell lysates were prepared from 15cm dishes of sub-confluent cells. The dishes were taken out of the incubator and placed on ice. The medium was aspirated off and the cells were washed twice with ice cold 1x PBS. Lysis was carried out directly on the dishes, by adding 2.5 ml of NP-40 lysis buffer mixed with 125µl of aprotinin onto each dish. The cells were lysed on ice for 30 minutes. The lysates were then scraped off the dishes and transferred into microcentrifuge tubes. The tubes were centrifuged at 10,000rpm for 15 minutes at 4°C. The supernatants were transferred into new microcentrifuge tubes, or combined into a 15ml conical tube if they originated from the same dish. The lysates were stored at -20°C until use.

2.2.3.3 Storage of cells

Cells were split and allowed to grow. After 2 days, one 15cm dish was washed briefly with trypsin, and then trypsinized with 1ml trypsin for 5 minutes at 37°C. The trypsinized cells were collected with 6 ml of FCS or DCS mixed fresh with 10% DMSO. The cells were aliquoted into cryogenic vials, put in -70°C overnight and transferred to liquid nitrogen storage the following day.

2.2.4 DNA manipulations

2.2.4.1 Agarose gel electrophoresis

DNA fragments were resolved in 1.0–1.5% agarose gels, made up and run in a 1x TAE buffer solution. Gels were routinely run at approximately 10V/cm. DNA fragments were visualised by UV transillumination, using ethidium bromide staining, which was at a concentration of 100ng/ml in the gel. Fragment size was checked by comparison with the 1kb ladder and the λHindIII and 100bp markers.

2.2.4.2 PCR amplification

PCR amplification of the T-Ag amino acids 1 to 136 for subcloning was carried out using the PJ-PS1N/PJ-136 and the NLEXA(F)/NLEXA(R) primer pairs (see Table 2.1). The reaction mixtures consisted of: 1x buffer, 3ng of pZipNeoSVLT vector DNA, 30pmoles of each primer, 6mM $MgCl_2$ (for *Taq*) or 1x $MgSO_4$ buffer (for Vent), 0.75mM dNTPs, and 2.5 units of *Taq* or Vent polymerase, in a final volume of 100µl. Control reactions without DNA were also setup to control for contamination. Each reaction was covered with 50µl of paraffin oil to prevent evaporation during thermal cycling. Using conditions that were specific for each primer pair (Table 2.1), 30 cycles of denaturation at 95°C for 30 seconds, annealling for 30 seconds, and extension at 72°C for 1 minute, were carried out.

2.2.4.3 DNA fragment purification

DNA fragments required for subcloning were excised from the agarose gels, and purified using the glassmilk Geneclean II kit from Bio101 according to the manufacturer's instructions.

2.2.4.4 Restriction enzyme digestion

Digests were performed using the incubation buffers provided with the enzymes, and according to the conditions recommended by the manufacturers.

2.2.4.5 DNA ligation

Ligation reactions consisted of 20-50ng of linearized vector and a 3-fold molar excess of insert DNA, in 1x ligase buffer (10x buffer is 660mM Tris-HCl, 50mM $MgCl_2$, 10mM DTT, 10mM ATP, pH 7.5) with 1 unit of T4 DNA ligase, in a total volume of 20µl. Ligations were incubated at 16°C overnight. "Vector-only" control ligations, using vector without insert DNA were setup to test the frequency of vector re-ligation.

2.2.4.6 DNA sequencing

Double-stranded sequencing of subcloned T-Ag fragments and library cDNA inserts, was carried out using the dideoxynucleotide chain-termination method and the Applied Biosystems "ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction" kit, according to the manufacturer's instructions. Sequencing reaction products were electrophoresed on the ABI 373XL automated DNA sequencer (Applied Biosystems) and separated by size. Data was collected using the 373XL Collection software and analysed using Sequencing Analysis version 3.0 and Sequence Navigator version 1.0.1 software.

2.2.5 Protein analysis

2.2.5.1 Protein quantification

The protein concentration of samples was determined in triplicate for each extract, using a Bio-Rad protein assay. 2µl of sample were added to a

microcentrifuge tube containing 800µl of 1x PBS and 200µl of the Bio-Rad reagent and mixed well. After 5-15 minutes, the absorbance at 595nm was measured in a Bio-Rad spectrophotometer. The protein concentration of the sample was calculated according to a standard curve prepared using a range of bovine serum albumin (BSA) concentrations.

2.2.5.2 Immunoprecipitations

Lysates prepared as described in section 2.2.3.2, were thawed on ice. Their protein concentration was determined, and 300µg of lysate were used for each immunoprecipitation. The primary antibody was first added to the lysate: 100-300µl of hybridoma supernatant or 10µl of purified antibody were added, and incubated on a wheel at 4°C for 1 hour (the antibodies used are described in 2.1.10). This was followed by the addition of 100µl of 10% (w/v) protein A-sepharose beads pre-washed twice with NP-40 lysis buffer, and incubation on the wheel at 4°C for 1 hour. The immunoprecipitates were centrifuged at 10,000rpm for 30 seconds at 4°C. The supernatant was discarded and the beads were washed twice with 500µl of NP-40 lysis buffer. Finally, the beads were resuspended in 20-40µl of Laemli sample buffer and stored at -20°C until use.

2.2.5.3 SDS-PAGE

All samples were boiled at 100°C for 5 minutes prior to loading. For direct immunoblots, 20-30µg of total lysate were loaded. The immunoprecipitations were boiled at 100°C for 5 minutes and then briefly centrifuged to sediment the beads. The supernatant was loaded onto the gel. Proteins were fractionated on 7.5%, 10% or 12% gels (see 2.1.4), alongside prestained broad range SDS-PAGE standards. Electrophoresis was carried out in 1x SDS-PAGE running buffer at 50 Volts overnight or at 150 Volts for approximately 5 hours.

2.2.5.4 Western blotting

After electrophoresis, proteins were transferred onto a hybond-C nitrocellulose membrane in 1x transfer buffer, using the Trans-Blot Cell wet tank blotting system from Bio-Rad. The transfer was carried out at 63 Volts for 4 hours at 4°C. The nitrocellulose filter was then processed for immunoblotting.

All of the incubations were carried out on a rocker. The filter was first blocked by incubation in 5% blotto for 1 hour at room temperature or overnight at 4°C with 0.02% sodium azide. The blocking solution was discarded, and the filter was incubated with the primary antibody diluted as required* in 5% blotto for 1 hour at room temperature. This was followed by two 10 minute washes with PBS/Tween. The filter was then incubated with a horseradish peroxidase-linked species-specific secondary antibody diluted 1:2,000 in 5% blotto, for 1 hour at room temperature. The filter was then washed four times in PBS/Tween (5 minutes each wash). Finally, the mixed ECL reagents were added on the filter for 1 minute without agitation, and then the filter was wrapped in cling film and exposed to X-ray film (Fuji-RX) for the appropriate length of time between 1 and 60 minutes, to achieve optimum signal and resolution.

(* antibody dilutions: PAb416 1:20, Bub1 and Bub3 1:1,000, cyclin B1 1:2,000, securin 1:2,000 and LexA 1:2,000)

2.2.6 Immunofluorescence and microscopy

Immunofluorescence was carried out jointly with Mr. A.Entwistle. Microscopy was carried out entirely by Mr. A.Entwistle.

For immunofluorescence, approximately 1-2x10^4 NIH 3T3 cells were cultured on sterile coverslips. The cells were fixed with 4% (w/v) paraformaldehyde dissolved in PBS for 20 minutes, and permeabilized with 0.2% (v/v) Triton X-

100 dissolved in PBS and 1% (w/v) BSA for 10 minutes. Cells were stained with PAb101 (1:10) coupled to IgG2a biotin (1:100), followed by Cy5 streptavidin (1:250), and with Bub1 (1:50) followed by FITC-conjugated anti-rabbit IgG (1:25). Co-localization of T antigen and Bub1 in early prophase was visualized with an LSM510 confocal laser scanning microscope (Zeiss UK). For the β-tubulin and Hsp40 immunofluorescence, the antibodies were used in 1:100 and 1:50 dilutions respectively.

For microscopy, dishes of NIH 3T3 cells were treated with nocodazole, 12 hours after plating from confluence, at a range of concentrations from 0.4 ng/ml to 4μg/ml for at least 11 hours and 10μg/ml of Hoescht 33258 for at least 2 hours. The cells were then transferred to the stage of an Inverted Axiovert 135 Microscope equipped with a humidified chamber maintained at 37°C with 5% CO_2, and timed sequences of image data were collected through phase contrast optics and a cooled CCD camera.

2.2.7 Flow cytometry

NIH 3T3 cells were cultured in 15cm dishes and synchronized by growing to confluence. When confluent, the cells were split and allowed to recover for 12 hours before the addition of a range of nocodazole concentrations from 20ng/ml to 80ng/ml. To avoid losing cells in G2 which are rounded up and detach easily, the medium was removed and transferred into a 50ml conical tube. The cells were trypsinized and collected in the same tube. The tube was centrifuged at 1,000rpm for 5 minutes and the supernatant was discarded. The cell pellet was washed twice with 10ml of ice cold 1x PBS. After the second wash, the cell pellet was well resuspended in 500μl of 1x PBS. A total of 10 ml of ice cold 70% ethanol were added dropwise into the 50ml tube while vortexing. The cells were stored at 4°C until use. The cells for flow cytometric analysis were stained with propidium iodide (PI) and

analyzed on a FACS machine using the CellQuest software (Becton-Dickinson). Flow cytometric analysis was kindly carried out by Dr. Derek Davies at the ICRF.

2.2.8 Kinase assays

Extracts

Cells were syncronized and treated with nocodazole as for flow cytometry. The cells were harvested in their medium and washed twice with ice cold 1x PBS. After the final wash, the cells were transferred into microcentrifuge tubes for easier handling, and lysed with 1ml of Hepes-modified Beach lysis buffer supplemented with 0.35 U/ml aprotinin, 1mM PMSF and 2.5μg/ml leupeptin, for 20 minutes on ice. The lysates were centrifuged at 13,000rpm for 20 minutes at 4°C and the supernatants transferred into new microcentrifuge tubes.

Imunoprecipitations

The protein concentration of the lysates was determined as described in 2.2.5.1. 200μg of lysate were used for histone H1 reactions, and 800μg for autophosphorylation reactions. The anti-Bub1 antibody was added to the lysates and incubated overnight on a rotary shaker at 4°C (15μl of antibody used for H1 reactions, and 50μl for autophosphorylation reactions). The following morning 100μl of 10% protein A-sepharose beads were added to each tube and incubated for 2 hours on a rotary shaker at 4°C. The immunoprecipitation reactions were then centrifuged at 13,000rpm for 30 seconds at 4°C. The supernatant was aspirated, taking care not to remove any of the beads. The beads were then washed twice with 500μl of the Hepes-modified Beach lysis buffer and once with 800μl of the kinase assay buffer.

Kinase reactions

20µl of substate mix were added to the washed beads. The reactions were incubated for 30 minutes at 37°C. 50µl of SDS sample buffer were then added to each reaction, and boiled for 5 minutes at 100°C. The reactions were stored in -20°C until use. Prior to loading the samples were boiled again for 5 minutes and centrifuged briefly to sediment the beads. H1 reactions were loaded on 12% SDS-PAGE gels, and autophosphorylation reactions were loaded on 8% gels. After electrophoresis, the gels were dried, and the results were visualized by autoradiography carried out by exposure of X-ray film (Amersham Hyperfilm) with an intensifying screen at -70°C.

CHAPTER THREE

Setup of the yeast two-hybrid system

This chapter describes how I setup and optimized the yeast two-hybrid system before using it to screen for T-Ag interacting proteins.

The setup of the system is mainly focused on selecting a suitable bait. This process involves a series of steps (Figure 3.1). Firstly, the subcloning of T-Ag into various LexA fusion vectors, and secondly, the characterization of these baits. The bait characterization includes three tests: The investigation of the intrinsic transactivation capacity of the various LexA-T-Ag baits, their expression into yeast, and their ability to enter the nucleus. These tests were repeated a number of times for all of the screens carried out. A representative result from each of these tests is presented in this chapter. The results of these tests indicated that the most suitable bait to use in my yeast two-hybrid screens was the inducible-expression LexA-T-Ag fusion.

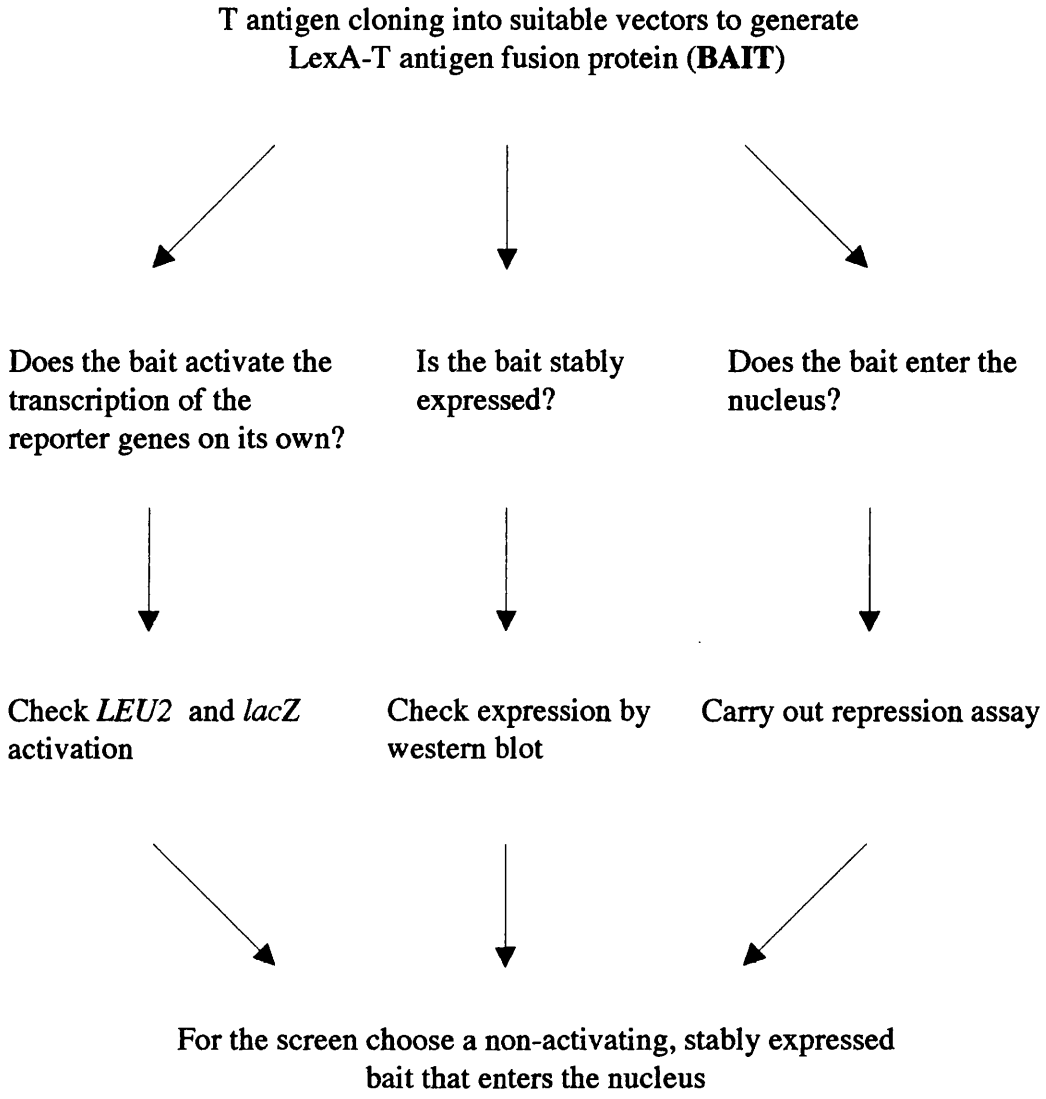


Figure 3.1 Flowchart of the bait selection process.

3.1 CLONING

A number of different versions of the yeast two-hybrid system exist. The version chosen for the T-Ag screens is called "the interaction trap" (Gyuris et al., 1993). The interaction trap uses the reconstitution of the chimeric LexA/B42 transcriptional activator to detect protein interactions, i.e. the protein of interest is expressed as a fusion with the DNA-binding domain of the bacterial repressor protein LexA, and the library cDNAs are fused to the bacterial B42 acid blob activation domain (Brent and Ptashne, 1985 ; Ma and Ptashne, 1987).

There are three different types of LexA cloning vectors available for the interaction trap, depending on the orientation of the LexA fusion and the promoter that drives its expression. All LexA plasmids carry the *HIS3* selectable marker for growth on media lacking histidine. In addition, all vectors are shuttle plasmids, i.e. they carry a yeast and a bacterial replication origin, allowing them to propagate in both yeast and bacteria.

The N-terminal domain of T-Ag from amino acid 1 to 136 was PCR amplified from a pZipNeoSVLT vector carrying the full coding sequence of T-Ag. T-Ag primers with different restriction end tags were used to subclone the PCR fragments into the different vectors (see Materials Table 2.1). Both sense and antisense strands of all the constructs were sequenced after subcloning.

3.1.1 C-terminal fusion vectors

C-terminal fusion are the standard vectors used for cloning baits in the interaction trap system. T-Ag was subcloned into the pEG202 and the pMW103 vectors (Appendix I). These vectors use the strong *ADH1* promoter to express LexA followed by T-Ag. T-Ag was PCR amplified from pZipNeoSVLT using the PJ-PS1N and PJ-136 primers, and the PCR product

was digested with BamHI and XhoI and inserted into the unique BamHI and XhoI sites of the polylinker, in frame after LexA. pEG202 and pMW103 are identical vectors carrying different antibiotic resistance genes.

3.1.2 N-terminal fusion vector

In order to address the possibility that cloning T-Ag to the C-terminus of LexA may block the N-terminal residues of T-Ag available for interaction, T-Ag was subcloned into the pNLexA vector (Appendix II). pNLexA uses the strong *ADH1* promoter to express T-Ag followed by LexA. T-Ag was PCR amplified from pZipNeoSVLT using the NLEXA(F) and NLEXA(R) primers, and the PCR product was digested with EcoRI and BamHI and inserted into the unique EcoRI and BamHI sites of the polylinker before LexA.

3.1.3 Inducible expression vector

To address the possibility that the continuous expression of T-Ag driven from the strong *ADH1* promoter may be toxic to yeast, T-Ag was subcloned into the pGilda vector (Appendix III). pGilda expresses the same fusion as pEG202 and pMW103, with the exception that the expression of the bait is under the control of a galactose-inducible promoter (*GAL1*). This way most of the yeast manipulations can be carried out on glucose media, when the bait is not expressed, and expression can be induced briefly during the screen, when selecting for interactors. T-Ag was PCR amplified from pZipNeoSVLT using the PJ-PS1N and PJ-136 primers, and the PCR product was digested with BamHI and XhoI and inserted into the unique BamHI and XhoI sites of the polylinker, in frame after LexA.

3.2 CHARACTERIZATION OF THE BAIT

A number of controls were carried out to select the most suitable LexA-T-Ag bait from the different constructs described in the previous section. The basic yeast strain EGY48 *(MATα, trp1, his3, ura3, leu2 :: 6LexAop-LEU2)* was used for all manipulations. EGY48 is defective in the production of tryptophan, histidine, uracil and leucine, so it requires these nutrients to be provided in its growth medium. This allows for the selection of cells transformed with plasmids carrying one of these auxotrophic markers, in media lacking the corresponding nutrient.

For reasons not known, it has been observed that some fusion proteins are expressed only in a small percentage of the colonies carrying the bait plasmid (Golemis and Brent, 1997). It is therefore important to assay more than one colony for each construct, and to characterize the same colonies for all three tests. Finally, one of these individually characterized colonies must be used to perform the library screens.

3.2.1 Transcription activation assays

The two-hybrid system used in this project, is based on the ability of the bait and a target protein which interact with each other, to activate the transcription of the *LEU2* and *lacZ* reporter genes. For this reason, a bait that can activate the transcription of these reporter genes on its own is not suitable for the screen. The *LEU2* reporter gene was provided by the EGY48 yeast strain, while the *lacZ* reporter was provided by the pJK103 plasmid (Appendix VI). EGY48 is a high sensitivity *LEU2* reporter strain, as it contains six LexA operator binding sites to direct transcription from the *LEU2* gene. The *lacZ* is a lower sensitivity reporter, as the pJK103 plasmid contains only two LexA operators.

EGY48 was transformed with the different LexA-T-Ag baits and with the pJK103 *lacZ* reporter plasmid. In addition, EGY48 was also transformed with the positive control plasmid pSH17-4 which expresses a transcriptionally active LexA-Gal4 bait (Golemis and Brent, 1992), and with the negative control plasmid pEG202-hsRPB7, which expresses a transcriptionally inactive LexA-RPB7 bait (Khazak et al., 1998).

The LexA bait vectors carry a *HIS3* marker and the pJK103 plasmid has a *URA3* marker, so EGY48 cells were firstly plated on glucose complete media lacking histidine and uracil (Glu/CM-His-Ura) to select for transformants. Six independent colonies were picked from each of the different LexA-T-Ag transformations, as well as from each control transformation, and all the transformants were assayed for their ability to activate *LEU2* and *lacZ* transcription.

Transformants were streaked onto two Glu/CM-His-Ura plates, one also lacking leucine (-Leu) and one containing X-Gal (Figure 3.2 a and c). However, it is important to assay the transformants on galactose media too, because the B42 activation domain of the libraries used for screening is Gal-inducible and therefore, selection of interacting proteins is carried out on galactose media. Therefore, the six transformants were also streaked onto media supplemented with 2% galactose and 1% raffinose (for brevity denoted Gal) as the carbon source instead of glucose (Figure 3.2 b and d). Yeast cells grow better on glucose because they can more readily metabolize it than galactose, so it should be noted that in all tests, growth and colour intensity was less in galactose compared to glucose.

The streaks were checked for growth and colour at frequent intervals for four days. The colour phenotype is visible faster than the growth phenotype, therefore, strongly activating baits usually show *lacZ* activity in the first 12-24 hours and *LEU2* activity after two days. Inactive baits suitable to use in a two-hybrid screen should remain white on X-Gal and not have

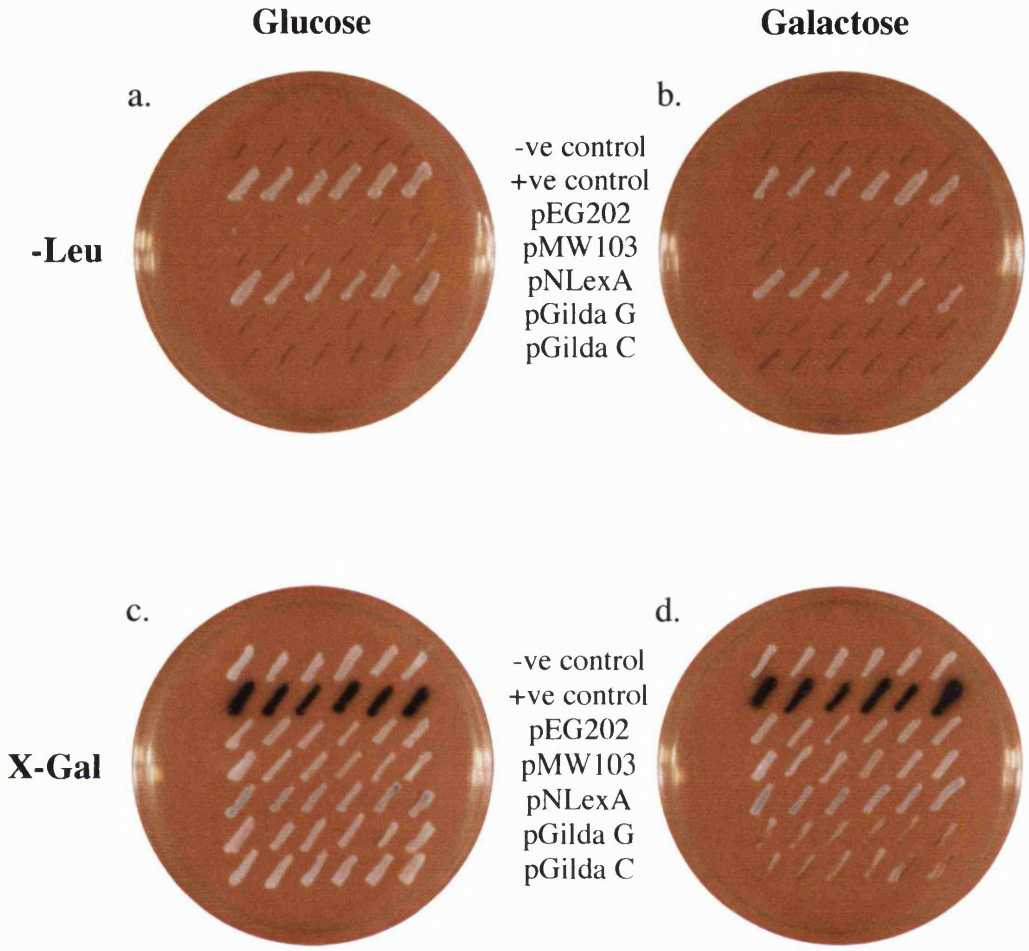


Figure 3.2 Transactivation assays of the five constructs (day 4 results). The four selective media are: a. Glu/CM-His-Ura-Leu, b. Gal/CM-His-Ura-Leu, c. Glu/CM-His-Ura/X-Gal and d. Gal/CM-His-Ura/X-Gal. -ve control is the LexA-RPB7 non-activating bait and +ve control is the LexA-Gal4 activating bait (pSH17-4 plasmid). G indicates pGilda plasmid provided by E. Golemis and C indicates pGilda plasmid purchased from Clontech.

grown on -Leu after four days (Golemis and Brent, 1997). On day 1, the pSH17-4 control had started to turn blue on X-Gal as expected, whereas the rest of the baits were white and none of them had grown on -Leu. On day 2, pSH17-4 was dark blue and had grown well on -Leu, and from the baits, pNLexA was slightly blue and had started to grow on -Leu. By day 4, pNLexA was light blue and had grown as well as the positive control. The rest of the baits showed no activity (Figure 3.2).

The results of these tests showed that the C-terminally fused T-Ag baits of pEG202, pMW103 and pGilda were all transcriptionally inactive. pEG202 and pMW103 showed slight growth on -Leu after four days, but visible only under the microscope, which should not interfere with the screen. Nevertheless, this indicated that the C-terminal inducible pGilda vector expressed the most suitable bait for screening, at least with respect to the transactivation assay.

The N-terminal bait pNLexA showed intrinsic transactivation capacity and was therefore unsuitable for screening. In order to use this bait, a strain that is not as sensitive to *LEU2* activation as EGY48 would have to be used. For this purpose, the EGY191 yeast strain (*MATα, trp1, his3, ura3, leu2 :: 2LexAop-LEU2)*, which carries only two LexA operator binding sites and is therefore less sensitive to reporter activation, was used to transform the pNLexA bait. The transactivation assay was performed again (data not shown), and even though the bait still exhibited transactivation, it was less than the positive control, and so could potentially be used in a library screen.

It is important to note the possibility that pEG202, pMW103 and pGilda showed no transcription activation, either because they were not expressing the LexA-T-Ag baits, or because the baits failed to enter the nucleus and therefore could not bind the LexA operators. To address these possibilities, firstly the expression of the baits was tested by western blotting.

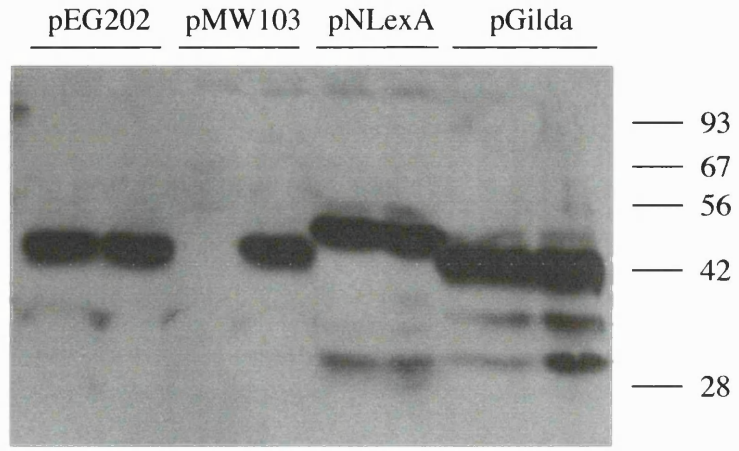
3.2.2 Expression of the fusion protein

Testing the expression of the fusion protein is an important step in bait characterization, since often there are baits whose expression in yeast is toxic and are not expressed at all, or in some cases the baits undergo proteolytic cleavage from yeast proteases.

From the six transformants of each of the constructs that were made, two were selected to test for the expression of the fusion protein. The colonies were grown in Gal/CM-His-Ura liquid media to keep the selection for the bait plasmid and the *lacZ* reporter plasmid. Protein lysates were made from cultures at an $O.D_{600}$ of 0.5-0.7 when the cells are in exponential phase and growth is optimum. The extracts were analyzed on a 12% SDS-PAGE polyacrylamide gel and immunoblotted with an anti-LexA antibody (Figure 3.3a).

pGilda showed stable expression in both colonies. The pEG202 baits were also expressed in both colonies, however, expression of only one out of the two pMW103 baits was detected. To confirm this variability, three more colonies of pEG202 and three of pMW103 were again assayed for expression of the fusion protein. None of the six colonies appeared to express any bait (data not shown). The baits were either not expressed at all, or they were entirely cleaved by yeast proteases. Thus, pGilda was found to be the most stable bait of the three C-terminal constructs. To further confirm the stable expression of the pGilda baits, three more pGilda colonies were probed with the anti-LexA antibody. The lysates were loaded onto a gel together with lysates of three LexA antibody controls (colonies carrying plasmids which express LexA only), and lysates of three colonies carrying the LexA-RPB7 bait (negative control in the library screens). The pGilda LexA-T-Ag bait was again stably expressed in all of the colonies (Figure 3.3b).

a. Anti-LexA antibody



b. Anti-LexA antibody

c. Anti-T-Ag antibody

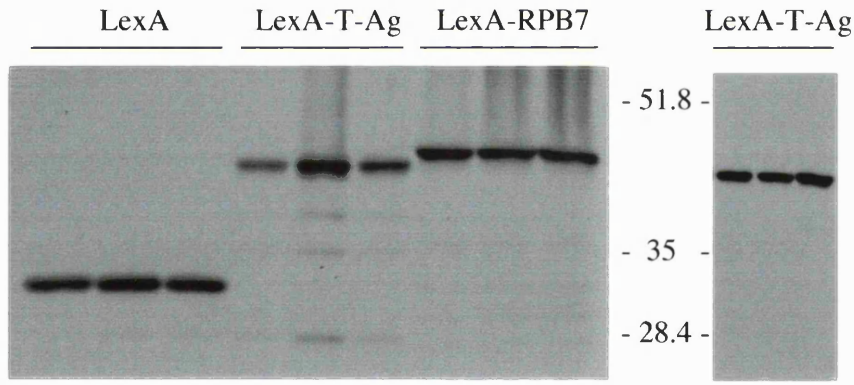


Figure 3.3 Expression of the fusion proteins.

Interestingly, the two baits expressed from the pNLexA plasmid (Figure 3.3a), migrated slower on the polyacrylamide gel, suggesting that either proteolytic cleavage had occurred in the rest of the baits, or simply the baits migrated differently in the gel. To investigate these possibilities the pGilda extracts were also immunoblotted with PAb416, an anti-T-Ag antibody whose recognition site maps to amino acids 82 to 130 of T-Ag (Harlow et al., 1981 ; Kohrman and Imperiale, 1992). The T-Ag blot confirmed that the important N-terminal T/t common region, which extends from amino acid 1 to 82 of T-Ag, was indeed intact, indicating that the difference in size between baits was due their differential migration (Figure 3.3c).

3.2.3 Repression assay

After the transactivation and expression assays it was decided that pNLexA (using the EGY191 strain) and pGilda (using the EGY48 strain) could be used in the library screens. The final test was to assay the ability of the LexA-T-Ag baits to bind the LexA operator binding sites.

This was done using a repression assay (Brent and Ptashne, 1984). This assay was carried out using the pJK101 *lacZ* reporter plasmid, which contains UAS from the GAL1 promoter (UAS_{GAL}) upstream of the *lacZ* gene and two LexA operator binding sites between the UAS_{GAL} and the *lacZ* gene. On galactose media and in the absence of LexA, the endogenous yeast Gal4 protein binds to the UAS_{GAL} and drives the transcription of the *lacZ* gene. Therefore, colonies turn blue as a result of β-galactosidase activity. In contrast, when LexA is present, it binds the LexA operators located between the UAS_{GAL} and the *lacZ* gene, and represses the transcription of *lacZ*. The transcription of *lacZ* is not completely blocked, it is however significantly decreased. On glucose media, regardless of the presence of LexA, the UAS_{GAL} is repressed and there is no *lacZ* transcription so colonies appear

white. It is important to carry out the repression assay for the LexA-T-Ag bait, as it has been shown that LexA DNA binding can be severely compromised depending on the protein it is fused to (Golemis and Brent, 1992).

Only the pGilda bait was assayed for repression, since the pNLexA bait had demonstrated intrinsic transactivation, and was therefore definitely binding the LexA operators. Three combinations of plasmids were used for the assay. EGY48 was transformed with the pJK101 plasmid and the pGilda plasmid, the pJK101 plasmid and the pRFHM1 plasmid (positive control for repression encoding LexA-Bicoid bait that binds LexA operators ; Golemis and Brent, 1992), and with the pJK101 plasmid alone (negative control for repression since no LexA is present). Each of the three transformations was plated on the appropriate medium to select for colonies carrying the corresponding plasmids.

Six colonies from each transformation were streaked onto two CM-Ura/X-Gal plates, one containing glucose and one with galactose (Figure 3.4). Only Ura was omitted from these plates to allow streaking of all three types of transformants on the same plates and eliminate variation. The omission of His from the media to select for the LexA plasmids is not necessary because the assay is very brief. The plates were examined for colour development at frequent intervals for the following 12 to 24 hours.

As expected, all colonies remained white on the glucose plate because the UAS_{GAL} was repressed on this medium. On the galactose plate, the colonies carrying pJK101 only, turned dark blue, and the colonies carrying pRFHM1, were light blue due to repression of β-galactosidase activity. The colonies carrying the pGilda plasmid were also light blue, indicating that the LexA-T-Ag bait is able to bind LexA operators. Interestingly, the pGilda colonies were even whiter than the pRFHM1 positive control, indicating that the LexA-T-Ag bait is an even stronger repressor than LexA-Bicoid.

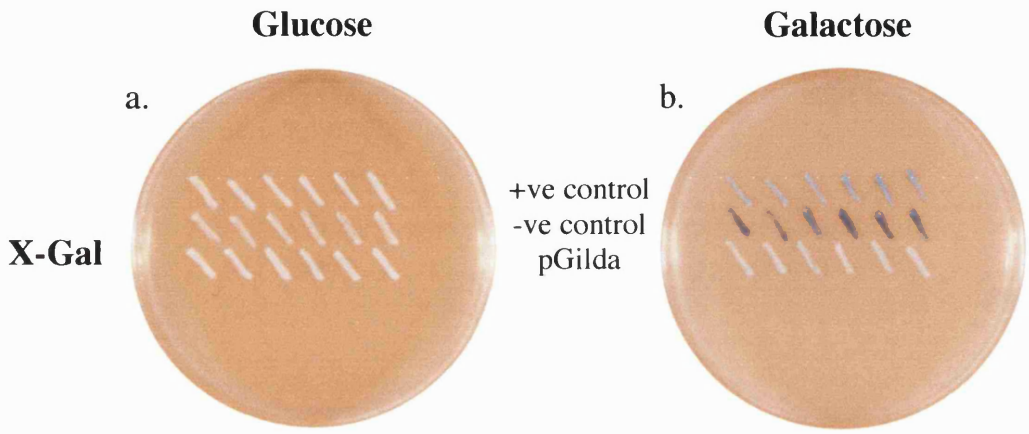


Figure 3.4 Repression assay of the pGilda construct. The two selective media are: a. Glu/CM-Ura/X-Gal and b. Gal/CM-Ura/X-Gal. +ve control is the LexA-bicoid bait (pRFHM1 plasmid) and -ve control is the pJK101 plasmid only (no bait).

3.3 DISCUSSION

The transactivation, expression and repression assays carried out demonstrated the suitability of the different LexA-T-Ag baits. In addition, the variability exhibited in the activation and expression activity of the different baits, emphasized the fact that characterization of individual colonies was very important prior to screening.

The *LEU2* and *lacZ* transcription activation assays, in addition to determining the suitability of the different baits, also highlighted an interesting point regarding the activation properties of the LexA-T-Ag bait, depending on the orientation of the fusion. When T-Ag is expressed at the C-terminus of LexA, the resulting fusion protein lacks transactivation activity. In contrast, the bait expressed from the pNLexA vector exhibits intrinsic transactivation. Since the sequence of the C- and N-terminal LexA-T-Ag fusions is identical, it is most likely that the two fusions adopt a completely different conformation which either enhances or blocks the regulatory properties of the LexA-T-Ag bait. Activation by LexA is very susceptible to even very small fusions, as it has been shown that even when the LexA moiety is expressed on its own, it acquires a weak transactivation ability due to the small extra peptide expressed from the polylinker sequence (Golemis et al., 1997).

Preliminary transactivation assays carried out using the N-terminal deletion T-Ag mutant *dl*1135 which has a deletion from amino acid 17 to 27 (which removes the predicted α-helix of the J domain) subcloned into pEG202, gave the same results as the pNLexA vector, i.e. the LexA-*dl*1135 bait showed intrinsic transactivation capacity. It is possible that a yeast repressor protein could be binding the deleted region, therefore, its presence is responsible for the transcriptionally inactive phenotype of the wildtype T-Ag versus the mutant T-Ag which lacks this region. Even though this data

strengthens the correlation between conformation and transactivation, it should be noted that these experiments were carried out in the initial phases of the project before the system and the bait characterization tests were optimized, and so further work is required to confirm these results.

Combined with the transactivation assays, the expression assay for the different baits indicated that the inducible expression pGilda bait was the most suitable bait for the screen, since it was expressed in all of the colonies tested. A slight difference in size was observed between the N-terminally fused and the C-terminally fused baits which migrated faster on the SDS-PAGE gels. Peptide cleavage sometime occurs in baits, however, the clipping of amino acids from the C-terminally fused baits by endogenous yeast proteases, cannot be explained due to the possible presence of yeast recognition sequences on T-Ag, since pGilda and pNLexA carry an identical T-Ag sequence. Proteins often run on SDS-PAGE gels at a different size than their predicted one, so the different migration exhibited by the baits is most likely a reflection of that.

The repression assay result was also interesting, which indicated that the LexA-T-Ag fusion had increased repression activity compared to the LexA positive control. This is especially interesting since the fusion of protein moieties to the LexA moiety has generally been shown to inhibit the ability of LexA to bind DNA (Golemis and Brent, 1992). This result could again be explained by the possible presence of a repressor element binding the T-Ag moiety, which could be directly repressing *lacZ* transcription.

Alternatively, the enhanced repression could be due to enhanced operator occupancy by the LexA-T-Ag bait. Operator occupancy could be increased either by enhanced affinity for DNA binding by the LexA-T-Ag fusion, or simply by an increase in amount of the LexA-T-Ag bait present in the nucleus. The first hypothesis requires that either T-Ag or LexA contributes to the enhanced affinity for DNA binding. The T-Ag moiety itself

cannot account for direct DNA binding since this activity is not present in amino acids 1 to 136 of the protein. Therefore, either the presence of T-Ag enhances the intrinsic DNA binding property of LexA, or the sequence combination of the LexA-T-Ag fusion acquires a *de novo* DNA binding activity.

The second hypothesis suggests that there is an increased amount of LexA-T-Ag present, which can saturate the LexA operators. Normally, in the interaction trap, the DNA binding domain-fused baits do not carry a nuclear localization sequence, while the activation domain-fused library proteins are transported to the nucleus by virtue of the SV40 NLS which is contained in the fusion cassette of the pJG4-5 library vector (Appendix IV). This is done so that there is an excess of library protein in the nucleus, and therefore an excess of interacting proteins, over the DNA-bound bait, which increases the probability of detecting an interaction. The LexA-T-Ag bait however, carries the SV40 NLS as part of the N-terminal T-Ag sequence (amino acids 126 to 132). This could result in a higher nuclear concentration of the LexA-T-Ag bait and subsequently, an increase in operator binding capacity that is not seen with other LexA fusion proteins.

In either case, this strong repressor activity could potentially create a problem for library screens. The library proteins may have to be expressed in high enough levels to allow them to shift the equilibrium from LexA-T-Ag/DNA bound bait or LexA-T-Ag/repressor-bound bait, to LexA-T-Ag/library protein-bound bait, in order for the reporters to be activated and any interaction to be identified.

Finally, the fact that T-Ag can function as a DnaJ protein could be a problem in itself for the library screens, since as a chaperone it could potentially bind a number of yeast proteins and increase the number of false-positive clones isolated.

CHAPTER FOUR

The yeast two-hybrid screens

This chapter describes the yeast two-hybrid screens that were carried out in order to identify novel proteins that interact with T-Ag. The initial unsuccessful screens are first discussed, and how the particular problems that were encountered improved the setup of subsequent screens (summarized in Table 4.1). The following sections describe the four screens that were performed after the optimization of bait selection as described in chapter 3. The pNLexA and the pGilda baits were each used to screen both a mouse embryonic library and a HeLa library. All screens were performed using the interaction trap system, either by the standard method of direct transformation, or by interaction mating. The various stages of the screens starting from the library transformation and through to the final specificity tests of the isolated clones are described (Figure 4.1). The results of these four screens from each stage are summarized in Table 4.2.

Bait vector	Library	Transformation method	Total transformants	Cells screened	*LEU2*+ colonies (library)	*LEU2*+ colonies picked	*LEU2*+/*lacZ*+ and/or Gal-dependent clones	PROBLEM
pEG202	HeLa	Direct	$5x10^4$	$2.5x10^8$	65	65	65	auto-activation (clones did not re-transform)
pEG202	HeLa	Direct	$4x10^6$	$2x10^7$	0	0	0	yeast not viable on galactose
				$1x10^8$	0	0	0	"
				$3x10^8$	0	0	0	"
pEG202	HeLa	Direct	$2.5x10^6$	$2x10^7$	0	0	0	"
pEG202	Mouse*	Direct	$1.5x10^6$	$6.2x10^7$	700	700	342	yeast did not express TAg
pEG202	Mouse*	Direct	$3.5x10^6$	$1x10^7$	70	70	70	auto-activation (clones did not re-transform)

Table 4.1 Summary of the unsuccessful screens (*GAL4 activation domain).

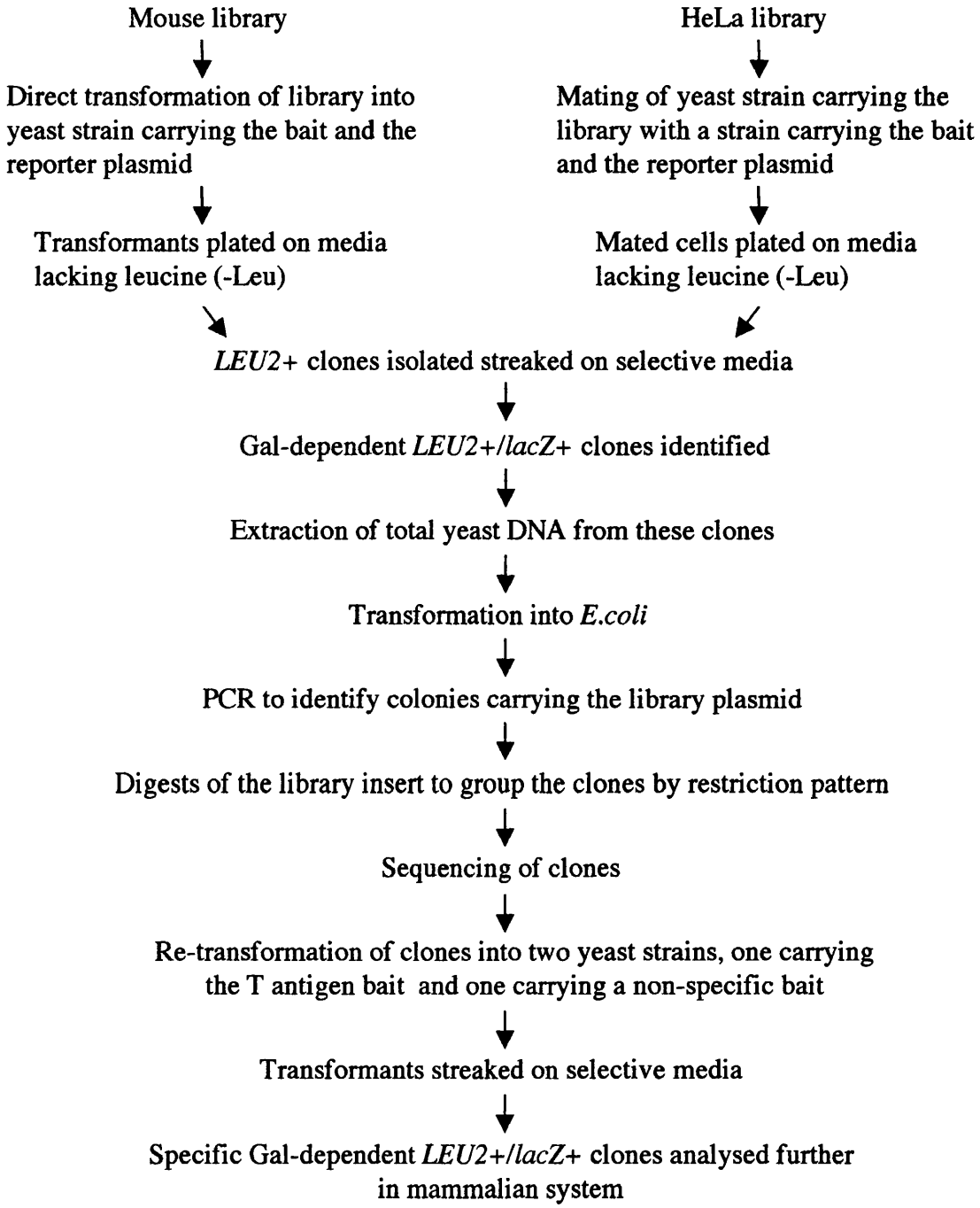


Figure 4.1 Flowchart of the yeast two-hybrid screen strategy.

Bait vector	Library	Transformation method	Total transformants	Cells screened	*LEU2*+ colonies (library)	*LEU2*+ colonies (empty vector)	*LEU2*+ colonies picked	Gal-dependent *LEU2*+/*lacZ*+ clones	Potential interactors
pNLexA	Mouse	Direct	$3x10^5$	$3x10^6$	270	244	270	0	0
pNLexA	HeLa	Mating	$1x10^6$	$1x10^6$	504	486	96	0	0
pGilda	Mouse	Direct	$3x10^5$	$9x10^6$	250	12	121	29	22
pGilda	HeLa	Mating	$3x10^5$	$3x10^5$	122	0	122	42	3

Table 4.2 Summary of the yeast two-hybrid screens.

4.1 UNSUCCESSFUL SCREENS

A number of yeast two-hybrid screens were initially carried out, which although did not identify any potential T-Ag interactors, formed a significant part of this project, and their outcome helped optimize subsequent screens. All these screens were carried out with the C-terminal fusion LexA-T-Ag bait expressed from the standard two-hybrid vector pEG202. The libraries screened, were the HeLa B42 library described previously, and the pPC67 mouse day 14.5 embryo GAL4 library (Appendix V).

Three problems were encountered with these screens (Table 4.1). Firstly, in two of the screens the bait exhibited intrinsic transactivation activity and no clones repeated their phenotype upon retransformation. Secondly, in another screen it was observed that yeast was not maintaining the expression of the LexA-T-Ag bait, and thirdly, in two of the screens yeast was not viable on galactose and therefore no colonies were obtained, a problem that was never encountered in any of the subsequent screens.

Intrinsic transactivation was seen both with the HeLa and the mouse library screens. All of the colonies picked from the screens had their DNA extracted, electroporated into *E.coli* and retransformed into yeast, and no colonies were found to repeat their phenotype. The significant variability that exists from one yeast colony to another had not been observed at the time, and therefore, the screens were not always performed using individually characterized colonies, i.e., even though the characterization tests were always carried out for the given bait, the actual library screens were performed with a freshly transformed colony. So one possibility is that the screens were performed with an intrinsically transactivating bait from the start. Alternatively, the bait gained transactivation potential following the library transformation, as it was later also seen with the pNLexA screens. This

emphasized the necessity of including an empty library control vector in all screens, which would indicate the background activity of the bait.

In one of the mouse screens, it was found that the colonies picked did not maintain expression of the LexA-T-Ag bait. It is highly unlikely that yeast would have just deleted the T-Ag sequence, since pEG202 is a high copy vector and that would have required multiple events. It is therefore more likely that even though the plasmid was retained, the bait was not expressed. This suggested that prolonged expression of the LexA-T-Ag bait may be inhibitory to yeast, and that a system where the expression of T-Ag is transient should be considered, like the pGilda vector.

A method used for the first mouse screen, where 700 colonies were isolated, was the "plasmid eviction" approach. Due to the large number of colonies, and the non-inducible nature of the GAL4 promoter, it was decided to assess the library-dependence of these colonies, by growing them on non-selective media. That is, the colonies were continuously streaked onto media containing tryptophan, which is the marker for the pPC67 library vector used, in order for the yeast cells to evict this vector since it would no longer be required for growth. Plasmids like pPC86 (the backbone vector for the PC67 mouse embryo library) that contain chromosomal *ars* (autonomously replicating segment) sequences are usually unstable, that is, after growth on non-selective media < 5% of the cells will still carry the plasmid (Tschumper and Carbon, 1983). This is the principle on which the eviction assay is based. However, this was not the case for the pPC86 vector and the eviction assay turned out to be an extremely time-consuming and inefficient process. The period of time required to evict the library plasmids from all of the 700 colonies was far longer than it would have taken to extract the DNA, transform *E.coli,* group the classes of clones and re-transform them into yeast. This screen emphasized the importance of using an inducible-expression library (Gal-inducible like the B42 libraries), which would facilitate the easy

scoring of library-dependent clones simply by streaking them onto the appropriate media.

In addition, it should be added that in all of the initial screens the bait and the reporter were introduced into yeast by means of a stepwise transformation, i.e. the bait plasmid was transformed first, transformants were selected and then the *lacZ* reporter plasmid was transformed. This was done to achieve a high transformation efficiency, however, it is possible that multiple transformations are stressful to yeast cells and can result in altered properties of cells, for example an increase of intrinsic transactivation ability. Therefore, in order to minimize yeast handling and in spite of the decrease in transformation efficiency, the simultaneous transformation of bait and reporter was used for subsequent screens.

Finally, it should be added that all the initial screens were very large-scale screens, based on the belief that the more primary transformants and the more cells screened, the more chance there would be of isolating interactors. In addition, all of the colonies were picked and pursued, even when the number was as high as 700. This was very time-consuming, and as it turned out picking such a high number of colonies, did not increase the chance of isolating true positive clones. It was therefore decided, that subsequent screens would be smaller scale, and that when large number of clones were isolated, only a representative number would be pursued to first determine the stringency of the screen.

4.2 pNLexA SCREENS

The low sensitivity EGY191 yeast strain (two LexA operators) was used for both pNLexA screens. The mouse embryonic library was screened by direct transformation and the HeLa library was screened by interaction mating. Both libraries are constructed in the pJG4-5 vector, which expresses cDNAs as translational fusions to the SV40 NLS, the B42 activation domain and the hemagglutinin (HA) epitope tag (Appendix IV).

Mouse day 19 embryo library (for detailed protocol see Methods 2.2.2.3)

An individual, characterized colony of EGY191, carrying the pNLexA bait plasmid and the pJK103 *lacZ* reporter plasmid, was used for the transformation of the library. Transformations were carried out using library DNA, except one which was carried out with the empty pJG4-5 library vector as control, to estimate the number of cDNA-independent false positive colonies. A total of $3x10^5$ library transformants were obtained, and to fully represent each independent transformant, $3x10^6$ cells were plated on media lacking leucine to test for LEU2 activation.

After four days, 270 colonies had grown. However, a comparable number of colonies carrying the empty vector (244) had also grown, meaning that there was a very high background of non-specific colonies. Nevertheless, it was decided to test the library colonies for any potential interactors that may exhibit *LEU2* and *lacZ* activation above background. All 270 colonies were therefore picked and streaked onto the 4 selective plates to assay their *LEU2* and *lacZ* phenotype (see 2.2.2.3). The LexA-T-Ag bait transformed with the pJG4-5 empty vector was used as a negative control (LexA-T-Ag/B42), and the LexA-RPB7/B42-RPB4 interaction was used as a positive control (Figure 4.2).

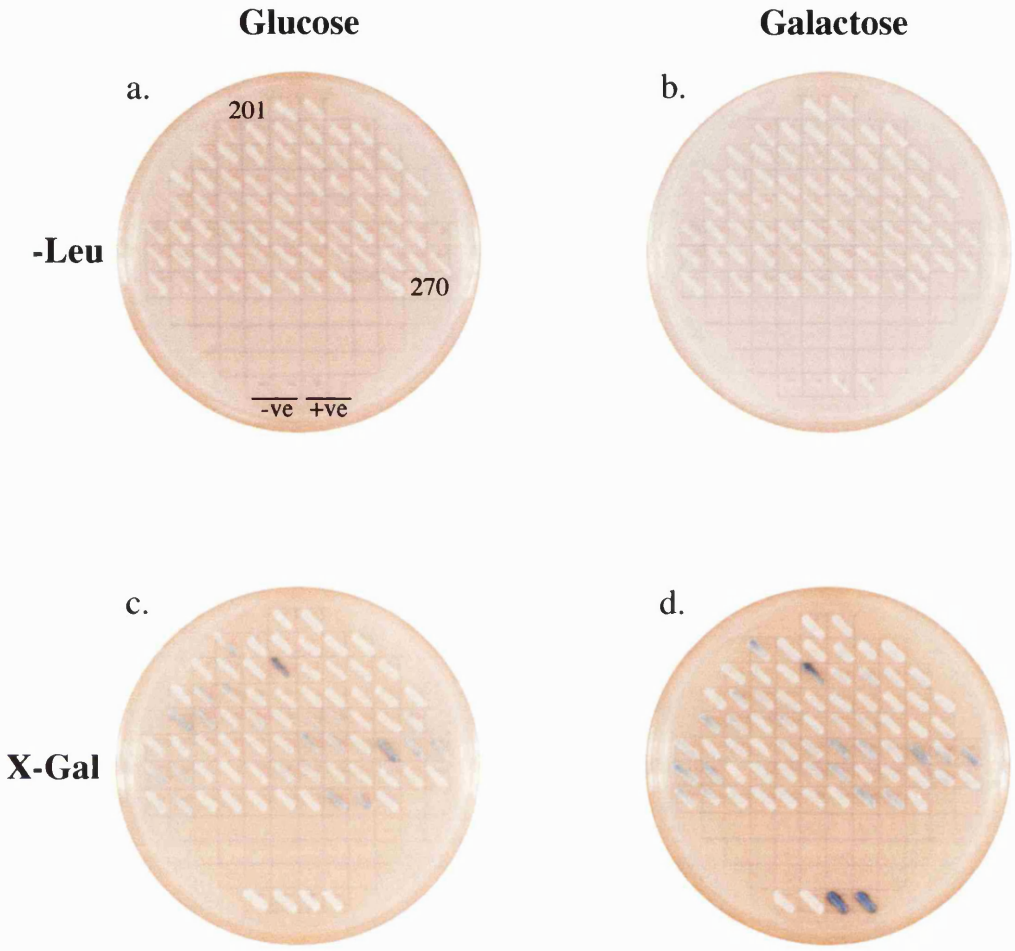


Figure 4.2 pNLexA mouse clones 201-270 on the 4 selective plates. The selective media are: a. Glu/CM-His-Ura-Trp-Leu, b. Gal/CM-His-Ura-Trp-Leu, c. Glu/CM-His-Ura-Trp/X-Gal and d. Gal/CM-His-Ura-Trp/X-Gal. The -ve controls are LexA-T-Ag/B42 clones and the +ve controls are LexA-RPB7/B42-RPB4 clones.

All of the 270 colonies grew as well on glucose as on galactose. Similarly, the colonies that turned blue, did so on both Glu/X-Gal and Gal/X-Gal media. This result indicated that the *LEU2*$^+$/*lacZ*$^+$ phenotype of these colonies was not galactose-dependent, and therefore not due to the presence of the library plasmid, but due to transactivation of the reporters by the LexA-T-Ag bait. Therefore, none of the 270 colonies picked were pursued any further. Interestingly, the negative control LexA-T-Ag baits did not activate the reporters, which suggests that the increase in intrinsic transactivation was somehow triggered by the library transformation.

HeLa library (for detailed protocol see Methods 2.2.2.4)

The screening of the HeLa library was performed by interaction mating (Bendixen et al., 1994 ; Finley Jr. and Brent, 1994). In a standard screening as described above, the library is transformed into a haploid yeast strain carrying the bait and the *lacZ* reporter. In the interaction mating screening, one haploid strain is transformed with the library, and another haploid strain of the opposite mating type (a or α) is transformed with the bait and the *lacZ* reporter. The two strains are mated to produce a diploid strain that carries the bait plasmid, the library plasmid and the LEU2 and *lacZ* reporters. Any two haploid strains can be used for interaction mating, as long as their mating types and nutritional requirements (auxotrophic markers) can be combined.

The strain carrying the pNLexA bait and the *lacZ* reporter was again EGY191. The strain transformed with the HeLa library was RFY206 *(MATa, trp1Δ::hisG his3Δ200 ura3-52 lys2Δ201 leu2-3)*. RFY206 was also transformed with the empty library pJG4-5 vector as a control.

A characterized colony of EGY191 was used to setup the culture for the mating. Two matings of EGY191 were setup, one with an aliquot of the pretransformed library strain, and one with an aliquot of the pretransformed control strain. The mating efficiency for pNLexA was estimated (see Methods

2.2.2.4) to be 25%, and $1x10^6$ transformants were obtained. In order to fully represent the transformants, a total of $1x10^7$ cells had to be plated. However, since $1x10^6$ cells is the maximum number of cells that can be plated onto a dish without cross-feeding between colonies being a problem, and the mating efficiency was 25%, forty dishes had to be plated to screen $1x10^7$ cells. Instead it was decided to start with a small scale screen first to get an indication of the number of colonies growing, and subsequently plate more cells if necessary. Therefore, $1x10^6$ cells were plated on media lacking leucine to test for LEU2 activation.

After four days a total of 504 colonies had grown. Similar to the mouse screen, a high number of control colonies had also grown (486). A representative group of 96 colonies (colonies that had appeared on the second, third and fourth day) were picked. When streaked on the 4 selective plates, none of the 96 colonies exhibited a Gal-dependent *LEU2*$^+$/*lacZ*$^+$ phenotype. It was highly unlikely that any potential interactors would exist in the remaining 408 colonies, and therefore the screen was not pursued any further.

4.3 pGilda SCREENS

The high sensitivity EGY48 yeast strain (six LexA operators) was used for both pGilda screens. As for the pNLexA screens, the mouse library was screened by direct transformation, and the HeLa library was screened by interaction mating.

Mouse day 19 embryo library

The screen was carried out as described for the pNLexA bait. A total of $3x10^5$ library transformants were obtained and $9x10^6$ cells were screened. After four days, 250 colonies had grown. In contrast, only 12 colonies carrying the empty library vector had grown, indicating that background transactivation was not induced by the pGilda bait. 121 colonies were picked and streaked onto the 4 selective plates, and 29 of these had a Gal-dependent *LEU2*$^+$/*lacZ*$^+$ phenotype (Figure 4.3).

To isolate the library plasmid, DNA was extracted from these clones and transformed by electroporation into JS4 or DH5α *E.coli* cells. Colony PCR amplification using library-specific primers (BCO1 and BCO2 ; Table 2.1) was carried out to identify *E.coli* colonies carrying the pJG4-5 library vector, since pGilda, pJK103 and pJG4-5 all carry the ampicillin resistance gene. Three colonies were PCR amplified for each yeast DNA transformed, based on the estimation that on average one in three colonies would carry the library vector, one would carry the bait vector and one would carry the *lacZ* vector (Figure 4.4a).

Some library plasmids were very hard to isolate. In this case, more than three, and in some cases more than ten or fifteen *E.coli* colonies had to be PCR amplified before one was found that carried the library plasmid. So even though it was expected that the percentage of library-containing colonies among the total number of colonies would be standard, there seemed to be a

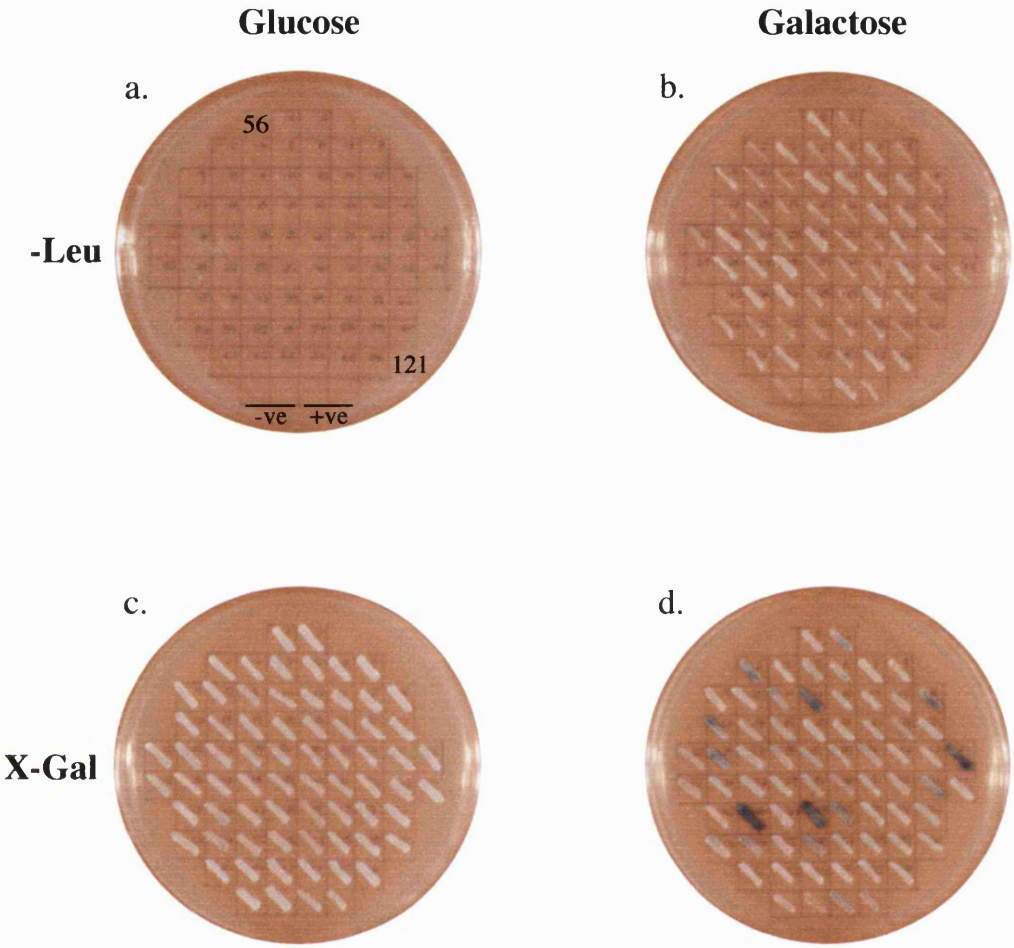


Figure 4.3 pGilda mouse clones 56-121 on the 4 selective plates. The selective media are: a. Glu/CM-His-Ura-Trp-Leu, b. Gal/CM-His-Ura-Trp-Leu, c. Glu/CM-His-Ura-Trp/X-Gal and d. Gal/CM-His-Ura-Trp/X-Gal. The -ve controls are LexA-T-Ag/B42 clones and the +ve controls are LexA-RPB7/B42-RPB4 clones.

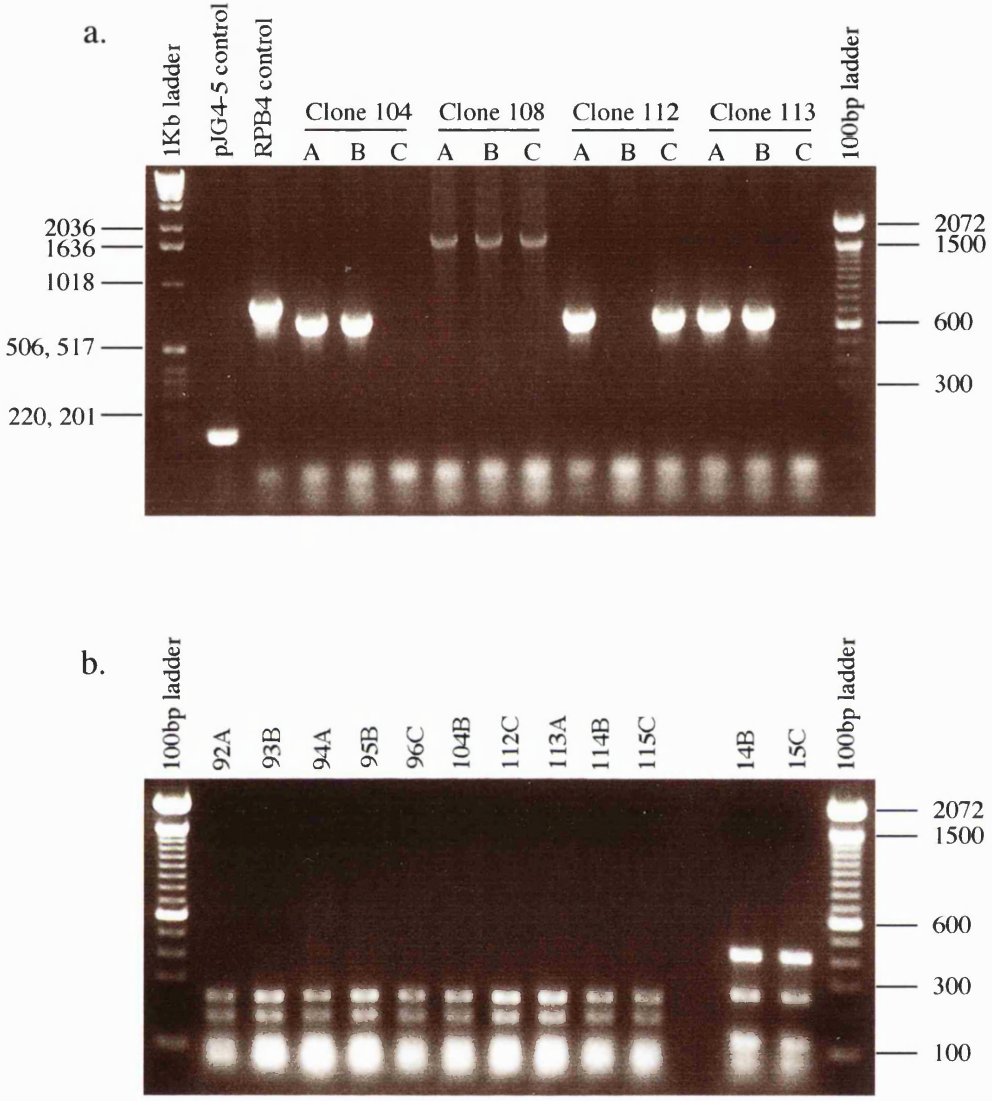


Figure 4.4 PCRs and digests of library cDNAs.

variability depending on the clone. This variability could possibly result from either the yeast or the bacteria. If the library protein is toxic or in any way unfavourable to yeast, the copy number of the plasmid could drop significantly. So when the total DNA is transformed into *E.coli*, there is a bias towards the bait and the *lacZ* plasmids. Furthermore, the B42 activation domain expressed by the library is derived from *E.coli* DNA and has been found to include a sequence which could act as a bacterial promoter. Therefore, it is possible that some of the library proteins are expressed in *E.coli* too, which again could be a problem if the protein is toxic to bacteria (I.Serebriiskii, personal communication). Another option would have been to carry out yeast colony PCRs directly to identify the different classes of interactors in order to avoid all the *E.coli* transformations of the same reisolates. However, yeast PCRs were found to be difficult to manipulate and not at all reproducible.

The 29 *E.coli* colonies carrying the library plasmids were identified, and their plasmid DNA was isolated. The JG4-5 SEQ primer (Table 2.1) was then used to sequence the cDNA inserts. All the inserts were successfully sequenced, except one, probably due to the presence of secondary structures which interfered with the sequencing reaction. Both standard nucleotide-nucleotide (BLASTn), and translated query-protein database (BLASTx) BLAST searches were carried out (www.ncbi.nlm.nih.gov/BLAST), which identified 22 classes of potential interactors among the 29 clones (Table 4.3). Some of these clones were among common false positives found in most yeast two-hybrid screens, like cytochrome oxidase, ribosomal proteins and collagen-related proteins, and so the potential classes of interactors are most likely to be less than 22.

Of these 22 clones, it was decided to pursue and test the specificity of the Hsp40 clone and the two B-cell translocation gene 1 (BTG1) clones. This decision was based on the function of these genes and their potential link to

Clone	No. of times isolated
Hsp40 DnaJ homologue subfamily B member 1	1
B-cell translocation gene 1,anti-proliferative (BTG1)	2
WD40 repeat protein 1 (Wdr1)	1
Mus musculus cDNA/human b-tubulin cofactor C	1
focal adhesion kinase (Fadk)	1
Homo sapiens calumenin (CALU)	1
Mus musulus cDNA/human SNAPC3 polypeptide	1
endogenous virus intracisternal A-particle	1
protein interacting with C kinase 1 (Pick1)	1
Mus musculus cDNA/rat rS-Rex-smRNA	1
similar to protein phosphatase 6, catalytic subunit	1
similar to methionine adenosyltransferase I, alpha	1
Mus musculus cDNA/5'UTR of image clone	1
tumorous imaginal disc protein Tid56-like	2
Mus musculus cDNA/hypothetical human protein	1
glutathione synthetase	1
unknown/hypothetical human protein	1
unknown	2
cytochrome c oxidase subunit II (Cox2)	1
ribosomal protein L7 (Rpl7)	1
skeletal muscle actin	1
alpha-1 type I procollagen	2
alpha-1 type II procollagen (Col2a1)	2
Clone unsuccessfully sequenced	1

Table 4.3 Summary of the 29 mouse clones isolated. In some cases, the BLAST search did not identify a known mouse sequence, so the closest rat or human homologue is shown. Some clones represented novel sequences indicated as unknown in the table.

T-Ag. Hsp40 is a DnaJ homologue (Silver and Way, 1993), which is a function shared by T-Ag, and BTG1 is a gene with an antiproliferative function (Rouault et al., 1992), thus involved in growth control like T-Ag. However, the decision to pursue only these two clones, does not in any way mean that the rest of the clones can be excluded as potential true interactors of T-Ag.

To verify the specificity of the Hsp40 and BTG1 interactions, the plasmids carrying Hsp40 and BTG1 were re-introduced into an EGY48 yeast strain newly transformed with the pGilda LexA-T-Ag bait and the pJK103 *lacZ* reporter plasmid, and were re-tested for association to see if the phenotype would be repeated. In addition, the plasmids were introduced into a yeast strain carrying the LexA-RPB7 control bait, to see if these clones interact non-specifically (see Methods 2.2.2.7).

Three independent transformants from each re-transformation were streaked onto the 4 selective plates. The BTG1 clone did not exhibit LEU2 or *lacZ* transcription in either of the yeast strains, whereas the Hsp40 clone exhibited T-Ag specific LEU2 transcription, but did not activate *lacZ* transcription (Figure 4.5).

It is important to note that the phenotype of the re-transformed clone is not always identical to the phenotype of the original transformant. When re-introducing the plasmid, it is unlikely that the exact same copy number will be introduced into the yeast cell as during the library transformation, and that may affect the intensity of the phenotype. In addition, when the colonies are first picked from the library screen and streaked onto the 4 selective plates, they are scored into three categories on the basis of growth (fast, medium and slow growers), and into four categories on the basis of colour (dark blue, blue, light blue and white). This was done because, in contrast to the high sensitivity *LEU2* reporter which has six LexA operators, the *lacZ* reporter has only two, and it is possible that there are interactions which can activate

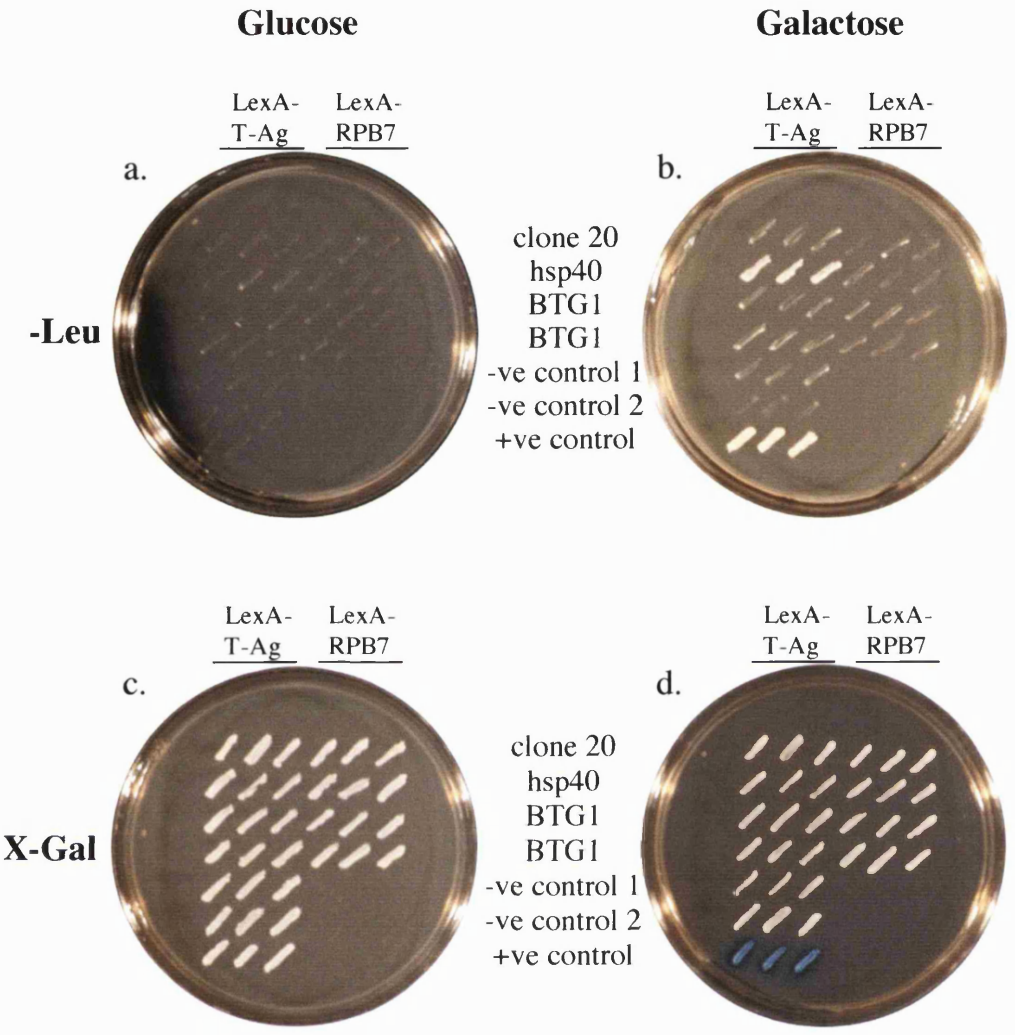


Figure 4.5 Mouse library pGilda re-transformants on the 4 selective plates. The selective media are: a. Glu/CM-His-Ura-Trp-Leu, b. Gal/CM-His-Ura-Trp-Leu, c. Glu/CM-His-Ura-Trp/X-Gal and d. Gal/CM-His-Ura-Trp/X-Gal. -ve control 1 is B42 and -ve control 2 is B42-RPB4. +ve control is LexA-RPB7/B42-RPB4. Clone 20 is the unsuccessfully sequenced clone that was re-transformed to determine specificity before any further characterization.

LEU2 transcription very strongly, but *lacZ* transcription very weakly or even not at all. This is why if a clone is a fast grower but is light blue or even white, as it was the case for the Hsp40 clone, it is still considered a potential interactor (Estojak et al., 1995). In fact, in almost all of the screens carried out, the dark blue and blue colonies turned out to be false positives.

In conclusion, based on the above observations, the retransformation and specificity tests have shown that Hsp40 is a true positive interactor of the LexA-T-Ag bait.

HeLa library

The screen was carried out as described for the pNLexA bait. The mating efficiency was 5%, and $3x10^5$ transformants were obtained. A total of $3x10^5$ cells were screened and 122 colonies grew on media lacking leucine. No colonies grew on the control plates. All of the 122 colonies were picked and streaked onto the 4 selective plates and assayed for growth and colour. Of these, 42 were found to exhibit a Gal-dependent *LEU2*$^+$/*lacZ*$^+$ phenotype.

As for the mouse screen, the yeast DNA from these 42 clones was isolated and electroporated into *E.coli*. PCR was used to identify the colonies carrying the library vector, as well as the size of the insert. It was interesting that out of the 42 clones, 39 appeared to have the same insert size. To determine if these 39 clones represented the same insert, the PCR products were digested with HaeIII and their digestion patterns were analyzed on agarose gels. Two more clones with the same insert size, different from the 39, were also digested (Figure 4.4b). This further suggested that the 42 clones represented three classes of potential interactors, one class isolated 39 times, one class isolated twice, and one class isolated once. Sequencing and BLAST searches determined that the clones were the cytoskeletal protein β-tubulin,

the transport vesicle coat protein COPII , and the mitotic checkpoint kinase BUB1 (Table 4.4).

Clone	No. of times isolated
β-tubulin	39
COPII	2
BUB1	1

Table 4.4 Summary of the 42 HeLa clones isolated.

It was decided to retransform and test for specificity, four of the β-tubulin clones, one COPII clone, and the one BUB1 clone (Figure 4.6). COPII exhibited non-specific growth on media lacking leucine and was not pursued any further. β-tubulin did not exhibit LEU2 or *lacZ* transcription in either yeast strain. The retransformations showed that only the BUB1 clone interacted specifically with the LexA-T-Ag bait. Again it is interesting that out of the 42 clones isolated from the initial transactivation tests, the BUB1 clone was the only one with a light blue/almost white colour phenotype.

In conclusion, the HeLa library screen identified BUB1 as a true T-Ag interactor. It should be noted, that unlike the rest of the screens, this screen did not produce any of the common false positives. In addition, no background was seen by the empty library control, and furthermore, β-tubulin was isolated many times which in general is an indication of a potential true interactor. Therefore, the possibility that despite its retransformation phenotype, the β-tubulin clone may also be a potential T-Ag interactor, was considered and is discussed in the following chapter.

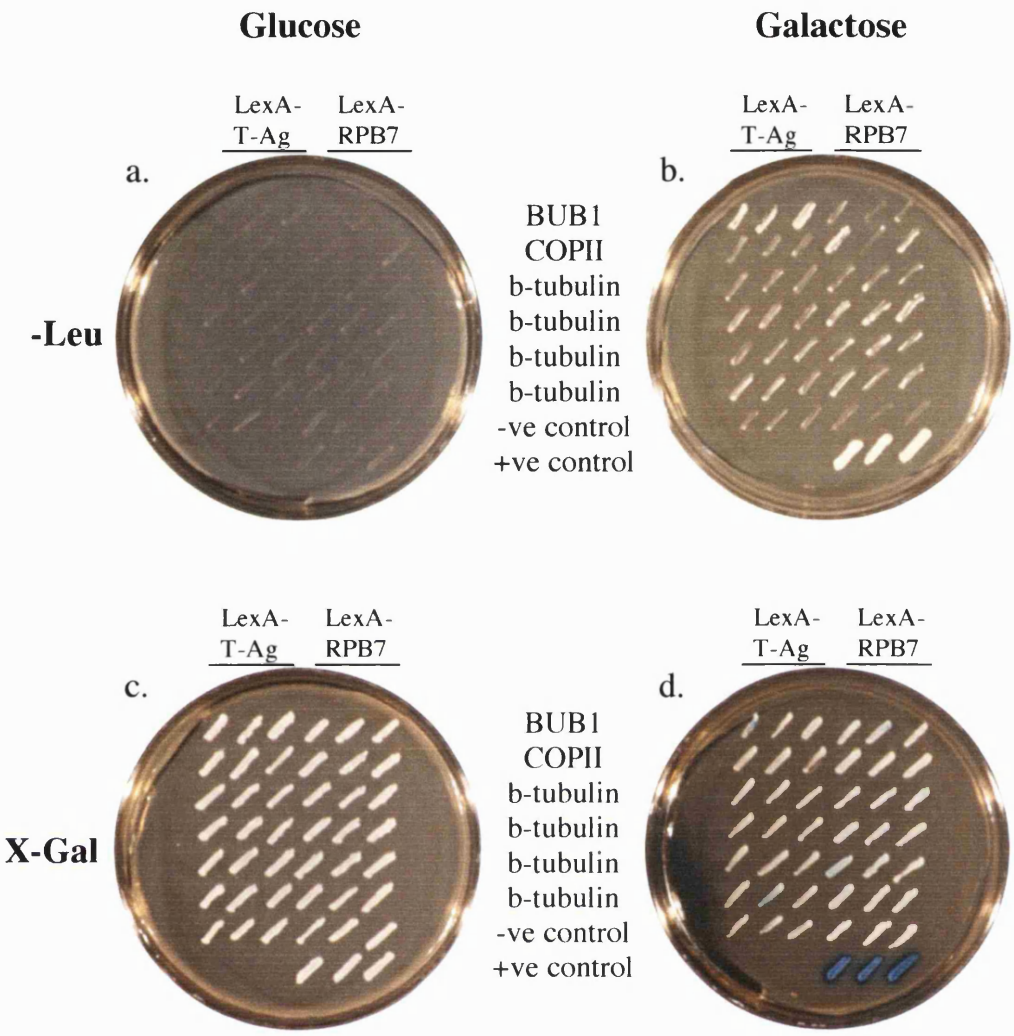


Figure 4.6 HeLa library pGilda re-transformants on the 4 selective plates. The selective media are: a. Glu/CM-His-Ura-Trp-Leu, b. Gal/CM-His-Ura-Trp-Leu, c. Glu/CM-His-Ura-Trp/X-Gal and d. Gal/CM-His-Ura-Trp/X-Gal. -ve control is B42 and +ve control is B42-RPB4.

4.4 DISCUSSION

In order to identify novel T-Ag interactors, a number of yeast two-hybrid screens were carried out. Following screen optimization and bait characterization, pNLexA and pGilda were used to screen a HeLa and a mouse embryo library. Several conclusions can be drawn from the results of these screens.

The orientation of the LexA fusion was important for the outcome of the screens. The pNLexA bait, although introduced into a less sensitive *LEU2* yeast strain to reduce background transactivation, exhibited an increase in activation ability following the library transformation. This is a problem that has been encountered with other baits used in two-hybrid screens, but no explanation has yet been found as to why this happens (Golemis and Brent, 1997). The pGilda bait on the other hand, was stable and did not exhibit any background activation, indicating that the C-terminal orientation of the fusion and the inducible nature of the bait were the most appropriate conditions to screen for T-Ag interactors.

The results of the screens also emphasized the difference between the direct transformation method and the interaction mating method. Interaction mating was shown to give better results in the screens, mainly indicated by the low number of false positive clones isolated, and the total absence of background when using the empty library control (see Table 4.2 for numbers). The advantages of using diploid yeast instead of haploid are numerous. In general, diploid cells are more robust and can better tolerate the various experimental manipulations. They can also better tolerate the expression of toxic proteins. In addition, the library is transformed by a "natural" method as opposed to transformation, and thereby causes less stress to yeast cells. Finally, it has been shown that the *LEU2* and *lacZ* reporters are less sensitive to transcription activation compared to haploid cells, which

contributes to the enhanced stringency of the screen and the reduction of background (Finley Jr. and Brent, 1997).

However, regardless of the LexA fusion or the method of screening, false positive clones were isolated in all of the T-Ag screens. It has been shown that there are false positive clones which include ribosomal, mitochondrial, and proteins like collagen, which are commonly found in almost all of the interaction trap two-hybrid screens (for a full list see www.fccc.edu/research/labs/golemis/InteractionTrapInWork.html), some of which were also isolated from the T-Ag screens (e.g. procollagen).

Any event that leads to the transcriptional activation of the reporters could be misjudged as a positive interaction. False positives can arise by many different ways, for example, by a mutation in the yeast strain during the screen that allows it to grow on galactose, or by the presence of library proteins that can interact with the LexA DNA-binding domain or directly bind and activate the promoters, or by "sticky" proteins that can interact non-specifically with any bait due to the presence of highly charged or hydrophobic residues. In addition, false positive colonies may arise independently of the library proteins. This is usually seen with baits that can weakly activate transcription of the reporters. During the screen, some cells acquire the ability to enhance this transactivation, possibly by spontaneously increasing the copy number and the expression of the bait (Golemis and Brent, 1992). To account for any mutational events that may have occurred in the original yeast strain, the plasmids isolated from positive clones should always be retransformed into a new yeast strain.

On the other hand, known or expected interactions of the bait are often not isolated. These are called the "false negatives" (Brent and Finley Jr., 1997 ; Vidal and Legrain, 1999). Both known or novel interactions could be missed in a two-hybrid screen for various reasons. It could be due to the misfolding or the inappropriate post-translational modification of the proteins,

or to the requirement of a third protein to act as a bridge between the bait and the prey. The directionality of the bait and prey is also critical, as it has been shown that some interactions can only be detected when the proteins of interest are fused either to the binding domain or to the activation domain, but not in both ways (Estojak et al., 1995). In addition, some potential binding partners of the bait could be toxic to yeast, and therefore, never be isolated or be under-represented in the library. Furthermore, some interactions might not be represented in some libraries due to the library construction strategy that was used. It is therefore not surprising that known N-terminal interactions of T-Ag, were not recovered from the screens. pRb in particular, the main N-terminal interaction of T-Ag, is likely not to have been picked in the screens due to the position of the T-Ag binding domains within the pRb protein. Human pRb has a 4.7 Kb mRNA transcript with 1.8 Kb of 3'UTR and encodes for a protein of 928 amino acids (Lee et al., 1987). The two pocket domains encompass mRNA nucleotides 1315 to 1854 (aa 393 to 572) and 2074 to 2457 (aa 646 to 773) and are both required for binding to T-Ag (Hu et al., 1990 ; Huang et al., 1990). Since the libraries screened were constructed using oligo-dT primers, in order for a pRb transcript containing both pocket domains to be represented in the library, a transcript of 3.4 Kb would have to be synthesized that does not contain any EcoRI or XhoI restriction sites. Since the average insert size of the libraries is 0.2-2.5 kb, a 3.4 kb transcript is under-represented, if present at all, therefore, it is unlikely that pRb would be isolated in the screens.

Due to the repression assay results which had indicated that the LexA-T-Ag bait binds strongly to the LexA operators, the colonies with the strongest phenotype were picked first, in the assumption that they would represent the strongest interactions with T-Ag. Also, it has been shown that generally the affinity of the two-hybrid interaction can be correlated to the *in vitro* affinity of the interaction (Estojak et al., 1995). However, the colonies

that grew on media lacking leucine after two days, and that turned dark blue were found to be false positives. Instead, both Hsp40 and BUB1 were slow growers that appeared on media lacking leucine on the fourth day, and just barely turned blue on X-Gal. This indicates that the basic "rules" of prey selection can sometimes vary depending on the bait, and cannot be generalized for all screens. The decision of which clones to select, therefore, is somewhat arbitrary, and it is up to the individual researcher and the time he or she can afford to spend on the screen, to decide how stringent the selection of clones will be.

Finally, the biological significance of the BUB1-T-Ag and the Hsp40-T-Ag interaction must be investigated outside yeast. The yeast two-hybrid system should only be regarded as a tool for identifying potential interactions, but its results should not be unequivocally extrapolated to the mammalian system. Sometimes the bait and the prey identified are not expressed in the same cell types or the full-length proteins cannot interact. Most importantly, it should be investigated whether the bait has any effect on the function of the prey identified. This further characterization of the isolated interactors was the next step of the project, and is discussed in the following chapter.

CHAPTER FIVE

The large T antigen interactors

This chapter describes the interactors isolated from the yeast two-hybrid screens. It focuses mainly on BUB1, which was isolated from the HeLa library screen. A description of BUB1 and its function is given, followed by the experiments carried out to confirm and characterize its interaction with T-Ag in mammalian cells. Finally, due to time constraints, only preliminary experiments were carried out for the Hsp40 and β-tubulin interactors, which are discussed briefly in the final section.

5.1 BUB1

5.1.1 BUB1 is a mitotic checkpoint protein

The BUB1 protein kinase is a mitotic checkpoint protein involved in monitoring spindle assembly (Roberts et al., 1994 ; Cahill et al., 1998 ; Ouyang et al., 1998). The spindle assembly checkpoint is a surveillance mechanism which monitors the proper assembly of the mitotic spindle and is essential for ensuring the accurate segregation of chromosomes, thus preventing any gain or loss of genetic material (aneuploidy). If there is a spindle defect, like the presence of a single chromosome which is not attached to the spindle, the checkpoint generates a signal that blocks cell cycle progression (Rieder et al., 1995).

Genetic screens in budding yeast have identified some of the components of this checkpoint machinery that arrests cells in response to spindle damage. These are the *Bub* (*Bub1* , *Bub2* , *Bub3*) and *Mad* (*Mad1*, *Mad2* , *Mad3*) families of genes (Hoyt et al., 1991 ; Li and Murray, 1991), and the *Mps1* gene (Weiss and Winey, 1996). The spindle checkpoint machinery is highly conserved, as homologues of these genes have now been identified in many organisms, including mice and humans (Li and Benezra, 1996 ; Taylor and McKeon, 1997 ; Cahill et al., 1998 ; Ouyang et al., 1998 ; Taylor et al., 1998 ; Martinez-Exposito et al., 1999).

The signal that arrests cells in mitosis in the presence of damage, is generated by the transient binding of these checkpoint components on the kinetochores, the structures on the chromosomes that attach to the microtubules of the spindle (Figure 5.1). In the presence of unattached chromosomes, Bub1, Bub3, Mad1 and Mad2 localize to the kinetochores forming a protein complex that may also contain Mad3 and other as yet unidentified components (Amon, 1999). In vertebrate cells, this complex also

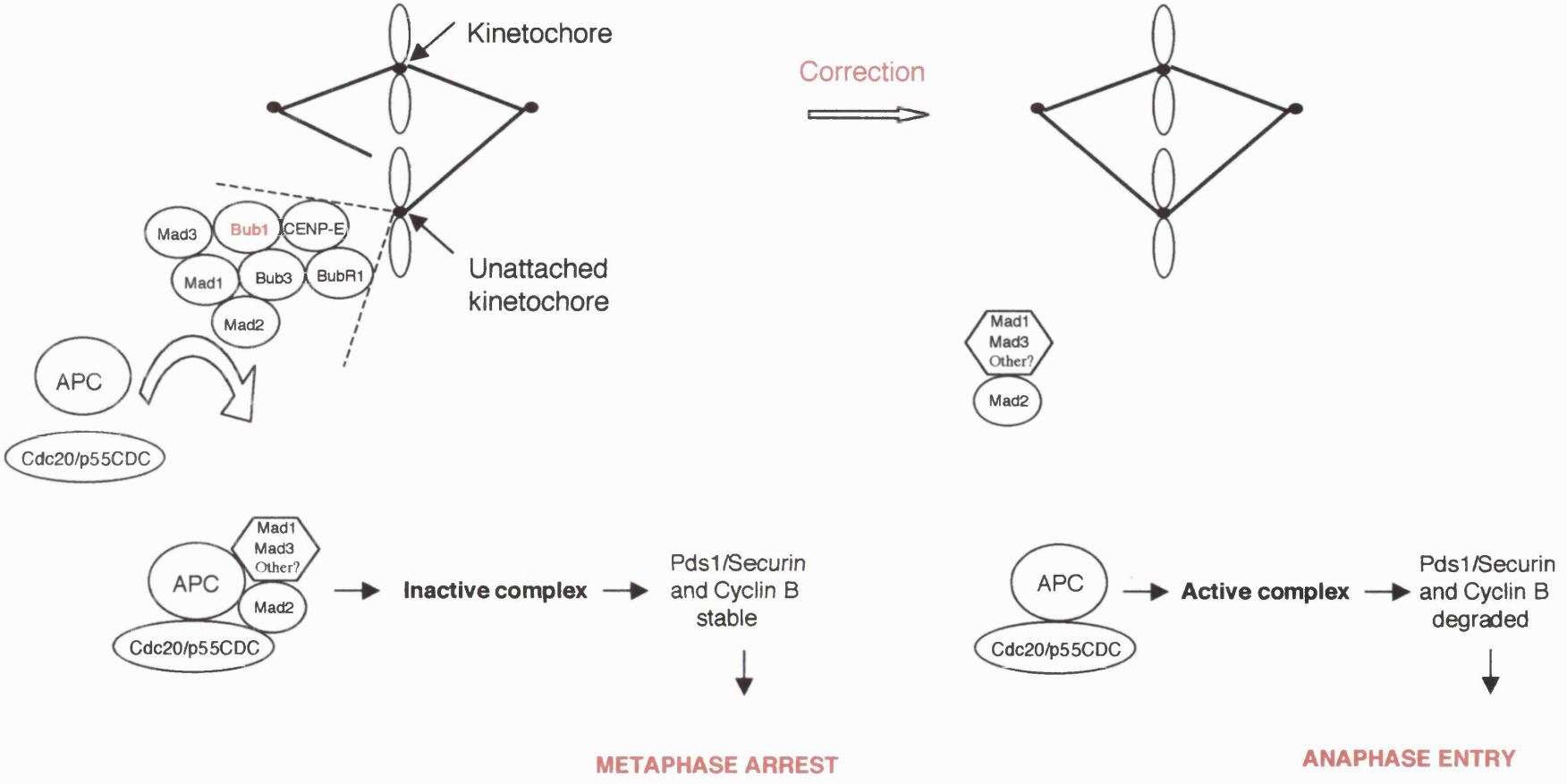


Figure 5.1 Schematic representation of the mitotic checkpoint. Adapted from Amon, 1999.

contains a Mad3/Bub1-related protein called BubR1 (Taylor et al., 1998 ; Chan et al., 1999), and the microtubule motor centromere protein-E (CENP-E ; Yen et al., 1992 ; Yao et al., 2000). The mechanism by which the formation of this complex is initiated is not fully understood, however, it is suggested that it is triggered by the lack of physical tension between the kinetochores and the microtubules when the chromosomes are not properly attached to the spindle (Li and Nicklas, 1995).

This Bub/Mad complex catalyzes the formation of a Mad2/cdc20/APC complex. cdc20 (p55CDC in mammalian cells) regulates the activity of the anaphase promoting complex (APC) by mediating its association with Mad2 (Kallio et al., 1998). Active APC causes the degradation of the sister chromatid separation inhibitor Pds1 (securin in mammalian cells), and the degradation of the mitotic cyclin B by the proteasome, so that cells can progress to anaphase and exit mitosis. When the checkpoint is activated, Mad2 binds the cdc20/APC complex, thus inhibiting its function and arresting the cells at metaphase (Li et al., 1997). When all the chromosomes are properly attached to the spindle microtubules, Mad2 dissociates from the cdc20/APC complex which becomes active again (Fang et al., 1998).

5.1.2 T-Ag interacts with Bub1

The BUB1 cDNA clone isolated from the yeast two-hybrid screen of the HeLa library, encoded the carboxy terminus of BUB1 (aa 601 to 1085), which encompasses the protein's conserved kinase domain (CD2, Figure 5.2). To validate and confirm that the yeast two-hybrid result was physiologically relevant, the interaction of T-Ag with Bub1 was demonstrated using the full-length endogenous proteins from mammalian cell lysates. Rat tsa and mouse NIH 3T3 were the two cell lines used for all experiments.

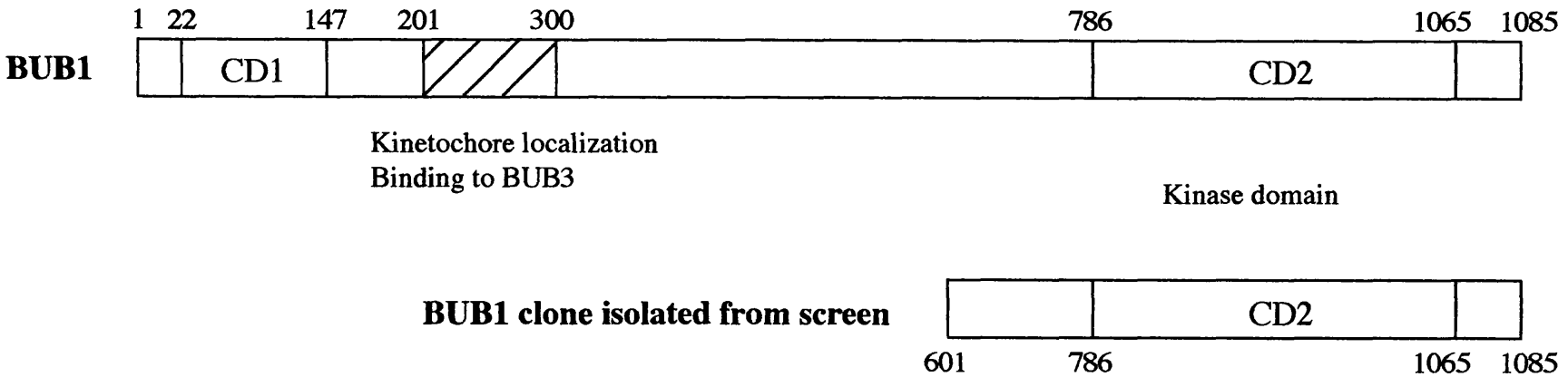


Figure 5.2 Schematic representation of the BUB1 protein and the BUB1 clone isolated from the HeLa screen. The diagram shows the positions of the conserved domains (CD) 1 and 2, and the kinetochore localization and BUB3 binding domain. CD2 corresponds to the protein's kinase domain (Taylor and McKeon, 1997 ; Taylor et al., 1998).

tsa cells are conditionally immortal cell lines derived by immortalizing rat embryo fibroblasts with the thermolabile *tsA58* mutant T-Ag (Jat and Sharp, 1989). These cell lines grow continuously at the permissive temperature (33°C), but undergo irreversible growth arrest in the G1 and G2 phases of the cell cycle upon shift to the non-permissive temperature (39.5°C), where the *tsA58* T-Ag is rapidly inactivated (Gonos et al., 1996). T-Ag and Bub1 co-immunoprecipitated only at the permissive temperature in tsa cells (Figure 5.3a). Direct immunoblotting showed that Bub1 is expressed at both temperatures in the tsa cells; this further verifies that the interaction is solely dependent on the presence of T-Ag (Figure 5.4). As a control for antibody affinity and protein-protein stability at 33°C and at 39.5°C, SV4 cells were used, which are immortalized with wildtype T-Ag and can proliferate continuously at both temperatures. T-Ag and Bub1 interacted at both 33°C and 39.5°C in SV4 cells. Also, to control for antibody specificity, an immunoprecipitation from SV4 cells was carried out using normal rabbit serum (NRS) and immunoblotted with PAb 416. T-Ag was not detected when NRS was used but was present when anti-Bub1 was used.

In these experiments, the Bub1 protein was visualized only after immunoprecipitation with an extreme C-terminal T-Ag antibody (PAb423). The Bub1 protein was not detected when cell lysates were immunoprecipitated with T-Ag mouse monoclonal antibodies with epitopes within the N-terminus (PAb416) and the central domain of T-Ag (PAb100). This is likely to be the result of reduced accessibility to the binding site, due to steric hindrance caused by the presence of bound Bub1. To address the possibility that this could also be due to the three antibodies having different binding affinities for T-Ag, duplicate immunoprecipitation experiments were carried out, which were also probed for T-Ag. All three antibodies were shown to immunoprecipitate comparable amounts of T-Ag (Figure 5.3b). These results are in agreement with the yeast two-hybrid result, which

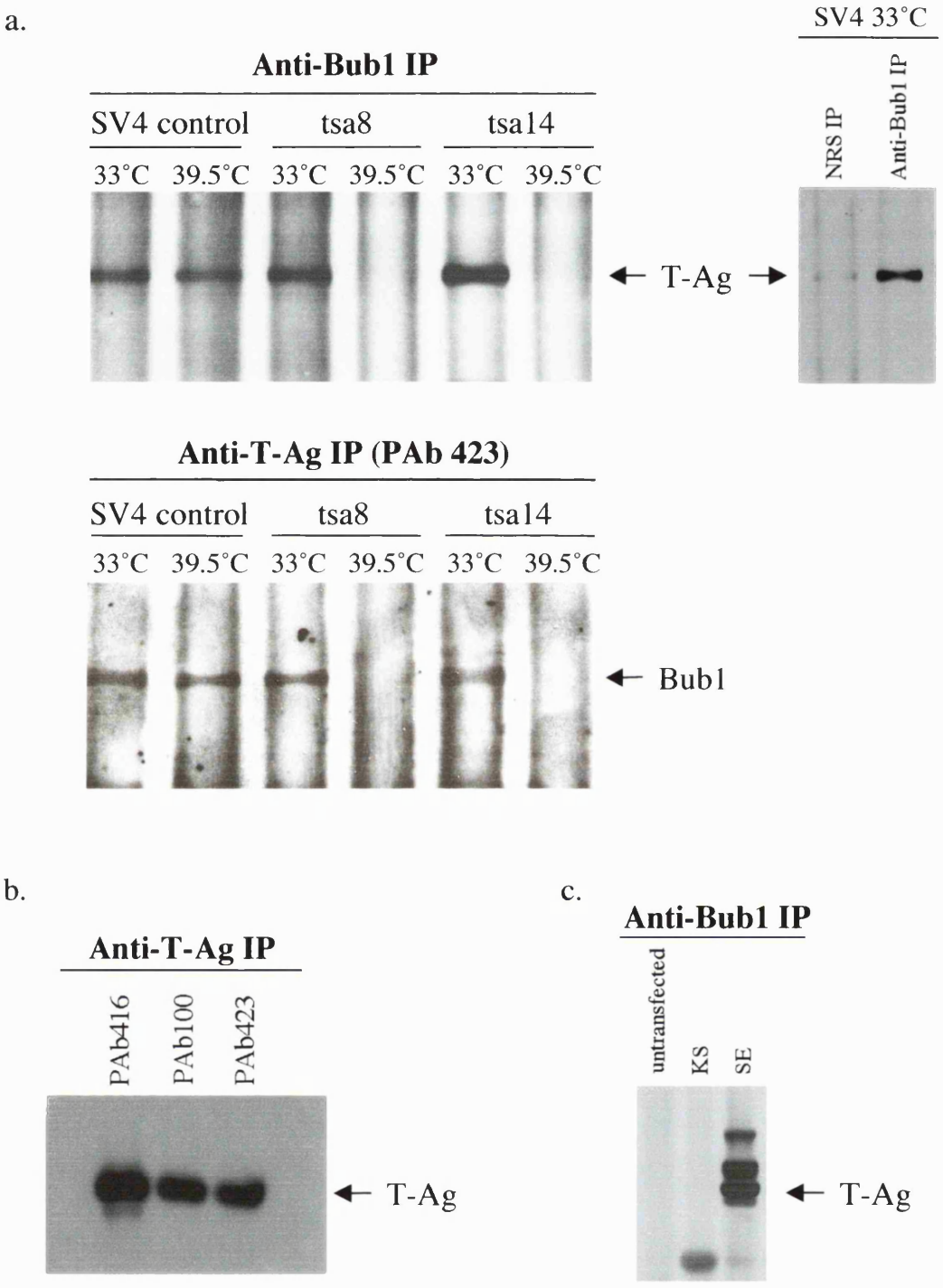


Figure 5.3 T-Ag/Bub1 co-immunoprecipitations. a. Reciprocal immunoprecipitations in the tsa cells, b. T-Ag immunoprecipitations in SV4 cells with 3 different antibodies raised against the N-, central and C-terminal domain of T-Ag, c. Immunoprecipitations in the NIH 3T3 cells. In all blots T-Ag was detected using PAb416 (NRS: normal rabbit serum).

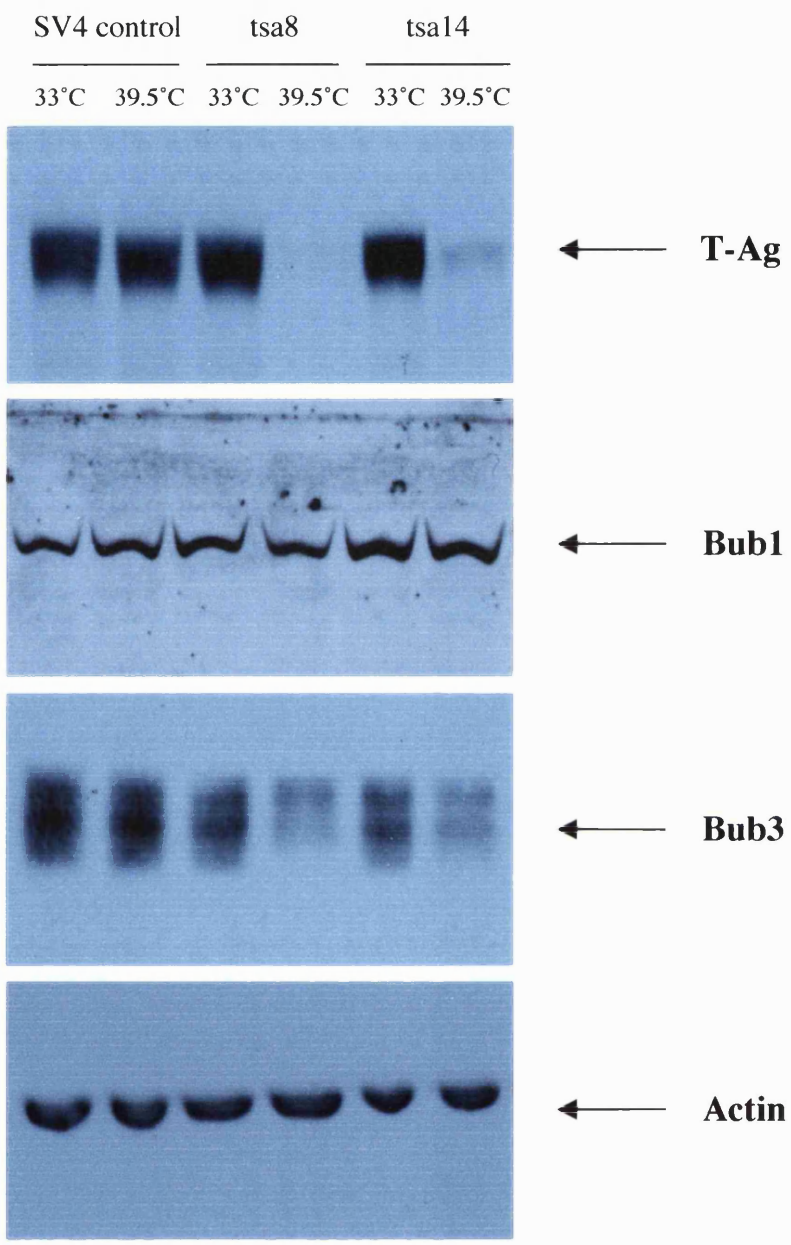


Figure 5.4 Expression of T-Ag, Bub1 and Bub3 in the tsa cells by direct immunoblotting. T-Ag was detected using PAb416. Actin expression was used as a control.

isolated BUB1 through its interaction with the N-terminal domain of T-Ag, thus supporting the fact that the presence of bound Bub1 in that region could prevent the proximal binding of T-Ag antibodies.

In order to confirm the interaction in another cell line, it was shown that T-Ag interacts with Bub1 in mouse NIH 3T3 cells which ectopically express T-Ag (SE cells). NIH 3T3 untransfected cells and cells transfected with pBluescript (KS cells) were also used as controls (Figure 5.3c).

If T-Ag and Bub1 are biological partners, T-Ag should associate with other proteins known to complex with Bub1. Therefore, the interaction of T-Ag with Bub3, another component of the spindle checkpoint, was also tested. Bub3 is found in a complex with Bub1 in yeast, mouse and human cells (Hoyt et al., 1991 ; Roberts et al., 1994 ; Taylor et al., 1998 ; Martinez-Exposito et al., 1999). The binding of Bub3 to Bub1 is believed to be critical for the localization of Bub1 to the kinetochores of unattached chromosomes, and therefore for the proper assembly of the mitotic spindle (Figure 5.2). It was found that T-Ag co-immunoprecipitated with Bub3, both in the tsa (Figure 5.5a) and the NIH 3T3 cells (Figure 5.5b), suggesting that T-Ag and Bub1-Bub3 are components of the same protein complex.

Interestingly, in the tsa cells, in contrast to Bub1 expression, Bub3 expression decreases at the non-permissive temperature, where T-Ag is inactivated, suggesting that the expression of Bub3 may have a T-Ag-dependent regulation in these cells (Figure 5.4). On the other hand, expression of neither Bub1 nor Bub3 showed any difference between the control NIH 3T3 cells and the T-Ag-expressing SE cells (Figure 5.6).

5.1.3 T-Ag affects the mitotic checkpoint and the activity of Bub1

As described above, progression through the spindle assembly checkpoint is dependent upon the presence of a functional mitotic spindle.

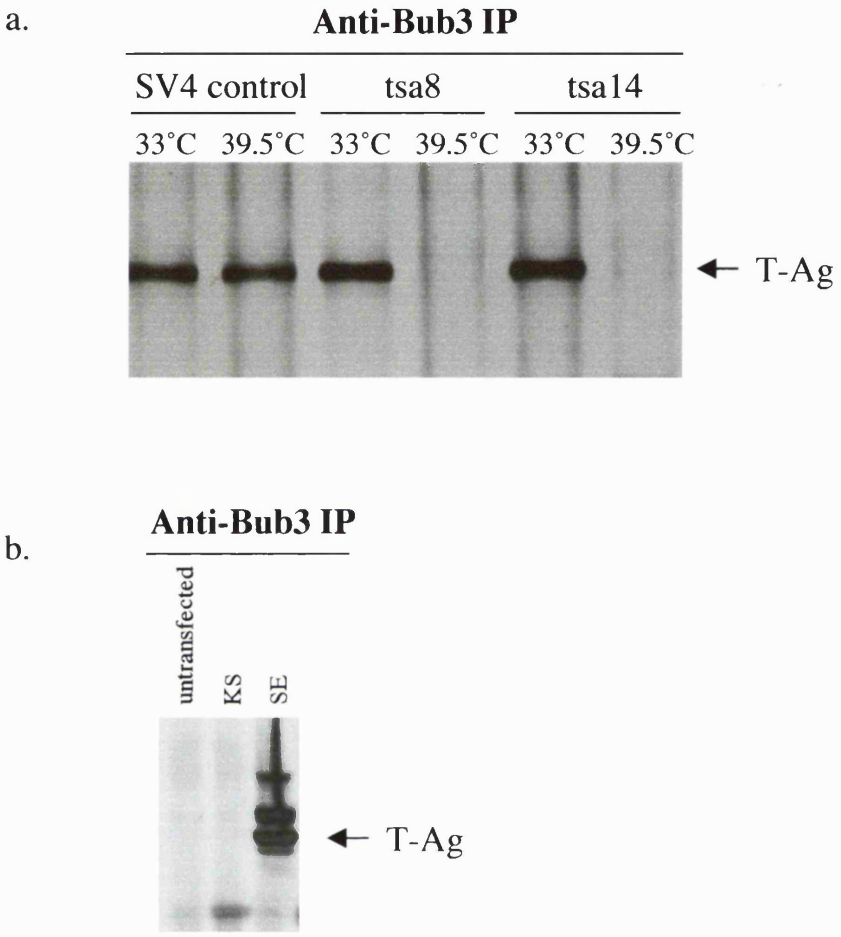


Figure 5.5 T-Ag/Bub3 co-immunoprecipitations. a. Immunoprecipitations in the tsa cells, b. Immunoprecipitations in the NIH 3T3 cells. T-Ag was detected using PAb416.

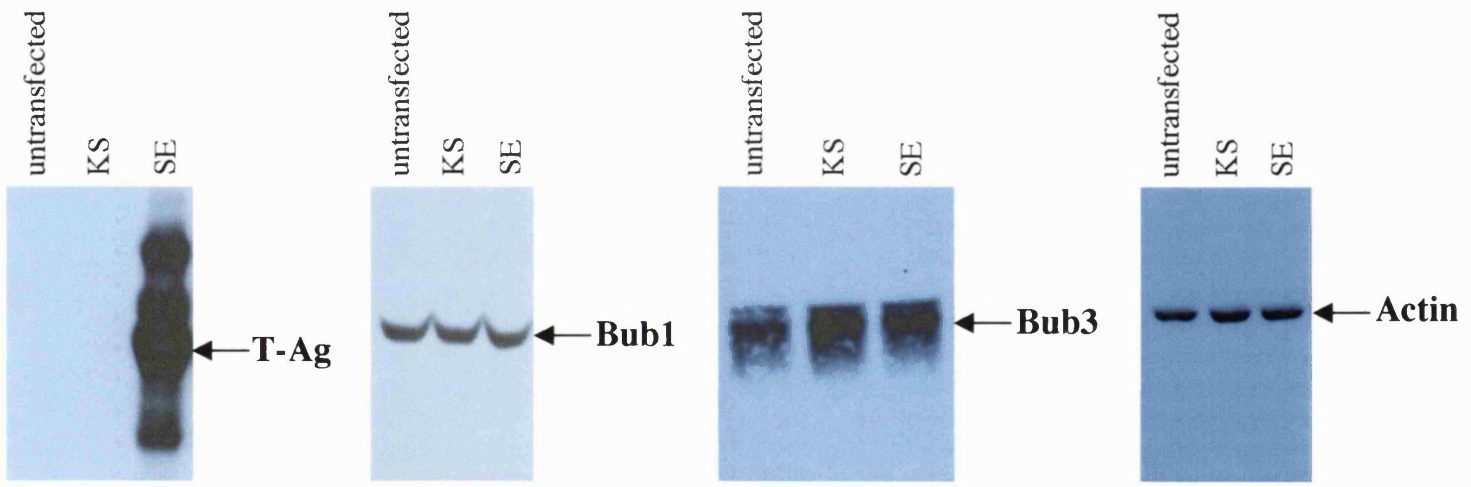


Figure 5.6 Expression of T-Ag, Bub1 and Bub3 in the NIH 3T3 cells by direct immunoblotting. T-Ag has two additional higher molecular weight forms, probably corresponding to different modification states of the protein. T-Ag was detected using PAb416. Actin expression was used as a control.

Disruption of the spindle by microtubule depolymerizing drugs such as nocodazole causes the cells to arrest in mitosis (Li and Benezra, 1996 ; Taylor and McKeon, 1997). If the interaction between T-Ag, Bub1 and Bub3 is functional, T-Ag expression should alter the normal response of the cells to nocodazole. Only the NIH 3T3 cells were used for this experiment. This is because the control for the tsa cell lines is to grow the cells at 39.5°C where T-Ag is inactivated. At this temperature however, the cells are growth arrested and so it is impossible to carry out any cell cycle experiment.

Thus, to test the response of cells to nocodazole, NIH 3T3 control cells (untransfected and KS) and SE cells were compared, after treatment with nocodazole at a range of concentrations for approximately 12 hours. All three cell types were able to undergo mitosis in the presence of 20ng/ml of nocodazole. At nocodazole concentrations of 80ng/ml or higher, all three cell types looked very similar in their morphology and were clearly arrested and contained condensed chromosomes. At the intermediate concentration of 40ng/ml, 40-60% of control cells (Figure 5.7 panels B, C, E and F) were rounded up and contained condensed chromosomes, indicating that division had been arrested at the spindle assembly checkpoint. In contrast, a smaller proportion of rounded cells - 10-30% - were observed in the SE cells (Figure 5.7 panels A, D, G and H). These also contained condensed chromosomes, but in 10-20% of these SE cells the chromosomes had split into two distinct groups indicating that mitosis was proceeding, albeit slowly (Figure 5.7 panels I - M). These results support the idea that T-Ag can affect the normal cellular response to nocodazole, i.e. the response to mitotic spindle damage.

The cellular localization of T-Ag and Bub1 in cells undergoing mitosis was also examined. Bub1 has already been shown to localize in the centromere/kinetochore region of condensed chromosomes in prophase and prometaphase but not during or after metaphase (Taylor and McKeon, 1997). Interestingly, Bub1 and T-Ag were found to co-localize within the nucleus, in

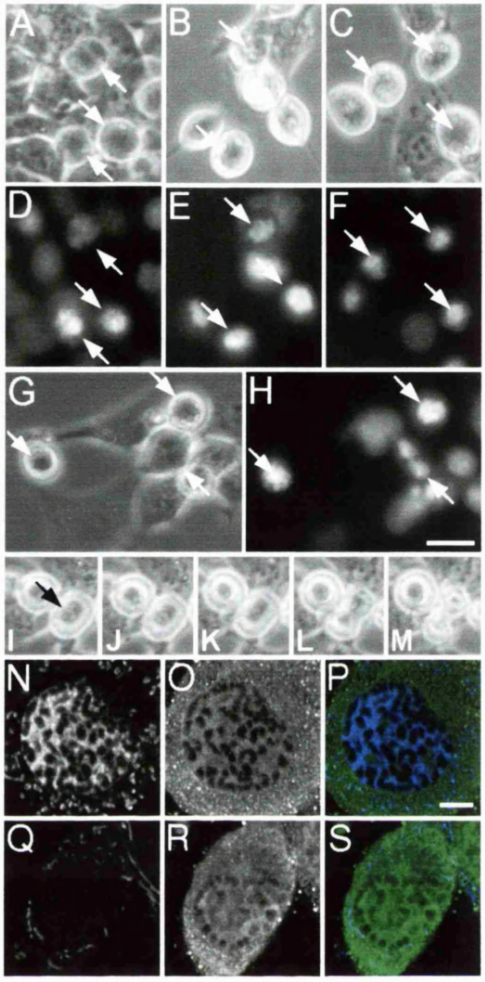


Figure 5.7 T-Ag co-localizes with Bub1 and affects the spindle assembly checkpoint. Panels A, D, G and H correspond to SE cells, panels B and E correspond to KS cells, and panels C and F correspond to untransfected cells. The cells shown were all treated with 40ng/ml nocodazole. White arrows pointing to the left indicate mitotic cells where the DNA had condensed and the condensates partitioned into two groups; white arrows pointing to the right correspond to mitotic cells where the DNA had condensed, but no partitioning was evident. Panels I-M show a timed sequence taken at 4 minute intervals of a small group of SE cells where one of the cells (black arrow) undergoes cytokinesis. Panels N-S show the distribution of T-Ag (N and Q), Bub1 (O and R) and their correspondance in early prophase (P and S) in SE cells (N-P) and KS cells (Q-S). Scale bars correspond to 20μm in panels A-M and to 5μm in panels N-S. Figure, legend and part of the corresponding paragraph in the text was provided by A.Entwistle.

the spaces between the condensing chromosomes, as cells entered prophase (Figure 5.7 panels P and S). As the cells progressed further into mitosis, no evidence of any co-localization between Bub1 and T-Ag was observed.

To further substantiate these observations, the cell cycle profiles of synchronized cells treated with nocodazole were analyzed by flow cytometry (Figure 5.8). Flow cytometry is a technique that determines the position of a population of cells in the cell cycle, by measuring the amount of DNA present in each cell (Crissman, 1995 ; Rabinovitch, 1995). To carry out the flow cytometric analysis for this experiment, the KS and SE cells were first synchronized and then treated with nocodazole. The cells were then stained with the DNA-binding dye propidium iodide (PI) and analyzed using a fluorescence-activated cell sorter (FACS) machine, which detects the fluorescence emitted from the DNA dye.

Synchronization can be achieved by various methods, including the use of drugs such as thymidine and hydroxyurea which block the cells at the G1/S border, or by growing to confluence which induces entry into G0. Different synchronization protocols were tried for the KS and SE cells, including a double thymidine block and a thymidine block followed by a hydroxyurea block, however these were not tolerated well by the SE cells. Synchronization was found to be optimum when the cells were synchronized by contact inhibition when growing to confluence.

After synchronization, when KS cells were treated with increasing concentrations of nocodazole, there was a gradual increase in the proportion of cells arrested in G2/M resulting in 85% of the cells being arrested in G2/M at 80ng/ml nocodazole. In the SE cultures, there was no significant increase in the proportion of cells in G2/M until 40ng/ml nocodazole. However, at 80ng/ml nocodazole the culture was fully arrested, with 84% of the cells arrested in G2/M. Interestingly, at 50ng/ml nocodazole, a significant difference was observed between the KS cells (71%) and the SE cells (56%).

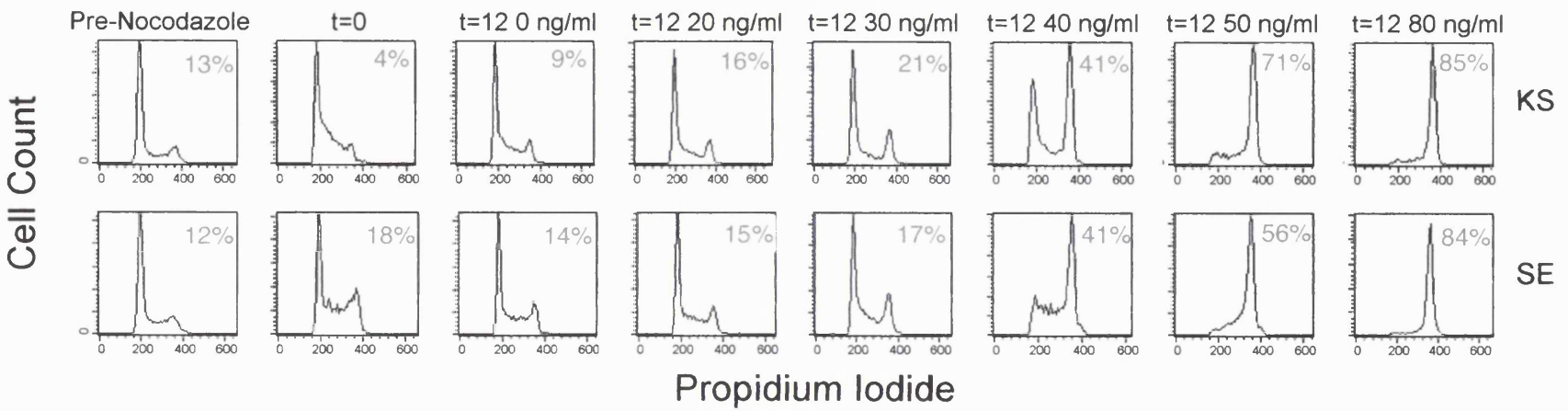


Figure 5.8 Flow cytometric analysis of NIH 3T3 cells. KS and SE cells were synchronized and treated with 0, 20, 30, 40, 50 and 80 ng/ml nocodazole for 12 hours. The percentage of cells arrested in G2/M is shown. The SE culture had more cells in the G2/M phase at the start of the experiment due to these cells exiting more rapidly out of G0 upon release from quiescence.

Taken together, these results indicated that expression of T-Ag in NIH 3T3 cells may be rendering them more refractory to the effects of nocodazole, permitting some cells to leak through the spindle assembly checkpoint in the presence of a disrupted mitotic spindle.

To analyze biochemically how T-Ag may render cells more refractory to the spindle checkpoint, the levels of cyclin B1 upon nocodazole treatment were examined. Cyclin B1 is the activator of the Cdc2 kinase which regulates passage through metaphase and accumulates when cells are arrested in G2. The expression of securin, the mammalian homologue of Pds1 that inhibits sister-chromatid separation at the mitotic checkpoint, was also examined (Figure 5.9). The failure to accumulate cyclin B1, as well as the premature degradation of securin, are both indicative of cells in which the mitotic checkpoint is defective (Zou et al., 1999 ; Michel et al., 2001). In accordance with the FACS analysis, a gradual and significant increase of cyclin B1 expression was observed in the KS cells treated with nocodazole, but not in the SE cells. Similarly, securin expression increased in the KS cells upon treatment with nocodazole, whereas no significant change in expression was observed in the SE cells. This further confirms that the cells expressing T-Ag exhibit the profile of mitotic checkpoint defective cells.

To provide some mechanistic insight into how T-Ag makes cells more refractory to the spindle checkpoint, the possibility that it has an effect on the kinase activity of Bub1 was investigated. The activity of the endogenous Bub1 has previously been found to be too low to be accurately detected in kinase assays (S. Taylor, personal communication), and therefore studies so far have either used purified recombinant Bub1 protein, or assayed ectopically expressed epitope tagged protein. It was decided however, to look at the physiological level of Bub1 kinase activity and investigate the possible effect of the expression of T-Ag.

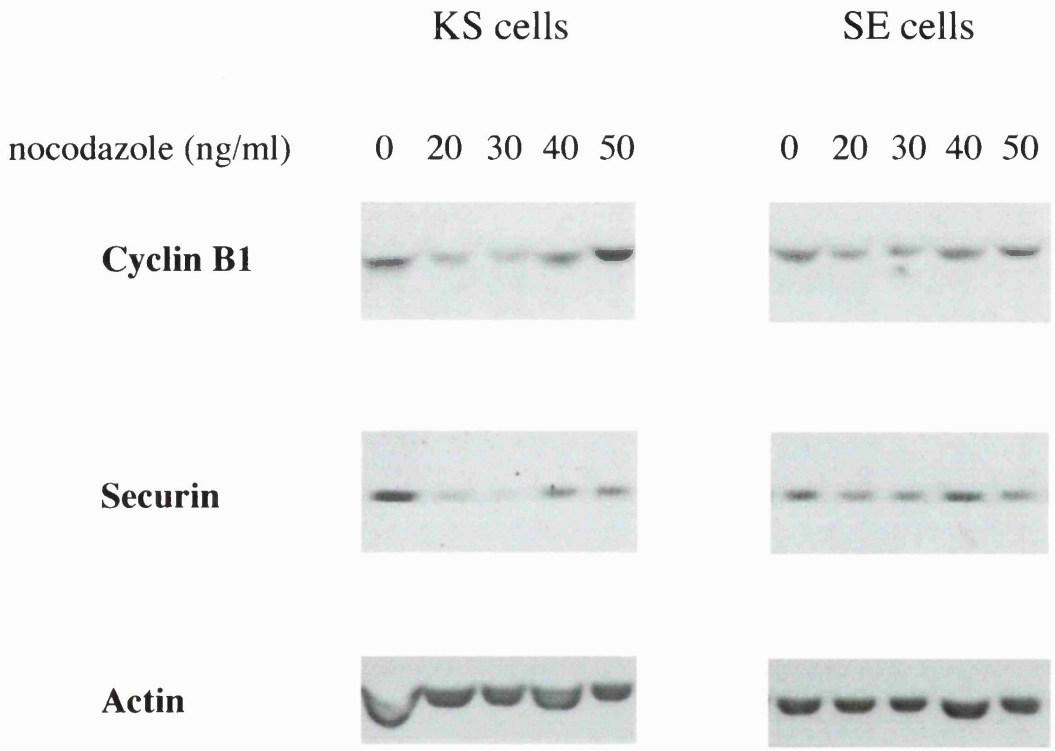


Figure 5.9 Cyclin B1 and securin expression in KS and SE nocodazole-treated cells. Actin expression was used as a control.

Therefore, endogenous Bub1 protein was immunoprecipitated from lysates of KS and SE cells and the kinase activity assayed using histone H1 as a substrate. An increase in activity was observed in the SE cells treated with nocodazole compared to the KS cells treated with nocodazole (Figure 5.10a). The autophosphorylation activity of endogenous Bub1 immunoprecipitated from the same cells was also examined. Autophosphorylation was only observed in Bub1 immunoprecipitates from SE cells treated with nocodazole and not KS cells (Figure 5.10b). To exclude the possibility that the lack of kinase activity was due to an unsuccessful immunoprecipitation from the KS cells, immunoblotting showed that equivalent levels of Bub1 were immunoprecipitated from both cell types (Figure 5.10c). Both these experiments indicate that T-Ag has a significant effect on the kinase activity and the autophosphorylation of Bub1.

Interestingly, in addition to autophosphorylation, the Bub1 immunoprecipitates from the SE cells contained four other phosphorylated proteins of approximately 30, 40, 45 and 90 kDa respectively (Figure 5.10b). Although the identity of these four proteins is not yet known, there was a clear increase in their phosphorylation, in the nocodazole-treated SE cells compared to the nocodazole-treated KS cells, especially for the 30 and 90 kDa proteins. This suggests that in addition to Bub1, the presence of T-Ag may also affect the kinase activity of Bub1-complexed proteins.

5.1.4 Discussion

Screening of the Hela library identified BUB1 as a potential T-Ag binding protein. This interaction was then confirmed outside the yeast system, and its functional significance was characterized.

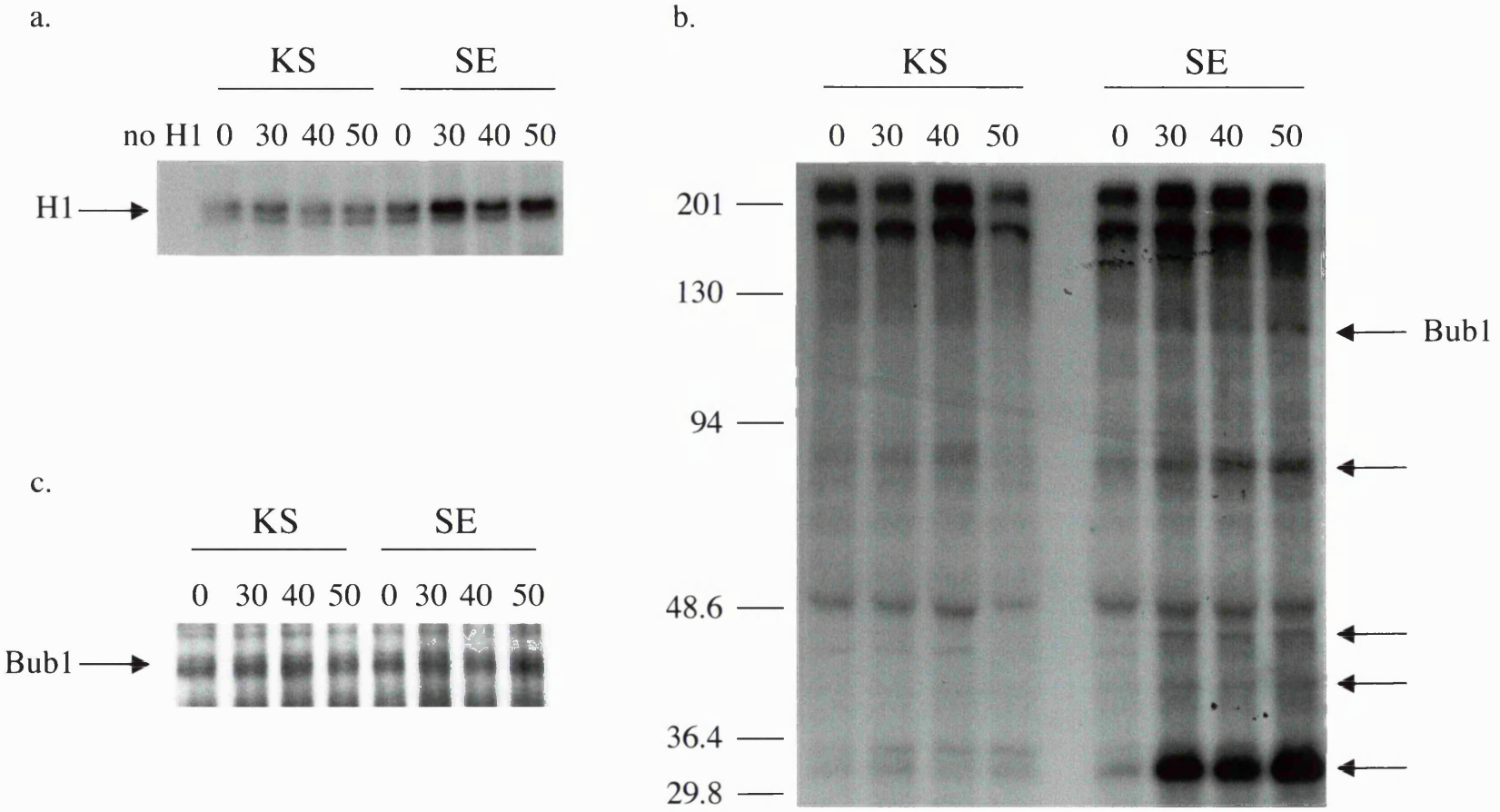


Figure 5.10 T-Ag affects the kinase activity of Bub1. a. Histone H1 assays for Bub1 immunoprecipitated from KS and SE cells treated with nocodazole. A control assay of a KS reaction without H1 substrate was also included. b. Autophosphorylation assay of Bub1 in the same cells. Arrows point to the proteins that are phosphorylated in the SE immunoprecipitates. c. Bub1 immunoblot of Bub1 immunoprecipitates.

The confirmation of the yeast two-hybrid interaction was done by T-Ag-Bub1 co-immunoprecipitations, using endogenous Bub1 protein from extracts of T-Ag-expressing rat tsa and mouse NIH 3T3 cells. T-Ag was found to co-immunoprecipitate with Bub1 in both cell lines. The reciprocal immunoprecipitations in the tsa cells, combined with the expression profile of the Bub1 protein in the same cells, further validated the interaction. The reciprocal immunoprecipitation however, was not successful in the NIH 3T3 cells. The cause for this is not known, however, it is a problem that is sometimes also seen in immunoprecipitations with known T-Ag-complexed proteins like pRb (S. Djelloul, personal communication).

The functional experiments carried out in order to assess the biological significance of the interaction, consisted of studying the cell cycle behaviour of mitotically stressed cells using nocodazole. The profile of T-Ag-expressing cells was compared to control cells and analyzed by microscopy. Flow cytometry was also carried out in order to quantify and substantiate the microscopic observations. Both experiments indicated that in the presence of nocodazole, a smaller percentage of T-Ag cells was arrested in mitosis compared to the control cells, suggesting that T-Ag allows some cells to overcome the spindle assembly checkpoint.

Finally, the most important experiment was to look at the direct effect of T-Ag on the function of Bub1. Bub1 is a highly conserved protein kinase. It has been shown that the kinase activity of Bub1 is essential for its checkpoint function in both yeast and mammalian cells (Roberts et al., 1994 ; Taylor and McKeon, 1997). Also, it has recently been suggested that phosphorylation of the Adenomatous Polyposis Coli (APC) protein by Bub1 may be important for the chromosomal stability of colon cells (Kaplan et al., 2001). Therefore, it was decided to investigate whether T-Ag can affect the phosphorylating activity of Bub1. Both an H1 assay and an autophosphorylation assay showed that the presence of T-Ag increased the

kinase activity of Bub1. The modification of Bub1 activity by T-Ag, may lead to a deregulated signaling cascade at the mitotic checkpoint, due to possible hyperphosphorylation of potential downstream targets of Bub1.

These results are further supported by previous T-Ag studies. It has already been shown that the presence of T-Ag can alter the kinase function of $p34^{CDC28}$ - complexes in yeast cells (Nacht et al., 1995). $p34^{CDC28}$ is a *S.cerevisiae* protein kinase that controls the G1 to S transition of the cell cycle. A T-Ag-glutathione S-transferase (GST) protein was shown to bind $p34^{CDC28}$ when expressed in yeast cells, and the co-immunoprecipitated complex retained kinase activity. Autophosphorylation reactions from cells expressing $p34^{CDC28}$ and T-Ag revealed the presence of a highly phosphorylated band at 60 kDa, which was only slightly phosphorylated in reactions containing $p34^{CDC28}$ only. This suggests that T-Ag can alter the kinase activity of the complexes it is bound with, as seen with the Bub1-complexes. The possibility that T-Ag can also alter the substrate specificity of these complexes cannot be excluded.

It should be noted here, that recent studies in Xenopus extracts suggest that the kinetochore localization and checkpoint functions of Bub1 are independent of its kinase domain (Sharp-Baker and Chen, 2001). It remains to be determined whether this observation is species-specific, or whether the kinase activity is involved in a different function of Bub1. Regardless however, of which function of Bub1 is regulated by its kinase activity, the results presented in this thesis suggest that T-Ag can affect that function.

In addition to the autophosphorylation, the Bub1 immunoprecipitates from the SE cells contained four other phosphorylated proteins of 30, 40, 45 and 90 kDa respectively. It is possible that these proteins are also components of the checkpoint machinery, and their identification should provide a further insight into how T-Ag makes cells leaky to the spindle

assembly checkpoint. A preliminary immunoblotting experiment using a Mad2 antibody suggests that the 30 kDa protein could be Mad2 - another key component of the mitotic checkpoint. A recent study shows that in HeLa cells even though Bub1 and Mad2 both localize to the kinetochore, they are not found in the same complex and could be part of distinct checkpoint pathways, one sensing tension and the other sensing spindle attachment respectively (Skoufias et al., 2001). If the 30 kDa protein is indeed Mad2, it could suggest that T-Ag affects both checkpoint pathways.

Various reports have indicated that the spindle checkpoint machinery is involved in malignancy. Abnormal checkpoint genes may be responsible for the genetic instability observed in various human cancers. In one report, two colorectal tumour cell lines that show high rates of aneuploidy, were shown to carry mutant alleles of the *BUB1* gene (Cahill et al., 1998). When these mutant versions of the *BUB1* gene were expressed exogenously, the cells showed disrupted mitotic checkpoint control. Furthermore, it has been found that cells lacking the breast cancer susceptibility gene *Brca2*, also carry mutations in *Bub1* which result in mitotic checkpoint inactivation and may cooperate with the Brca2 deficiency for the progression to tumourigenicity (Lee et al., 1999b). In addition to *BUB1* mutations in human cancers, it has also been shown that reduced expression of the MAD2 protein causes chromosomal instability and is linked to tumourigenesis (Li and Benezra, 1996 ; Wang et al., 2000 ; Michel et al., 2001), and a recent study shows MAD1 as the target of the human T-cell leukaemia virus type 1 in virally induced leukaemias (Jin et al., 1998). This data is very intriguing, especially in view of the fact that expression of T-Ag in human cells can induce chromosomal aberrations and aneuploidy, and cause genomic instability (Ray et al., 1990 ; Stewart and Bacchetti, 1991). Furthermore, the ability of T-Ag to cause karyotypic instability has been found to correlate with its ability to deregulate normal mitotic checkpoints. T-Ag-expressing cells were shown to

proceed into S phase, even when mitotic spindle assembly was compromised (Chang et al., 1997). It is therefore possible, that T-Ag can cause aneuploidy by the deregulation of mitotic checkpoints through its interaction with the Bub/Mad complexes.

Interestingly, studies have shown that the N-terminal domain of T-Ag alone can induce this genome destabilization (Woods et al., 1994), which is the domain that interacted with BUB1 in the yeast two-hybrid screen. In addition, it has been suggested that the genome destabilizing activity can be narrowed down between amino acids 128 and 147 of the N-terminus. It would be interesting to examine if the exact binding site of Bub1 in the N-terminus of T-Ag coincides with that region. In any case, the interaction of T-Ag with Bub1, may offer a novel explanation for the occurrence of T-Ag-dependent aneuploidy.

5.2 Hsp40

Hsp40, a homologue of the bacterial DnaJ chaperone, is a clone that was isolated as a potential T-Ag interactor from the mouse embryo library. Molecular chaperones are proteins with very diverse functions in the cell (Kelley, 1998). They assist other proteins in their assembly and disassembly and their translocation and transport across the cell. They are also involved in the degradation and the disruption of multiprotein complexes. All DnaJ proteins have in common a domain of approximately 70 amino acids called the J domain, which mediates their interaction with their DnaK chaperone partners, the Hsp70 proteins. This interaction stimulates the ATPase activity of Hsp70 which is required for their association with different protein substrates (Greene, 1998 ; Kelley, 1999).

As discussed in chapter 1, T-Ag also carries a domain homologous to the J domain in its N-terminus, which can functionally substitute the J domain of the *E.coli* DnaJ chaperone (Kelley and Georgopoulos, 1997). Interestingly, T-Ag has been shown to bind the Hsc70 protein in its N-terminus (Sawai and Butel, 1989 ; Sawai et al., 1994). Hsc70 is a mammalian DnaK homologue, very similar to Hsp70, and its interaction with T-Ag is dependent upon the presence of the T-Ag J domain (Campbell et al., 1997).

Preliminary co-immunoprecipitations in the tsa and the NIH 3T3 cells and immunofluorescence experiments, suggested that Hsp40 and T-Ag may interact in these cells. Based on the function of Hsp40 DnaJ proteins and their interaction with Hsp70 proteins, one could speculate that the interaction of Hsp40 with T-Ag is mediated by their respective binding to Hsp70, since the Hsp40 clone isolated from the screen carried the whole coding sequence of the gene, that is, included the J domain. This would mean that in the yeast two-hybrid screen, a yeast Hsp70 protein was a "bridging" protein that

enabled the interaction of T-Ag and the library-encoded Hsp40 to be identified.

However recently, it was found that even though the N-terminus of T-Ag alone (containing aa 1-136) can associate with the mammalian Hsc70, it can only transiently associate with its yeast counterpart (Sullivan, 2001). This non-stable binding between T-Ag and the yeast Hsc70 could suggest that Hsp40 was isolated in the two-hybrid through a non-specific interaction with T-Ag, since as a molecular chaperone it has the ability to associate with a wide variety of substrates. However, since numerous DnaK homologues exist in yeast, it is quite possible that the T-Ag-Hsp40 interaction was not mediated by Hsc70, but by another DnaK protein, with which T-Ag can stably interact. Alternatively, the transient binding of T-Ag and the yeast Hsc70 may have been stabilized *in vivo* in yeast, and further facilitated by the overexpression of the library-encoded Hsp40. Another possibility is that during the screen, T-Ag, Hsp40 and its DnaK partner, were part of a larger multimeric complex in yeast, in which T-Ag and possibly other yeast proteins were treated as "substrates" for the DnaJ-DnaK chaperone pair.

Further work is required therefore, in order to confirm the nature of the interaction between T-Ag and Hsp40 and to determine whether their interaction is specific.

5.3 β-tubulin

Preliminary experiments were also carried out for β-tubulin, isolated from the HeLa library screen, which also suggested its interaction and co-localization with T-Ag. β-tubulin is a major cellular protein which, together with a-tubulin, forms the microtubule structures of the cytoskeleton (Downing and Nogales, 1998). These preliminary experiments were carried out even though this clone did not exhibit the *LEU2*$^+$/*lacZ*$^+$ phenotype upon re-transformation, and furthermore, as a cytoskeletal protein it would be normally regarded as a false positive. The experiments were done for two reasons. Firstly, it was significant that β-tubulin was picked 39 times out of the 42 clones that were isolated. If the library screen was carried out by filter probe hybridization or antibody detection, this would simply reflect the high representation of this clone in the library. In the two-hybrid, however, it could also reflect the high affinity of this clone for the LexA-T-Ag bait.

Secondly and most importantly, the decision to choose β-tubulin for further characterization was based on the existence of previous reports which showed a correlation between T-Ag and tubulin. More than 20 years ago a study indicated that the expression of T-Ag in mouse cells was associated with an increased stability of tubulin (Wiche et al., 1979). Later, it was shown by specific protein extraction and solubilization techniques, that a multi-protein complex which contained T-Ag, p53 and other cellular proteins, existed in various SV40-transformed mouse cells. The purification of these proteins revealed that they included a- and β- tubulin. Reciprocal co-immunoprecipitations confirmed that T-Ag and p53 specifically interacted with tubulin (Maxwell et al., 1989 ; Maxwell et al., 1991). These findings are interesting because they propose a different function for T-Ag and p53, since their association with the microtubules could indicate that they may be

actively involved in their intracellular transport and signal transduction (Maxwell, 1991).

A hypothetical model that could potentially link all of the yeast two-hybrid isolated T-Ag interactors together is the possible involvement of all interactors in the assembly of the mitotic spindle. β-tubulin is the major component of the microtubules, which are attached to the kinetochores during spindle formation, and at which the Bub1 protein is recruited. It has also been suggested that chaperone proteins are actively associated with the microtubule network, since in yeast the formation of the mitotic spindle requires the presence of Hsp70 (Ursic and Culbertson, 1991). Prior to undertaking the task of confirming this theory however, the interaction of T-Ag with Hsp40 and β-tubulin must first be confirmed and the preliminary experiments must be repeated with the appropriate controls.

5.4 DISCUSSION

The yeast two-hybrid screens identified a number of clones and potential T-Ag interactors. One of these clones, BUB1, was selected for further characterization, and two more clones, Hsp40 and β-tubulin were preliminarily characterized. However, the decision to pursue only these three clones, was solely based on time considerations, and does not exclude the rest of the clones as potential T-Ag interactors.

For example, the tumorous imaginal disc Tid56-like protein isolated from the mouse embryo screen is also a clone that would be interesting to further characterize. In addition to the fact that it was isolated two times from the screen, its function also suggests that it could be a true T-Ag interactor. Tid56 is a *Drosophila* protein encoded by the *l(2)tid* tumour suppressor gene, localized to the mitochondrial matrix, and a member of the DnaJ family of chaperones (Kurzik-Dumke et al., 1995). The human homologue of *l(2)tid*, *TID1*, encodes the hTid-1 protein (Syken et al., 1999). hTid-1 is also a DnaJ protein, and like Hsp40, it is found in a complex with its DnaK partner Hsp70. hTid-1 has been found to be involved in the regulation of the apoptotic response and interestingly, to bind the E7 oncoprotein of HPV (Schilling et al., 1998 ; Syken et al., 1999).

The decision of which other clones to characterize must however, be made with caution. It should be noted that almost 80% of the library fusions in a two-hybrid screen encode proteins that do not normally exist, due to non-coding, antisense or out of frame DNA sequences (Vidal and Legrain, 1999). However, to complicate matters even more, it has been suggested that sometimes even out of frame fusions may encode bona fide interactors through a frame-shifting event (Fromont-Racine et al., 1997). In conclusion, the decision of whether or not to pursue and further characterize the isolated clones of a screen can be somewhat arbitrary.

CHAPTER SIX

Final discussion

6.1 SUMMARY OF RESULTS

The aim of this project was to identify novel proteins which interact with the N-terminal domain of T-Ag, using the yeast two-hybrid system. The biggest part of the project was the setup and optimization of the yeast two-hybrid. The interaction trap was used, which is a two-hybrid system based on the reconstitution of the LexA-B42 chimeric transcriptional activator.

The optimization of the screen consisted of testing the intrinsic transactivation properties, the expression and the repression activity of the T-Ag bait. It was determined that the most suitable bait for screening was the inducibly-expressed T-Ag bait fused to the C-terminus of LexA and expressed from the pGilda vector, because it lacked reporter transactivation activity, it was stably expressed and it could bind the LexA operators.

Numerous T-Ag interactor hunts were carried out during the course of this project, which identified a number of potential T-Ag interactors. Screening of a mouse day 19 embryo library identified 22 potential classes of T-Ag interactors including Hsp40, and screening of a HeLa library identified 2 potential interactors, BUB1 and β-tubulin. The interaction of T-Ag with the BUB1 mitotic checkpoint kinase was further characterized. T-Ag was found to interact with Bub1 in tsa and NIH 3T3 cells, to co-localize with Bub1 in early prophase, to render cells more refractory to the spindle assembly checkpoint, and to affect the kinase activity of Bub1.

6.2 FUTURE DIRECTIONS

There are numerous experiments that can be carried out in order to further analyze the interaction between T-Ag and Bub1. Most importantly, future experiments must focus on elucidating the mechanism by which T-Ag deregulates the function of the Bub/Mad complex.

For checkpoint activation at the kinetochores, Bub1 must be bound to Bub3 (Taylor et al., 1998) and Mad1 must be bound to Mad2 (Chen et al., 1998 ; Chen et al., 1999). In addition, the trimeric Bub1/Bub3/Mad1 complex has also been found critical for the checkpoint (Brady and Hardwick, 2000). Based on these findings, the role of T-Ag in the formation of these complexes can be investigated. Co-immunoprecipitations in control and T-Ag-expressing cells will determine whether the presence of T-Ag perturbs the interaction of Bub1, Bub3 and Mad1. All these co-immunoprecipitations would be very interesting to carry out using extracts of nocodazole-treated cells, to look at the association of T-Ag with these proteins upon activation of the spindle checkpoint.

The possible interaction of T-Ag with BubR1 could also be investigated. BubR1 is a protein highly homologous to Bub1, and another component of the Bub1/Bub3 complex (Taylor et al., 1998). In HeLa cells specifically, it has been shown that BUBR1 is associated with p55CDC (the mammalian Cdc20) and may be directly targeting the function of the APC (Wu et al., 2000). Also, the checkpoint in the HeLa cells is dependent upon the presence of a BUBR1, BUB3, CDC20 and MAD2 complex (Sudakin et al., 2001). The disruption of this complex by T-Ag could also be examined, especially in view of the T-Ag-Bub3 interaction described in chapter 5. In addition, upon checkpoint activation BubR1 has been found to be phosphorylated (Li et al., 1999). As with Bub1, the phosphorylation status of BubR1 may be studied using kinase assays from KS and SE cells.

In immunofluorescence experiments, T-Ag and Bub1 were found to co-localize in the nuclei of early prophase cells. This co-localization can further be optimized by specifically staining the kinetochores with a CREST auto-immune serum.

Furthermore, as previously discussed, the expression of T-Ag and specifically of the N-terminus is correlated with the occurrence of chromosomal aberrations and aneuploidy. In order to map the exact binding site of Bub1 on the T-Ag protein, a series of N-terminal T-Ag mutants can be generated and tested for association with Bub1. This experiment will also examine the possibility that the genome destabilizing activity present between amino acids 128 and 147 of the N-terminus is due to the binding of Bub1. One way of doing this would be to create a library of T-Ag mutants and carry out a reverse two-hybrid experiment which detects the dissociation between two proteins.

As far as future work on other T-Ag-interacting proteins is concerned, the interaction with Hsp40 and β-tubulin must first be confirmed and mapped, before further characterizing other proteins, such as the Tid56-like protein isolated from the mouse screen.

Finally, alternative strategies can also be used to identify other novel targets of the T-Ag N-terminus, such as expression library screening, or 2-D gel analysis of T-Ag immunoprecipitations from control and T-Ag-expressing cells.

6.3 CONCLUSIONS

The experience drawn from this project on the setup of the yeast two-hybrid has highlighted the importance of choosing a suitable bait, using the right vectors, the most suitable transformation method, individually characterized colonies for screening, and carrying out small-scale screens. In addition to the setup, the analysis has to be stringent too. As previously discussed, many of the isolated interactions from a two-hybrid screen may not normally occur in nature, and even if they do, they may not be of any functional significance. Therefore, two-hybrid screening must be carried out with caution, as must be the interpretation of the results.

This research has identified an important novel interaction between the viral oncogene T-Ag and the Bub1 mitotic checkpoint protein kinase. T-Ag-expressing cells were more resistant to the spindle assembly checkpoint, and demonstrated that T antigen co-localizes with, and affects the autophosphorylation and the kinase activity of Bub1. Since T-Ag has been shown to cause aneuploidy and malignant transformation in mammalian cells, the leakiness of the mitotic checkpoint induced by T-Ag is in accordance with this observation.

Therefore, this research suggests a possible new role for T-Ag, placing it directly in association with the mitotic checkpoint machinery, as well as providing further evidence to support the link between mitotic checkpoints, genomic instability and tumourigenesis. More importantly, further elucidation of the function of T-Ag, in addition to the identification of defects in other mitotic checkpoint genes in human cancers, could help identify potential targets for new cancer therapies.

In addition to cancer, defects in the mitotic machinery and increased aneuploidy are common traits in aging. A recent study investigating the expression profiles of young versus old cells, has shown the down-regulation

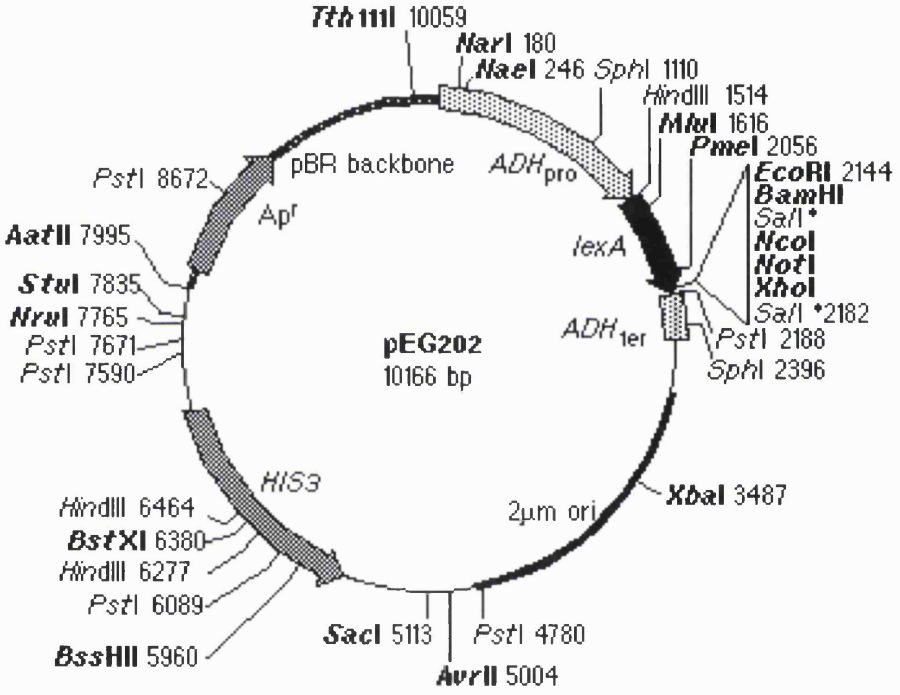
with age of various genes involved in the spindle checkpoint, including the microtubule motor CENP-E and various proteasome components of the APC (Ly et al., 2000).

Further research on T-Ag may also elucidate the occurrence of a number of human cancers due to SV40. Interestingly, SV40 sequences including T-Ag coding sequences, have been found in human bone tumours, various brain tumours and mesotheliomas (Carbone et al., 1996 ; Martini et al., 1996 ; Carbone et al., 1999 ; Testa and Giordano, 2001). These sequences are thought to have been introduced in humans in the late 1950s and early 1960s by the administration of polio vaccines contaminated with SV40. This occurred because at the time, polio vaccines were prepared using primary cultures of monkey kidney cells, some of which hosted the SV40 virus (Butel and Lednicky, 1999).

In conclusion, even though T-Ag has been the subject of extensive research and a lot is already known about its function, future work will further clarify its function, and aid the understanding of the normal cell cycle regulation and the process of aging, as well as contribute as a powerful tool in the fight against cancer.

Appendix I

Restriction map and multiple cloning site of the pEG202 bait cloning vector. Unique restriction sites are in bold. The *Sal*I sites (*) may be used as a unique site for cloning. Map taken from www.fccc.edu/research/labs/golemis/plasmids.

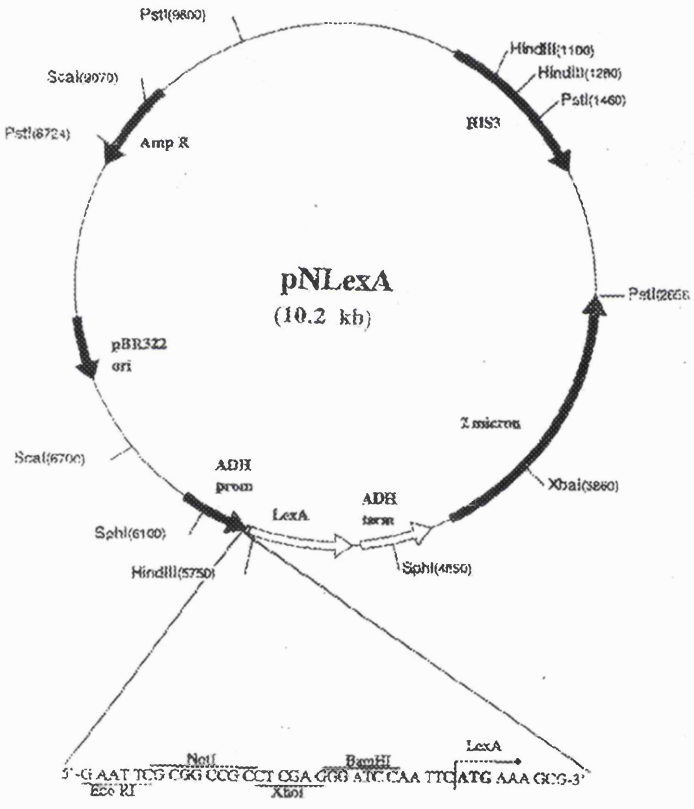


Polylinker sequence

		SalI*	NotI	SalI*
EcoRI	BamHI	NcoI	XhoI	
GAA TTC CCG GGG ATC CGT CGA CCA TGG CGG CCG CTC GAG TCG AC				

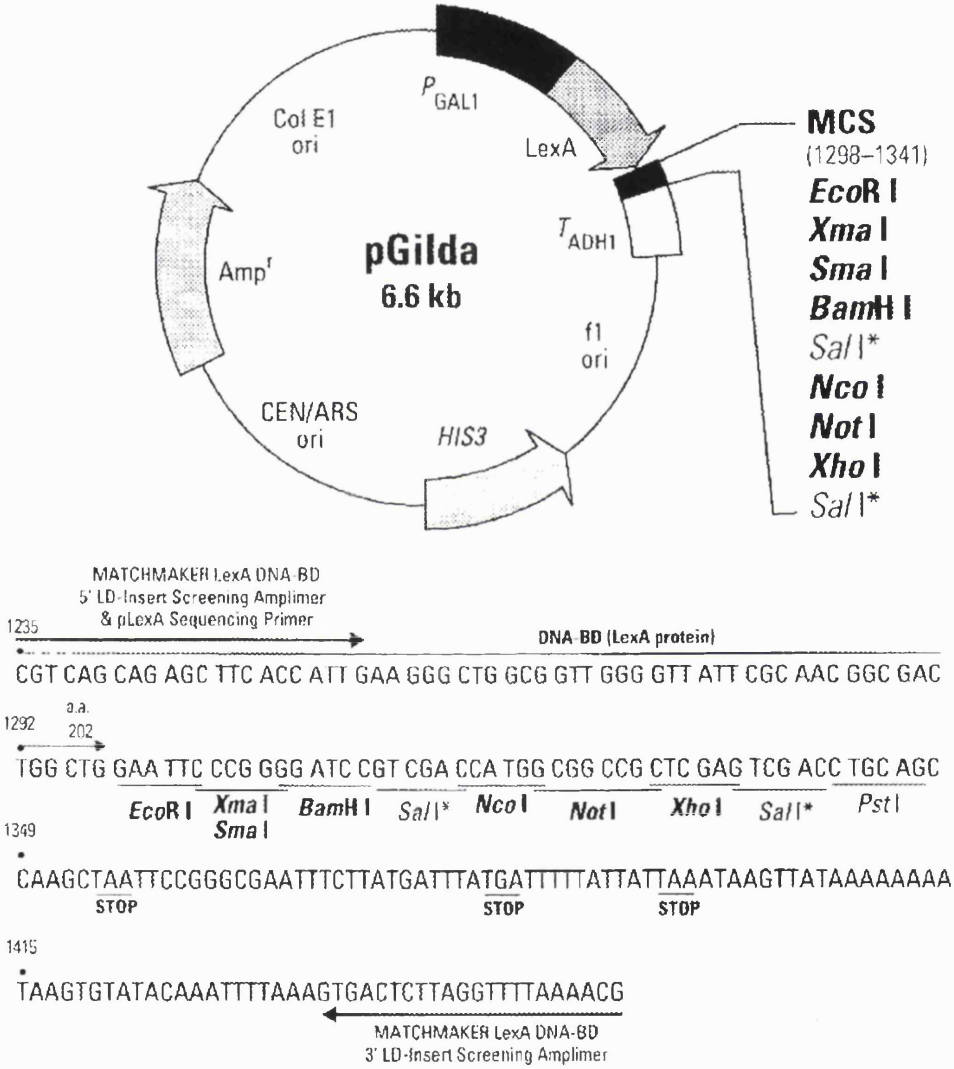
Appendix II

Restriction map and multiple cloning site of the pNLexA bait cloning vector. Map taken from OriGene Technologies, Inc.



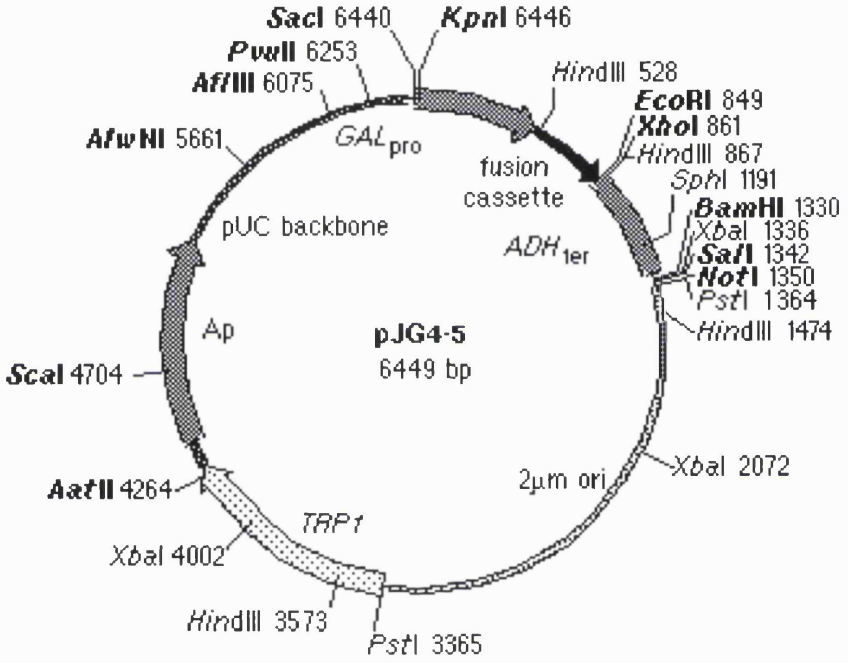
Appendix III

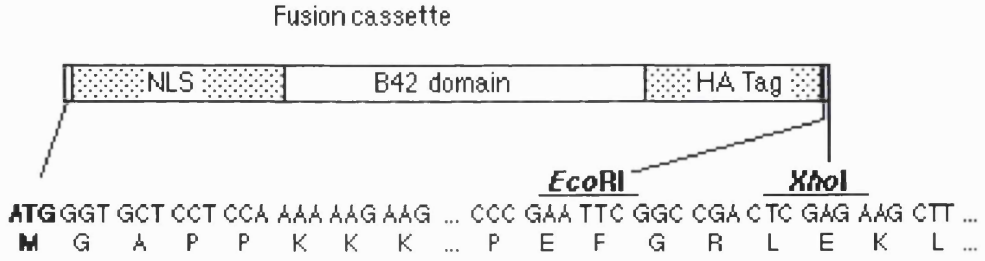
Restriction map and multiple cloning site of the pGilda bait cloning vector. Unique restriction sites are in bold. The *Sal*I sites (*) may be used as a unique site for cloning. Map taken from www.clontech.com.



Appendix IV

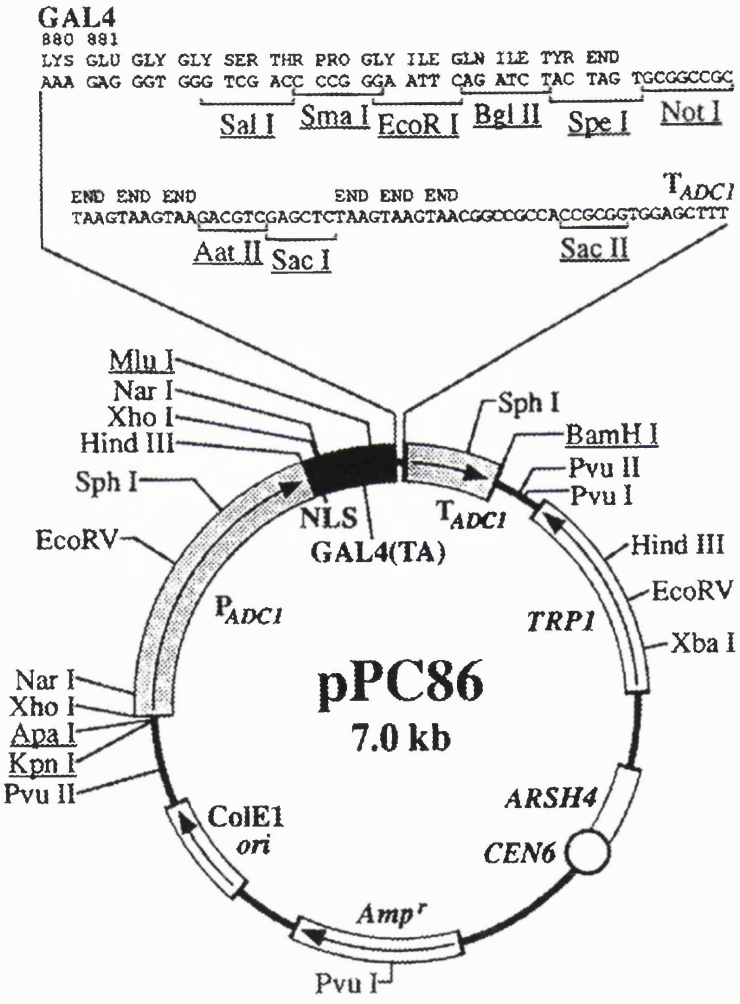
Restriction map of the pJG4-5 library vector. Unique restriction sites are shown in bold. Library cDNAs are cloned between the *Eco*RI and *Xho*I sites. Map taken from www.fccc.edu/ research/labs/golemis/plasmids.





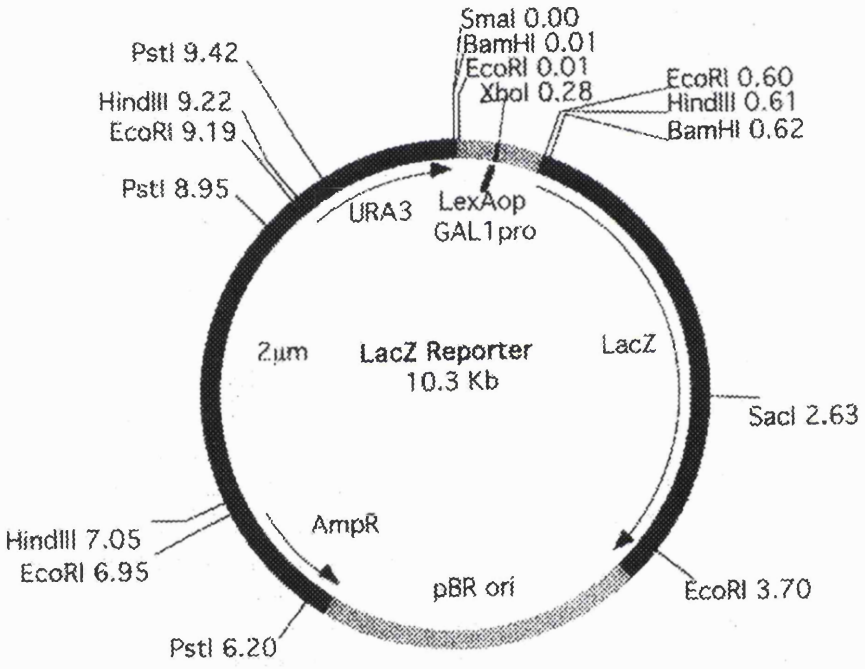
Appendix V

Restriction map and multiple cloning site of the pPC86 library vector. Unique restriction sites are underlined. Map taken from Chevray and Nathans, 1992.



Appendix VI

Restriction map of the LacZ reporter plasmid. The pJK103 reporter plasmid has a LacZ reporter backbone with two operator sites for LexA binding within the *GAL1* promoter. pJK101 used in repression assays is similar to pJK103, except for the *GAL1* UAS which is upstream of the two LexA operator sites. Map taken from Golemis and Brent, 1997.



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