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**Co-Expression and Functional Assay of Human
Rb and E2F1 Proteins in Yeast**

by

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DECLARATION

I the undersigned hereby declare that the work contained within this dissertation is my own and has not in its entirety or in part been submitted to any university for a degree.

All works cited have been acknowledged by complete references.



Alex Wollenschlaeger

December 1999, A.D.

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"Try not. Do or do not. There is no try."
Yoda, a long time ago in a galaxy far, far away.

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CHAPTER ONE

ABSTRACT

The retinoblastoma protein (Rb) was the first tumour suppressor to be discovered and, consistent with this role, has been found in aberrant form in a diverse array of human neoplasias. A principal target of Rb is the cell cycle-regulating transcription factor family E2F. The members of this family are responsible for activating transcription of a range of genes necessary for transition of the G₁/S boundary. Through its interaction with the E2F family and other cell cycle regulating proteins Rb is able to abrogate transition of the G₁/S boundary and consequently halt growth. Mutations of Rb that lead to functional inactivity of the protein result in uncontrolled passage through the cell cycle, which ultimately contributes to cancer development.

Clearly, a simple assay of Rb functional activity would be beneficial for diagnosis and prognosis of human malignancies. The principal aim of this study was to develop such an assay in the budding yeast *Saccharomyces cerevisiae*, using the ability of Rb to bind the E2F transcription factor family, specifically E2F1 in this case. Strains of *S. cerevisiae* were engineered to contain an E2F response element borne in the *CyC1* promoter upstream of the bacterial *lacZ* reporter gene. In addition, inducible yeast expression vectors for both human Rb and E2F1 were constructed.

Initial experiments demonstrated that the modified *S. cerevisiae* strains were indeed capable of detecting the presence of E2F activity. Further investigation demonstrated that this response could additionally be attributed to an endogenous yeast E2F-like activity, and that the endogenous and ectopic E2F activities could be distinguished. Nevertheless, the presence of this endogenous activity complicated interpretation of the experimental data and further experiments involving Rb expression were performed in the absence of ectopically expressed human E2F1. Still, this approach yielded some interesting results. The Rb protein was apparently able to disrupt binding of the endogenous yeast E2F-like activity to the E2F recognition element. This could be expected since certain protein components of the Rb-pathway seem to be highly conserved between man and yeast, and the E2F family is a target of

Rb. Conservation of Rb-pathway components proved to be the downfall of a yeast-based functional assay for Rb using this approach, and should serve as a caveat for further studies.

CHAPTER TWO

LITERATURE REVIEW

1. Introduction

The mammalian cell cycle is a plethora of chemical reactions existing in a delicate state of equilibrium that serves to prevent the cell from dividing at inappropriate times. Precise regulation of the cell cycle is of paramount importance to survival of the organism. The evolutionary conservation of multiple components is an indicator of the necessity of efficient cell cycling. Since the discovery of the first components of the cell cycle machinery in the 1970s our knowledge of the cell cycle has increased dramatically. The situation is such that we are now able to develop a clearer picture of the events that govern the fate of cells as they age and divide. Understanding this complex mechanism is of incalculable importance for more effective disease diagnosis and prognosis as components of the cell cycle machinery are among the most frequently altered in the development and pathogenesis of human cancers. Tumour suppressors and oncogenes are essential components of the human cell cycle, and it is their involvement in the cell cycle that is responsible for the unchecked growth that follows alterations of these genes (Weinberg, 1996).

Rb, p53, or p16 are mutated in the majority of human neoplastic disorders. The retinoblastoma susceptibility gene, *RBI*, and its corresponding protein, Rb, are integral components of the cell cycle (Weinberg, 1995). Abnormalities of the gene or gene product have potentially drastic consequences for the cell, and it is for this reason that the activity of the protein is subject to strict regulation. Work by a number of labs around the world in recent years has resulted in giant leaps in the understanding of how it is that this molecule is capable of having such far-reaching effects on the fate of the cell.

2. The Mammalian Cell Cycle

The two most important events in the mammalian cell cycle are the replication of the genome, S phase, followed by mitosis, M phase. Two gap phases designated G_1 and G_2 , during which the cell grows in size, and prepares for mitosis, respectively, separate S and M phases. The cyclins were the first components of the cell cycle machinery to be

discovered. They function by complexing with cyclin dependent kinases (CDKs), which are then activated, and these complexes subsequently phosphorylate a variety of target proteins. Unlike the cyclins, CDKs are constitutively expressed in the cell, but are only active when complexed with the appropriate cyclin (Musunuru and Hinds, 1997, and references therein).

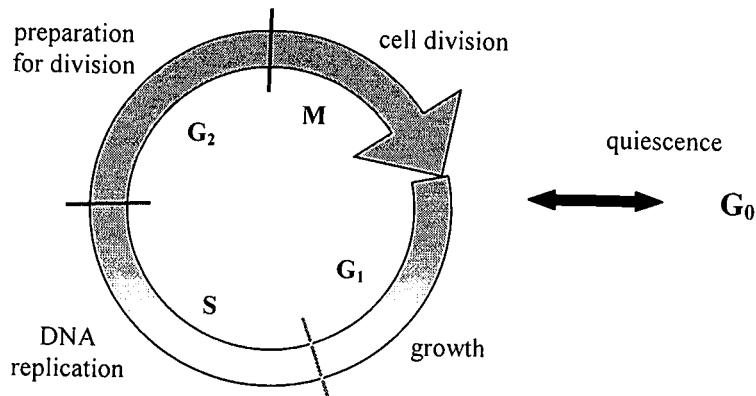


Figure 1. The cell cycle. The cell cycle consists of periods of DNA replication (S phase) and cellular division, or mitosis (M phase), separated by gap phases (G₁ and G₂), during which the cell grows and prepares for mitosis, respectively. The cell is additionally capable of exiting the cell cycle after mitosis to enter a quiescent state known as G₀ (Musunuru and Hinds, 1997).

The 8 known CDKs (CDK1-8) are capable of forming active cyclin-CDK complexes with 9 cyclins (cyclin A through I) in various defined combinations, each of which is active during specific times in the cell cycle. The cyclins can be divided with respect to their expression. G₁ cyclins include cyclins D and E, which complex with CDK4 and CDK6, and subsequently drive the cell cycle through the G₁ phase. Cyclins A and B are referred to as mitotic cyclins, due to their peak of expression during the mitotic period of the cell cycle, and are principally responsible for the activation of CDK1 (Musunuru and Hinds, 1997, and references therein).

During the G₁ phase of the cell cycle, following mitosis, cyclin D-CDK4 and cyclin D-CDK6 are the first complexes to appear in mid-late G₁. These are succeeded by cyclin E-CDK 2 in late G₁, followed by transition of the G₁/S boundary. During S phase the formation of cyclin A-CDK2 is established, followed by cyclin A-CDK1 in preparation for mitosis. The cyclin B-CDK1 complex appears at the G₂/M transition and both cyclin A and B are degraded following mitosis. The appearance and disappearance of specific cyclin-CDK complexes results in phosphorylation of defined target proteins at specific times during the cell cycle. After the cyclin-CDK complexes have performed their required

activities they must subsequently be deactivated. Degradation of the responsible cyclin is one such method, which is facilitated by ubiquitin-dependent proteolysis or destabilization of the protein by the presence of the so-called PEST amino acid sequence (Musunuru and Hinds, 1997, and references therein).

3. Inhibitors of the Cyclin-CDK Complexes

3.1 The CIP/KIP Family

In addition to the phosphorylation-dependent regulation of CDKs, there is regulation through the action of activators and inhibitors. The inhibitors of the many cyclin-CDK complexes can be separated into two distinct families based on the structural properties of the proteins. The first family, referred to as the CIP/KIP family, is characterized by a distinct N-terminal polypeptide sequence that provides the CDK binding and inhibitory activities. The principal members of this family include the p21, p27 and p57 proteins (Musunuru and Hinds, 1997, and references therein). The protein nomenclature is derived from the molecular mass, in kilodaltons (kDa), of each protein.

The most important member of the CIP/KIP family is p21. The protein was isolated almost simultaneously by five groups studying different aspects of the human cell cycle (Musunuru and Hinds, 1997, and references therein). This molecule is a versatile, almost universal, CDK inhibitor that has been demonstrated to complex and subsequently inhibit CDK2, CDK3, CDK4, CDK6, and to a lesser extent CDK1 and CDK5 (Harper *et al.*, 1995). p21 shares a homologous N-terminal region with the remaining CIP/KIP members that binds an extensive range of cyclin-CDK complexes (Chen *et al.*, 1995; Luo *et al.*, 1995). In addition, p21 contains a C-terminal domain specific for binding PCNA (proliferating cell nuclear antigen) thereby inhibiting the DNA replication activating activity of this mitogen (Flores-Rozas *et al.*, 1994; Waga *et al.*, 1994). The protein is activated in a p53-dependent manner following DNA damage, but is also up-regulated by a p53-independent mechanism by TGF- β (transforming growth factor β), PDGF (platelet-derived growth factor), FGF (fibroblast growth factor), and EGF (epidermal growth factor) (Musunuru and Hinds, 1997, and references therein).

The remaining members of the CIP/KIP family are less non-specific than p21 in their choice of substrates. p27 is capable of inhibiting cyclin-CDK complexes containing cyclins D, E and A (Polyak *et al.*, 1994). The protein contains the N-terminal homology sequence

of p21 but not the terminal sequence and is subsequently incapable of binding PCNA (Luo *et al.*, 1995; Toyoshima and Hunter, 1994). p27 is also activated by TGF- β in addition to activation by cell-cell contact. Crystal structure analysis has revealed that the mechanism for p27, and by extrapolation for the remaining CIP/KIP proteins, is dependent on two interactions with the target protein complex. The first involves three completely conserved amino acids for binding to the cyclin component of the cyclin-CDK complex, while the second interaction is with the active site of the CDK component (Russo *et al.*, 1996). p27 is subsequently removed from the cell via a ubiquitin-mediated proteolytic pathway (Pagano *et al.*, 1995). The p57 protein also contains the N-terminal domain shared by p21 and p27, and in addition the C-terminal sequence is related to that of p27. The protein can be seen as a highly conserved relative of p27, but with an additional central region containing distinct domains of unknown function. p57 is able to bind G₁-phase and S-phase complexes including cyclin D-CDK4 and cyclin A/E-CDK2 (Musunuru and Hinds, 1997, and references therein).

3.2 The INK4 Family

Additional CKIs specific for distinct CDK species are also present in the cell. This group of CDK inhibitors is referred to as the INK4 family and they are characterized by their ability to directly bind and subsequently inhibit specific cyclin-dependent kinases, specifically CDK4 and CDK6 (Musunuru and Hinds, 1997). Since the mechanism involves direct binding to CDKs, the inhibitory activity can be seen as competitive inhibition, with the INK4 proteins competing with cyclins for CDK binding. Physically the members of the INK4 family, p15, p16, p18 and p19, share a conserved domain consisting of four tandemly repeated ankyrin motifs. It is this ankyrin tetramer that is responsible for the CDK4/6 binding affinity (Musunuru and Hinds, 1997, and references within).

That the INK4 family targets CDK4 (and CDK6) is not serendipitous, as this is the CDK that is considered to be most essential for passage through the cell cycle. The targeted cyclin D-CDK4 and cyclin D-CDK6 complexes are (apparently solely) responsible for the initial phosphorylation of the retinoblastoma tumour suppressor protein (Lundberg and Weinberg, 1998). Essentially, the presence of four proteins responsible for the inhibition of the same group of proteins seems redundant, but the crux may lie in the different expression patterns of the members. Examination of *p18* and *p19* mRNA levels revealed a tissue specific pattern of expression leading to the assumption that the four INK4 proteins

are responsible for controlling specific CDK activity in distinct tissues (Guan *et al.*, 1996; Musunuru and Hinds, 1997, and references within; Okuda *et al.*, 1995).

The KIP/CIP and INK4 families thus differ mechanistically in that the KIP/CIP family binds the cyclin-CDK complex, while the INK4 proteins compete with cyclins for CDK binding (Musunuru and Hinds, 1997). Effectors of INK4 function are not well documented, but it appears that p15 is induced by TGF- β , resulting in growth arrest. Another important difference between the two groups of inhibitors is their prevalence in the genesis and development of cancers. Mutations of p16 are common in cell lines derived from a wide range of cancers, although the protein seems to be present in a more normal state in primary tumours. The *p15* gene is juxtaposed to the *p16* gene on chromosome 9 making determination of p15 abnormality in conjunction with p16 difficult. The KIP/CIP proteins on the other hand do not seem to be involved in the development of cancer (Musunuru and Hinds, 1997).

Recently, the *CDKN2* locus, which codes for p16, was found to additionally encode a second protein, p19^{ARF} (ARF refers to alternative reading frame) (Stott *et al.*, 1998). (Note that this protein is distinct from the p19 CDKI protein mentioned earlier). p19^{ARF} is transcribed from the same locus as p16, but in a different reading frame. The p19^{ARF} protein is capable of inducing cell cycle arrest at either the G₁/S or G₂/M boundaries. The mechanism involves stabilization of p53 and MDM2, which subsequently leads to an increase in the levels of p21. A homologue of human p19^{ARF}, also referred to as p14^{ARF}, has additionally been found in murine tissues (Chin *et al.*, 1998; Stott *et al.*, 1998). There is evidence to suggest that p19^{ARF} plays an important role in the blast crisis of murine primary pre-B cell transformants. This crisis occurs via a p53-dependent mechanism where the murine p19^{ARF} protein is thought to stabilize p53 and MDM2 and consequently upregulate apoptosis (Radfar *et al.*, 1998).

4. The Rb Tumour Suppressor

4.1 Physical Characteristics of the *RBI* Gene and Rb Protein

The retinoblastoma gene was first cloned by Lee *et al.* in 1987 (1987a). The gene, designated *RBI*, occupies 180 kilobases (kb) at band 14 on the long arm of chromosome 13. *RBI* contains 27 exons, which together are transcribed to yield an mRNA of 4.7 kb. A large untranslated region is situated at the 3' end and the coding region results in a 928

amino acid protein subsequent to translation. The protein, Rb, exists in both hypo- and hyperphosphorylated forms and this, coupled to the nuclear localization and intrinsic DNA binding activity of the protein allude to its cell cycle regulatory function (Lee *et al.*, 1987b). The DNA binding activity of the Rb protein is found exclusively in the C-terminal region (Wang *et al.*, 1990). This DNA binding activity is dependent on the phosphorylation status of Rb (Wang *et al.*, 1990).

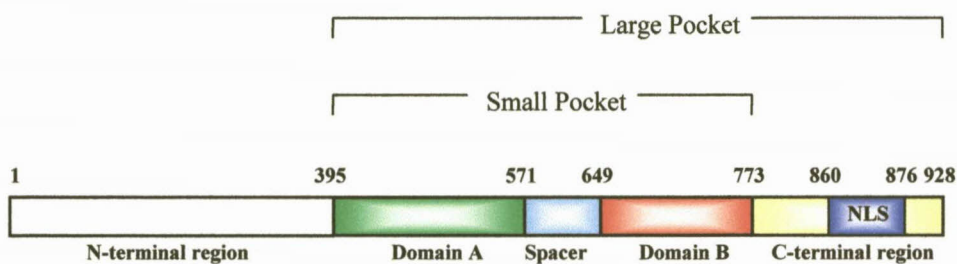


Figure 2. The retinoblastoma tumour suppressor protein, Rb. The A/B region, or small pocket, is essential for interaction with cellular transcription factors, like E2F, growth suppression, tethering of Rb to nuclear structures, and G₁ phosphorylation. The spacer region has no function, other than to ensure correct folding of the protein, while the C-terminal region displays non-specific DNA binding and contains a nuclear localisation signal (NLS). Functional activity within the N-terminal region remains to be elucidated

4.2 Phosphorylation of the Rb Protein

The Rb protein has growth-regulatory effects on the cell, which manifest in early G₁ phase of the cell cycle (Stokke *et al.*, 1993). Here the protein is found principally in the hypophosphorylated state, which is assumed to be the active state. It remains in this underphosphorylated state for the duration of the G₀ and G₁ phases while remaining localized to the nucleus. Phosphorylation of Rb accompanies its passage from the G₁ phase to (eventually) the M phase and this results in a substantial increase in molecular weight, from ~105 to 115 kDa (Thomas *et al.*, 1996). During this transition the protein is associated with the mitotic spindles and microtubule nucleation centers. The transition from hypo- to hyperphosphorylated states at the G₁/S boundary is accompanied by an altered affinity for the nuclear compartment (Mittnacht and Weinberg, 1991). It seems that the hyperphosphorylated form has a higher tendency to localize to the nucleus (Templeton, 1992).

The transition from hypo- to hyperphosphorylated form at the G₁/S boundary is of quintessential importance to the cell cycle. Buchkovich *et al.* (1989) found that Rb is present in the hypophosphorylated form only in the G₀ and G₁ phases of the cell cycle, while in the S and G₂/M phases the hyperphosphorylated form predominates. Three phosphorylation phases are required for the transition; the first in mid G₁, then during S phase, and finally during G₂/M (DeCaprio *et al.*, 1992). The transition of the G₁/S boundary is facilitated by the action of at least two different cyclin-CDK complexes. Cyclin E-CDK2 is only capable of phosphorylating Rb after the action of cyclin D-CDK4/6, which is thought to be responsible for the initial phosphorylation event (Lundberg and Weinberg, 1998). This event, catalyzed by cyclin D-CDK4, is essential for proper passage of the cell across the G₁/S boundary (Connel-Crowley *et al.*, 1997). This phosphorylation process has been faithfully reproduced in *Saccharomyces cerevisiae*, where Rb is phosphorylated by the yeast's own G₁ cyclins, as well as by ectopically expressed mammalian G₁ cyclins (Hatakeyama *et al.*, 1994; Hinds *et al.*, 1992). In addition to being functionally dependent on cyclin D-CDK mediated phosphorylation, Rb contributes to the stability of the cyclin D-CDK complex (Bates *et al.*, 1994). There has been speculation as to whether the cyclin component assists in targeting cyclin-CDK complexes to specific substrates. Cyclin E contains a substrate-targeting domain, which includes the conserved VXCXE motif, that specifically directs cyclin E-CDK activity to Rb. This region is related to the LXCXE motif, which is essential for the interaction of Rb and several cell cycle regulators and oncoproteins (V represents valine, which, like leucine in the LXCXE motif, is an aliphatic amino acid)(Lee *et al.*, 1998; Slansky and Farnham, 1996). Mutation of this conserved region results in an inability of the cyclin E-CDK2 complex to efficiently phosphorylate its major (only?) target protein, Rb (Kelly *et al.*, 1998).

The cysteine residue at position 706 of Rb appears to be vital to functional activity of the protein (Kaye *et al.*, 1990; Saijo *et al.*, 1994). Replacement of this residue by the non-conserved amino acid phenylalanine resulted in an inability of the Rb protein to undergo the transition to the hyperphosphorylated form, as well as inhibition of oncoprotein binding (Kaye *et al.*, 1990). In a follow-up study, replacement of Cys706 by tyrosine once again resulted in abnormal phosphorylation and oncoprotein binding deficiency (Saijo *et al.*, 1994).

Hyperphosphorylation of Rb additionally leads to loss of the ability of Rb to localize to the nucleus (Hinds *et al.*, 1992). This oscillation in the phosphorylation state of the retinoblastoma gene product implicates the protein as a cell cycle-regulated molecule. The retinoblastoma protein is one of the most important regulators of the cell cycle. It effectively serves as the "gate keeper" at the G₁/S boundary (DeCaprio *et al.*, 1989). Not only does the phosphorylation state of the protein oscillate, but also the total Rb levels in the cell are susceptible to variation. There is up to a 10-fold decrease in the level of Rb in the G₀/G₁ phase relative to that in the G₂/M phase (Hu *et al.*, 1991).

4.3 The Involvement of Rb in Apoptosis

In addition to its function as a cell cycle regulator, Rb is involved in the process of apoptosis, or regulated cell death (Herwig and Strauss, 1997). This effect is demonstrated by the extensive apoptosis observed in the peripheral and central nervous system of mouse embryos following targeted disruption of *RB1*. This effect seems to occur via p53-dependent and independent mechanisms (Macleod *et al.*, 1996). Rb is associated with cessation of the cell cycle in G₁ phase following DNA damage by ultraviolet (UV) radiation of human and mouse fibroblasts. Rb is rapidly dephosphorylated following UV irradiation, with the concomitant cessation of growth. p53 accumulates subsequent to growth arrest, implying a p53-independent pathway (Haapajarvi *et al.*, 1995). Rb is capable of inhibiting IFN- γ -induced apoptosis via both p53-dependent and independent pathways (Berry *et al.*, 1996).

The involvement of Rb in apoptosis is possibly also related to its interaction with the E2F1 transcription factor (see below). Rb thus has additional anti-apoptotic effects on the cell, based on its ability to restrain the apoptotic-promoting ability of E2F1. Rb participates in apoptosis via E2F1-dependent and -independent mechanisms (Macleod, 1999). Macleod (1999) suggests that the ability of Rb to constrain S phase entry is, possibly, independent of its ability to inhibit apoptosis.

4.4 The Interaction of Viral Oncoproteins with Rb

The retinoblastoma tumour suppressor protein is capable of binding the E2F transcription factor and subsequently repressing its activation activity (Arroyo and Raychaudhuri, 1992; Shan *et al.*, 1992). The region of Rb required for binding of E2F1 is referred to as the binding pocket or pocket domain. By necessity, E2F1 interacts solely with the

hypophosphorylated form of Rb, the same form that is the active form of the protein. The interaction of Rb and E2F1 can be abrogated by the adenovirus E1A transforming oncoprotein, which recognizes the identical binding region of Rb (Chellappan *et al.*, 1991; Hiebert *et al.*, 1992; Huang *et al.*, 1992; Whyte *et al.*, 1988). The sequence of E1A required to dissociate the Rb-E2F1 complex is also crucial to the transforming properties of the protein. The Rb pocket region is responsible for the interaction of Rb with the simian virus SV40 large T antigen, which is also capable of abrogating Rb-E2F association (Bartek *et al.*, 1992; DeCaprio *et al.*, 1988; Huang *et al.*, 1992). In addition to the binding pocket, sequences in the C-terminal region of the retinoblastoma protein are required for the interaction with E2F1 (Hiebert *et al.*, 1992; Huang *et al.*, 1992). The result of the interaction of viral oncogenes with the Rb-E2F complex is the release of E2F and consequently transcriptional activation of E2F regulated genes (Arroyo and Raychaudhuri, 1992).

The oncoproteins that share the ability to bind the pocket protein family also share a common motif. Rb, p107 and p130 are able to bind proteins that contain an LXCXE motif, where L refers to leucine, C to cysteine, E to glutamate, and X to any amino acid residue. This LXCXE motif has been found in all the proteins that associate with the pocket region of the pocket protein family of proteins, including the simian virus SV40 T antigen, the human papilloma virus (HPV) E7 protein, the adenovirus E1A protein, the E2F and DP proteins (Lee *et al.*, 1998; Slansky and Farnham, 1996). Binding to LXCXE is thought to involve a highly conserved groove in the B domain of the binding pocket. The A domain appears to be involved in structural stabilization of the folded B domain. The binding site shows marked similarity to the CDK2 binding site for cyclin A, and the TBP (TATA-box binding protein) binding site of TFIIF (Lee *et al.*, 1998).

5. The Pocket Proteins p107 and p130

Rb is the primary member of a group of proteins referred to as the pocket proteins, so named because all three family members contain the highly conserved pocket region first identified as the binding region for viral oncoproteins (Mulligan and Jacks, 1998). The p107 and p130 proteins are named with respect to their molecular masses in kilodaltons. The three proteins resemble each other physically, but closer inspection has revealed that they differ in their interactions with cellular components.

Both p107 and p130 were first identified by virtue of their interaction with cyclins, and their ability to bind the adenovirus E1A oncoprotein and/or SV40 large T antigen (Ewen *et al.*, 1991; Hannon *et al.*, 1993; Li *et al.*, 1993). The p107-encoding gene maps to 20q11.2 and results in a protein with significant homology to the Rb protein (Ewen *et al.*, 1991). The p130 encoding gene maps to 16q13 and encodes a protein of 1139 amino acids with a 4.85 kb cDNA (Hannon *et al.*, 1993; Li *et al.*, 1993). The protein was simultaneously discovered as a 130 kDa protein bound to the adenovirus E1A protein and through its interaction with CDK2 and various cyclins, especially D-type cyclins (Hannon *et al.*, 1993; Li *et al.*, 1993).

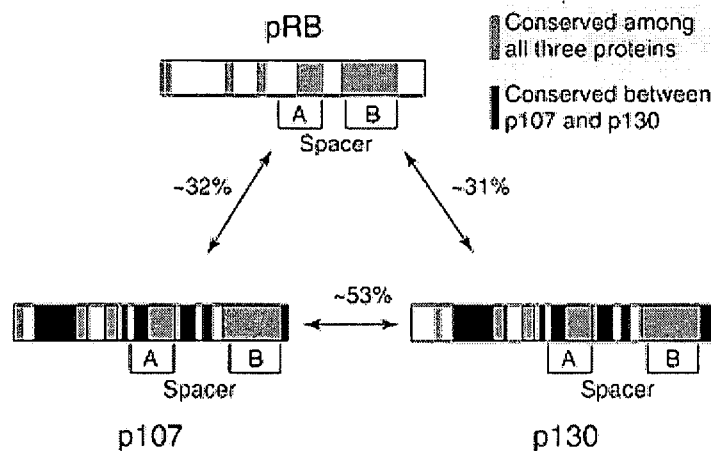


Figure 3. The pocket protein family. Rb, p107, and p130 are evolutionary conserved proteins, with p107 and p130 showing more homology than either protein with Rb. The A, B, and Spacer domains, which make up the pocket region, are conserved among all three proteins. (see text for details of pocket domain interactions (Mulligan and Jacks, 1998).

p107 and p130 share a higher percentage identity between them than either protein does with Rb (Li *et al.*, 1993; Mulligan and Jacks, 1998). The p107 and p130 proteins share common regions that are completely lacking in Rb, like the spacer region between the A and B regions that comprise the A/B pocket. This conserved spacer region allows p107 and p130 to interact with cyclin A-CDK2 and cyclin E-CDK2 (Mulligan and Jacks, 1998). Interestingly, Philips *et al.* (1998) suggested that exclusively Rb, and not p107 or p130, is capable of direct repression of cyclin A expression in quiescent cells. As is the case with Rb, p107 and p130 are capable of binding viral oncoproteins via the A/B pocket (Ewen *et al.*, 1991; Chittenden *et al.*, 1993). E2F1, E2F2 and E2F3 preferentially bind Rb, while p107 and p130 are found in association with E2F4 and E2F5 (Beijersbergen and Bernards, 1996; Hijmans *et al.*, 1995; Moberg *et al.*, 1996; Mulligan and Jacks, 1998; Musunuru and Hinds, 1997; Shirodkar *et al.*, 1992). p130 appears to play an essential role in the entry of

cells into the G₁ phase of the cell cycle from the quiescent G₀ phase (Chittenden *et al.*, 1993; Moberg *et al.*, 1996; Vairo *et al.*, 1995). In G₀ p130 is the dominant pocket protein and it interacts preferentially with E2F4, thereby repressing E2F4 mediated transcriptional activation (Chittenden *et al.*, 1993; Moberg *et al.*, 1996). As the cell enters the G₁ phase, E2F4 exchanges p130 for Rb and p107 and the levels of the complexes of these two pocket proteins are increased (Moberg *et al.*, 1996).

The presence of three proteins that share binding specificity for the E2F proteins implies a certain degree of functional compensation. In resting murine T lymphocytes (i.e. cells in G₀) the E2F4 transcription factor is found in complex with the p130 pocket protein (Hurford *et al.*, 1997; Mulligan *et al.*, 1998). Cells lacking p130 (p130 *-/-* cells) display normal arrest in G₀, but in this case the level of p107-E2F4 is elevated, implying that p107 is capable of functionally compensating for p130 (Mulligan *et al.*, 1998). In cells lacking both p130 and p107 (p130 *-/-*, p107 *-/-*) E2F4 is found complexed with Rb. These findings provide evidence for the functional overlap that exists within the retinoblastoma family.

6. The E2F Transcription Factor Family

6.1 Physical Characteristics

Possibly the most important property of the retinoblastoma tumour suppressor protein is its ability to bind the E2F family of transcription factors. E2F is responsible for activation of several genes required for progression of the cell into the S phase of the cell cycle. The E2F family has eight members at present, namely E2F1 to E2F6 and DP1 and DP2. E2F is the general name given to the heterodimeric transcription factor, composed of one of E2F1 to E2F6 in complex with a dimerization partner, either DP1 or DP2 (Beijersbergen and Bernards, 1996; Trimarchi *et al.*, 1998). The E2F and DP proteins are individually capable of activating transcription, but they also interact in a synergistic fashion through physical interaction to activate transcription from promoters with E2F binding sites (Bandara *et al.*, 1993).

The various complexes differ in their binding characteristics with the members of the Rb family of proteins. E2F1, E2F2, and E2F3 are found mainly in complex with Rb, p130 with E2F4 and E2F5, while p107 interacts with both E2F4 and E2F5 in addition to some binding activity with E2F1 (Beijersbergen and Bernards, 1996; Hijmans *et al.*, 1995; Moberg *et al.*, 1996; Mulligan and Jacks, 1998; Musunuru and Hinds, 1997; Shirodkar *et*

al., 1992). In contrast, E2F6 is incapable of binding any member of the Rb family and it appears to function exclusively as a transcriptional repressor (Trimarchi *et al.*, 1998). It is thought that a possible mechanism of this repression of transcription is through the sequestration of DP dimerization components from other E2FDP complexes. Alternatively, the E2F6 protein could possibly act as a molecular chaperone of the DP-1 and -2 proteins while they are not in complex with E2F1 to E2F5 (Trimarchi *et al.*, 1998).

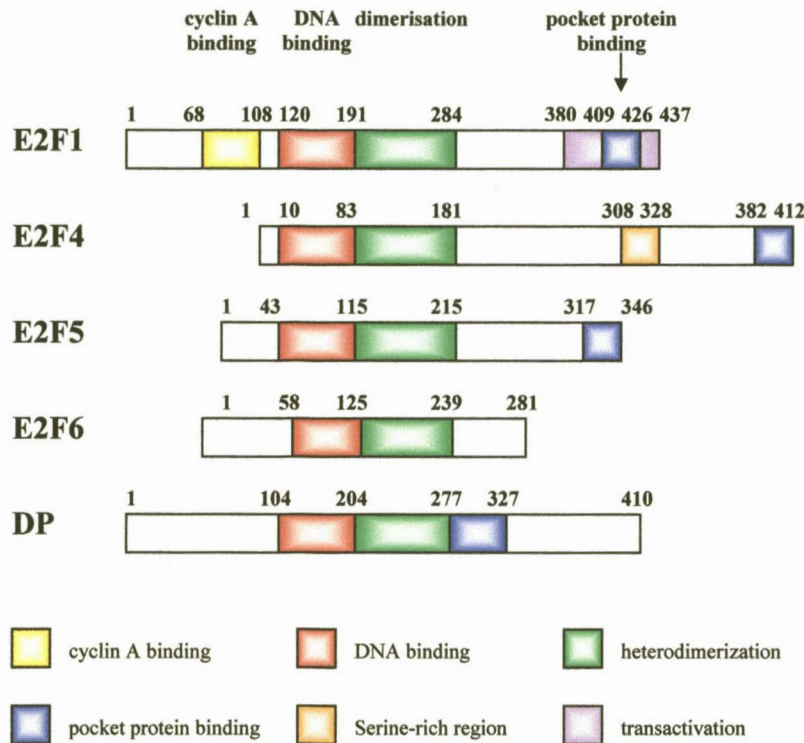


Figure 4. The E2F transcription factor family. E2F1 is representative of E2F1-3. DP1 is representative of DP1-2. Each of E2F1-6 is capable of complexing with either DP1 or DP2. Conserved domains and other regions of importance are indicated. With the exception of E2F6, the E2F proteins are able to bind the pocket protein family, namely Rb, p107, and p130, through the pocket protein binding region, which is located within the transactivation domain responsible for transcriptional activation. E2F6 has low DNA binding affinity, and lacks the C-terminal transactivation region, as well as the pocket-protein binding region, and is consequently incapable of regulating transcription from E2F response element-containing promoters (Hijmans *et al.*, 1995; Slansky and Farnham, 1996).

6.2 Differential Regulation by E2F Proteins

The different E2F proteins are capable of activating transcription from various groups of genes. E2F1 is the best-characterized member of the E2F family and a number of genes are activated in a specific fashion by this transcription factor. Genes encoding the S-phase proteins DNA polymerase α , thymidylate synthase, PCNA, and ribonucleotide reductase are activated by E2F1. Dihydrofolate reductase, thymidine kinase, and cell cycle regulatory

genes, namely *b-myb*, *c-myc*, and the cyclin A gene are also specifically activated by E2F1 (DeGregori *et al.*, 1995). E2F binding sites are found in the promoters of the genes of the G₁ cyclins D₁ and E, and both promoters are activated by E2F proteins. The cyclin E gene is under cell cycle-dependent regulation, which is mediated by E2F sites (Ohtani *et al.*, 1995). In addition, there appear to be feedback loops involved in the regulation of both cyclin D₁ and cyclin E, both involving E2F1 (Fan and Bertino, 1997; Ohtani *et al.*, 1995).

Studies on overexpression of the various E2F genes have shown that the different members are responsible for the activation of distinct target genes. The E2F1 protein is additionally capable of inducing apoptosis, an activity unique to this protein. Despite being extensively structurally related to E2F1, E2F2 and E2F3 are incapable of this effect. The induction of programmed cell death appears to be the result of specific activation of an apoptosis-promoting activity (DeGregori *et al.*, 1997).

The p53 tumour suppressor protein is one of the principal components of the apoptosis machinery of the cell. This protein has the ability to interact with the E2F1-DP1 complex. p53 acts as a transcriptional inhibitor of E2F1, and E2F1 and DP1 are able to downregulate p53-dependent transcription. E2F1 and DP1 are additionally capable of inhibiting p53 transcriptional activity, through physical complex formation with p53, in a mdm2-independent manner (O'Connor *et al.*, 1995). p19 is capable of interacting with the mdm2 protein, stabilizing it, and promoting apoptosis (Radfar *et al.*, 1998). The *INK4b*, p19 encoding, gene promoter contains binding sites for E2F1, alluding to a link between E2F1, p19, and apoptosis induction. Interestingly, the transactivation domain of E2F1 is not required for this effect (Macleod, 1999). It thus seems that in the presence of Rb, the E2F1-Rb complex is responsible for the active repression of apoptosis-promoting genes (Macleod, 1999). It is this ability of E2F1, to promote S-phase entry as well as apoptosis, that has led to the understanding that in certain situations, E2F1 is capable of acting either as a tumour suppressor, or as an oncoprotein (Macleod, 1999). The ability of E2F1 to act as either tumour suppressor or oncoprotein is dependent on tissue type and timing of expression (Macleod, 1999) (see also figure 6).

Promoter regulation from E2F sites is affected by the physical structure of the promoter. It has been suggested that the mechanism of transcriptional activation by the E2F family is dependent on the ability of the protein family to bend DNA at the promoter region (Cress

and Nevins, 1996). This ability of E2F proteins to bend DNA probably involves the so-called "marked box"; an evolutionary conserved region of the E2F family.

E2F is capable of exerting complex regulatory effects on the cyclin D-CDK4/CDK6 activity, or G₁ cyclin-dependent kinase activity, of the cell. E2F1 overexpression in Rb-deficient cell lines leads to an inhibition of G₁ cyclin-dependent kinase activity and transcriptional induction of p16 (Khleif *et al.*, 1996). Thus, in normal cells, the cyclin D-CDK4/CDK6 activity phosphorylates Rb, resulting in E2F induction of p16 and consequent abrogation of cyclin D-CDK4/CDK6 activity.

In addition to its role in the activation and repression of genes necessary for cell cycle progression, E2F1 is involved in the process of myogenesis. myoD and myogenin are basic helix-loop-helix transactivators responsible for the activation of genes necessary for myogenesis. E2F1 is capable of abrogating these interactions in the absence of Rb, but in the presence of Rb this inhibition is relieved (Wang *et al.*, 1996a). E2F1 appears to have different functions before and after the restriction point in the G₁ phase of the cell cycle. E2F1 transactivation-defective mutants, containing aberrations in distinct regions of the protein, were individually responsible for growth arrest either before or after the restriction point. Qin and Barsoum suggest that before the restriction point, E2F1 inhibits withdrawal from the cell cycle and entrance into a differentiation pathway, but after the restriction point, E2F assumes the well-known transcriptional regulatory activity (Qin and Barsoum, 1997).

Mammalian Cdc6 is the homologue of yeast Cdc6, a protein that is responsible for the initiation of DNA replication. In mammalian cells the protein is expressed exclusively in proliferating cells and not quiescent cells, and during the transition to the proliferating state the Cdc6 gene is regulated by E2F (Yan *et al.*, 1998). E2F1 is additionally involved in the entrance of quiescent cells, that is cells in G₀, to re-enter the cell cycle. In experiments where the E2F1 gene was constitutively expressed, serum withdrawal led to incomplete entrance of the cell population into G₀. Serum addition resulted in a rapid, asynchronous entry of G₀ cells into S phase following a short G₁ phase, consequently leading to cells with decreased volume. The S phase duration was extended in cells constitutively expressing E2F1 (Logan *et al.*, 1996).

To further complicate matters, Qin and Barsoum (1997) have suggested that E2F1 is capable of exerting different effects on the cell cycle at different times. They purport that before the restriction (R) point, E2F allows the withdrawal of the cell from the cell cycle, an ability that appears to be independent of binding proficiency of the protein. The alternative function of the E2F1 protein is the well-characterized adroitness of the protein to act as a transcriptional promoter of S phase promoting genes.

7. The Interactions of Rb, E2F and Cell Cycle Regulatory Proteins

7.1 Rb and E2F

Phosphorylation of the Rb protein at specific residues results in abrogation of the Rb-E2F complex. There are several regions of Rb involved in protein-protein interactions, namely the A/B pocket, which binds proteins with an LXCXE motif, the C pocket, which binds the c-Abl tyrosine kinase, and the large A/B pocket through which the E2F family is bound. Phosphorylation of Rb at two distinct locations is required for abrogation of E2F binding. The first is the C-terminal region where phosphorylation of several of the seven sites is required. Secondly, phosphorylation of two serines in the insert domain in the presence of the N-terminal region of the protein is capable of disrupting the complex (Knudsen and Wang, 1997).

E2F1 is capable of acting as both a transcriptional activator and repressor of the aforementioned genes required for passage of the cell across the G₁/S boundary and through the S phase of the mammalian cell cycle (Zacksenhaus *et al.*, 1996). During the early stages of G₁ the retinoblastoma protein is in the active growth-suppressing hypophosphorylated state, and is consequently found in complex with the E2F1 transcription factor. At this stage of the cell cycle Rb-E2F1 complex formation results in concomitant sequestration of E2F1 from E2F1 binding sites in the promoters of the aforementioned genes. This results in an inability of E2F1 to directly activate transcription from these promoters. In addition Rb is capable of binding E2F1 already bound to promoters to form an Rb-E2F1 complex that is subsequently able to actively repress the transcriptional activity of the respective genes (Sellers *et al.*, 1995; Qin *et al.*, 1995; Weintraub *et al.*, 1992; Weintraub *et al.*, 1995). The ability of Rb to repress transcription in an E2F-dependent manner is largely (wholly?) due to the presence of Rb itself. In a study of various promoter elements, Rb was found to repress transcription mediated by Sp1, AP-

1 and p53. In this mechanism, the transcription factor provides the binding activity via the appropriate binding promoter binding site, while Rb itself is responsible for transcriptional repression of the regulated gene (Adnane *et al.*, 1995).

Additionally, it appears that the phosphorylation events on Rb that accompany S-phase entry and completion, are separable. Using phosphorylation-defective mutants and ectopic E2F expression, Chew *et al.* (1998) were able to show that additional phosphorylation of Rb is required to complete S-phase, following successful entry. S-phase entry is accompanied by derepression of E2F regulated genes, but this effect of Rb phosphorylation is unnecessary for S-phase completion. It, therefore, appears that Rb may have additional properties pertaining to completion of S-phase, in addition to those already well characterized for S-phase entry.

7.2 Rb, E2F, and Histone Deacetylase

The mechanism of active repression by the Rb-E2F complex appears to involve the action of histone deacetylase enzymes. This protein catalyses the removal of acetyl groups from histone proteins, which serves to strengthen the interaction of histones and DNA in nucleosomes. This tighter binding precludes the binding of activators to their target binding sites thus abrogating transcriptional activation (DePinho, 1998). All three members of the pocket protein family, namely Rb, p107, and p130, are able to form ternary complexes consisting of a pocket protein, an E2F protein, and the histone deacetylase HDAC1 (Brehm *et al.*, 1998; Ferreira *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998). HDAC1 binds the pocket proteins through interaction with a conserved LXCXE motif situated in the A/B pocket domain. Formation of these ternary complexes is dependent on the presence of the pocket protein component (Brehm *et al.*, 1998; Ferreira *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998).

As would be expected, the phosphorylation of p107 by cyclin D-CDK4 and the binding of viral oncoproteins to the p107 A/B pocket result in disruption of the p107-E2F4-HDAC1 complex (Ferreira *et al.*, 1998). The addition of trichostatin A, an inhibitor of HDAC1, alleviates deacetylation-dependent transcriptional repression. It must be noted, however, that transcriptional repression through deacetylation is not the sole mechanism available to the cell as a basal level of repression persists following inhibition of deacetylase activity,

indicating an additional histone deacetylase-independent mechanism (Brehm *et al.*, 1998; Ferreira *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998).

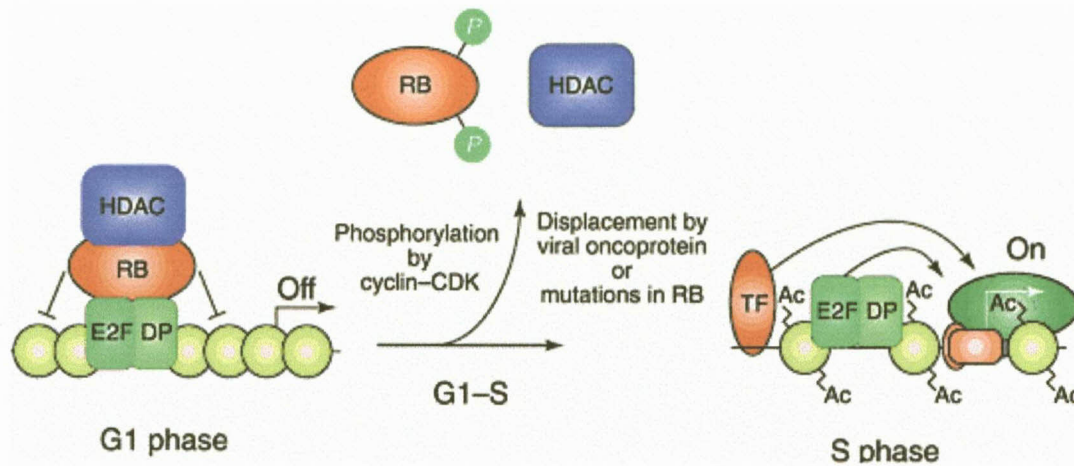


Figure 5. Rb modulates chromatin structure through sequestration of the histone deacetylase HDAC1. In the early G₁ phase, Rb is associated with the E2F1-DP1 transcription factor complex. This Rb-E2F1-DP1 complex is bound to the E2F response element found in the promoter of several genes essential for cell cycle regulation. Rb is additionally associated with HDAC1, which catalyses deacetylation of chromatin, which consequently adopts a closed chromatin structure. As the cell passes through the G₁ phase, Rb is phosphorylated by G₁-cyclin/CDK complexes, leading to disruption of the transcriptional repressor HDAC1-Rb-E2F1-DP1 complex. This complex can also be disrupted by specific viral oncoproteins that target the binding pocket of Rb. The surrounding chromatin is subsequently acetylated, permitting access to the transcription machinery (Brehm and Kouzarides, 1999).

This mechanism of repression, involving histone deacetylation, has been observed in the TGF β -mediated repression of *cdc25A* in keratinocytes. *cdc25A* is a tyrosine phosphatase responsible for the activation of G₁ CDKs. Following exposure to the anti-mitogenic cytokine TGF β , *cdc25A* activity is reduced, ultimately leading to cell cycle arrest. *cdc25A* is repressed by the E2F4-p130 complex, through recruitment of HDAC1, which leads to a condensed chromatin structure (Iavorone and Massagué, 1999, and references therein). Additionally, it appears that the Rb protein is responsible for chromatin modulation of the HLA-DR-encoding promoter in a mechanism independent of transcription (Osborne *et al.*, 1997). The gene for HLA-DR, which codes for the heavy chain subunit of the human major histocompatibility complex class two (MHC II), is regulated in an interferon γ (IFN γ) dependent mechanism that appears to additionally involve Rb.

Acetylation of the histones by histone acetyl transferase results in a decrease in binding affinity of histones for DNA, allowing access to transcription factors and subsequent activation of transcription (Bestor, 1998; DePinho, 1998). There is evidence to suggest that

the methylation state of DNA also impacts upon the deacetylation of histones and consequently the transcriptional activity of underlying genes (Bestor, 1998). Transcriptional silencing can be achieved through methylation of cytosine residues at the 5-position. It appears that these methylation patterns are capable of recruiting histone deacetylase and repressing transcription in a histone deacetylase-dependent manner.

7.3 Rb, E2F, and the Human SWI/SNF Complex

Rb and E2F1 are capable of interacting with chromosomal DNA via the mammalian SWI/SNF complex (Trouche *et al.*, 1997). The SWI/SNF complex was originally isolated from yeast as a complex that modulates chromosomal structure and is capable of activating transcription from the underlying genes. Two components of the human SWI/SNF complex are the hBRM and hBRG1 proteins. These human SWI/SNF proteins are found in association with relaxed chromatin, while being excluded from regions of condensed chromatin. These regions are enriched for the presence of hSWI/SNF proteins, which are associated with the nuclear matrix (Reyes *et al.*, 1997). hBRM potentiates transcriptional activation through a cooperative interaction with the glucocorticoid receptor (Muchardt and Yaniv, 1993). This interaction is dependent on the presence of the glucocorticoid receptor DNA binding domain and two distinct regions of the hBRM protein. It was subsequently demonstrated that the Rb and hBRM proteins interact to potentiate glucocorticoid receptor-mediated transcriptional activation (Singh *et al.*, 1995).

The remaining Rb family members, p107 and p130, are also capable of interacting with hBRM and hBRG1. This interaction appears to be dependent on the presence of an intact binding pocket, as co-expression of E1A drastically reduces Rb/p107/p130-mediated effects (Strober *et al.*, 1996). The transcriptional arrest observed at the G₂/M boundary is in part ascribable to the exclusion of hBRM and hBRG1 from the nuclear structure during early M phase. hBRM and hBRG1 are phosphorylated during mitosis and consequently unable to associate with the condensed chromosomal DNA. hBRM protein is found at lower levels during mitosis, unlike hBRG1, which is unaffected. The ability of hBRM and hBRG1 to interact with hSNF5, a human homologue of yeast SNF5, is retained after their phosphorylation, but the complex is no longer able to localize to the nuclear compartment (Muchardt *et al.*, 1996).

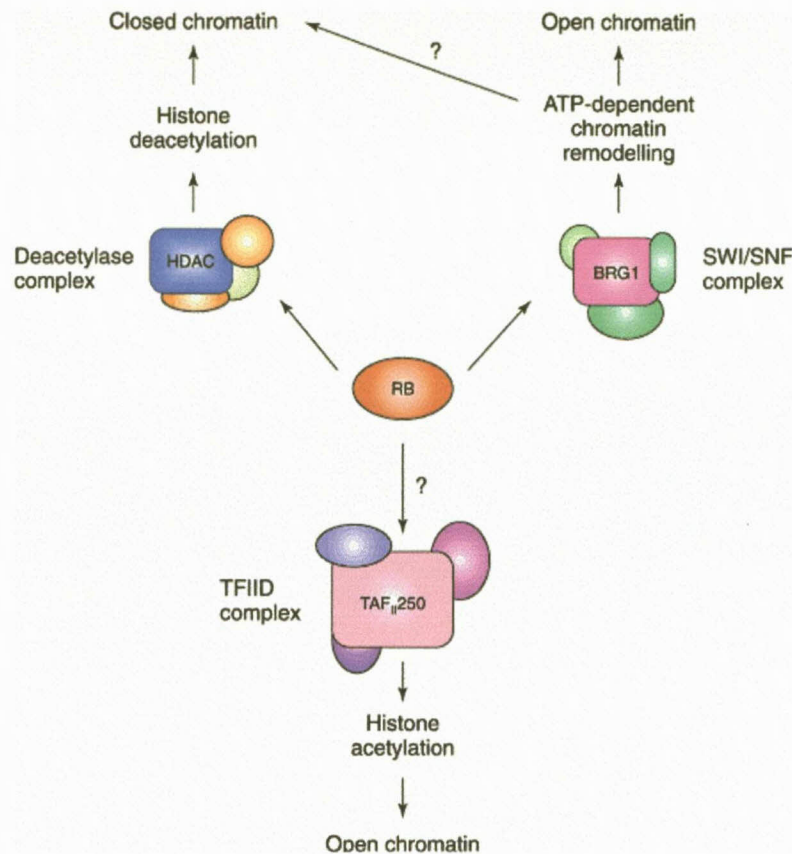


Figure 6. Rb serves as a central point for the modulation of chromatin structure. Rb sequesters HDAC1 histone deacetylase to promoters via its interaction with the E2F1-DP1 transcription factor complex. The chromatin assumes a closed structure following histone deacetylation. Rb is able to directly interact with the TAF_{II}250 subunit of TFIID, and consequently regulate its interaction with the RAP74 subunit of TFIID, and by inference, regulate the assembly of the transcription complex. TAF_{II}250 contains an additional histone acetylase activity that promotes a open chromatin structure, but the interaction of Rb with TAF_{II}250 at this level remains to be elucidated. Rb is additionally capable of interacting with the BRG-containing SWI/SNF complex that modulates chromatin structure via an ATP-dependent mechanism (Brehm and Kouzarides, 1999; Siegert and Robbins, 1999).

The hBRG1 and hBRM proteins are however not essential for survival, as examples of SWI/SNF-type complexes have been found that lack both of these proteins (Wang *et al.*, 1996b). In these instances the SWI/SNF-type complexes are composed of additional proteins, termed BRG1 associated factors (BAFs). These BAFs were isolated by their ability to coimmunoprecipitate with antibodies directed against BAF47/INI1, a SNF5 homologue also found to interact with and activate human immunodeficiency virus (HIV) integrase (Wang *et al.*, 1996b). The hSWI/SNF proteins are functionally controlled through phosphorylation and degradation, where cells blocked at the G₂-M boundary contain hSWI/SNF complexes with hBRG1 phosphorylated at two sites, and no hBRM. *In vitro*, active hSWI/SNF can be deactivated by phosphorylation and restored by dephosphorylation (Sif *et al.*, 1998). The E2F1 protein contains distinct binding sites for complexes of Rb and hBRM. The hBRM component is essential for complete inactivation

of E2F1 function by Rb and requires the LXCXE motif of the hBRM protein. This mechanism of E2F1 repression is supported by the ability of Rb to bind both hBRM and E2F1 simultaneously, and also the observation that E2F1 and hBRM are found in complex *in vivo* (Trouche *et al.*, 1997).

The Rb-E2F1 complex thus serves as an active repressor of transcription, with E2F1 providing the binding activity, and Rb providing the repressor activity of the complex. In this way transcription from genes containing E2F binding sites is downregulated prior to transition of the G₁/S boundary. Transforming growth factor β (TGF- β) is capable of causing growth arrest in G₁ in most cell types. The mechanism of this activity seems to be by the active repression of E2F responsive genes through the action of E2F4-Rb and E2F4-p107 complexes (Li *et al.*, 1997).

7.4 E2F1 and Sp1

Sp1 is a cellular transcription factor first isolated as a protein capable of binding the simian virus SV40 early promoter. The protein is responsible for the expression of several genes. The factor is not essential for cell growth or differentiation, but is involved in early embryonic development (Marin *et al.*, 1997). E2F is capable of interacting with Sp1 during promoter regulation. The thymidine kinase gene has juxtaposed binding sites for E2F and Sp1 separated by 10 base pairs. The transcription factors interact directly through the carboxy-terminal region of Sp1 and an amino-terminal region of E2F1. This region is conserved in E2F2 and E2F3, and consequently both these proteins are capable of the interaction with Sp1. The abrogation of the interaction of either transcriptional regulator with its binding site is capable of altogether abolishing activation (Karlsreder *et al.*, 1996). The cyclin D1-encoding gene is also under the control of E2F and Sp transcription factors. The E2F1 and Sp1, Sp2, or Sp3 proteins interact with the promoter of the cyclin D1 gene to repress activity. The E2F1 DNA binding activity, the Rb binding domain, and the amino terminal Sp1 binding region are all required for full repression of the promoter. Interestingly, p107 can also interact with E2F4 and Sp1, but in this instance the cyclin D1 gene promoter is activated, demonstrating the variable effects of the various E2F- and Rb-family members on the cell cycle (Watanabe *et al.*, 1998).

Furthermore, the interaction between Sp1 and E2F1 is synergistic. The factors are capable of activating transcription from each other's binding site (Lin *et al.*, 1996). There is

however conflicting evidence for the synergistic interaction of Sp1 and E2F. Blau *et al.* (1996) classified E2F1 as a transcriptional activator incapable of synergy with Sp1. Sp1 is however not the only transcription factor with which E2F1 is able to interact. The cell cycle-dependent element (CDE) is contiguous with the cell cycle genes homology region (CHR), and the CDE-CHR element is present in the promoters of vital cell cycle genes, for example those encoding cyclin A, Cdc2 and Cdc25. E2F1 has the potential to interact with the CDE-CHR binding factor-1 (CDF-1) protein. The various interactions of these proteins contribute to the timing of cell cycle-regulated transcription from responsive promoters (Lucibello *et al.*, 1997).

8. Repression of Promoters by the Rb-E2F Complex

As the cell passes through the cell cycle Rb becomes progressively more phosphorylated through the action of specific G₁ cyclin-CDK complexes. This abrogates the repressor activity of the Rb-E2F complex, and E2F is subsequently available to perform its transcriptional activating function. Following the transition of the G₁/S boundary, the level of E2F1 in complex with Rb drops, as a consequence of the phosphorylation of Rb, and a p107-E2F1-cyclin A complex is formed (Shirodkar *et al.*, 1992).

Additional E2F pocket protein interactions are essential for proper cell cycling. In quiescent cells the E2F4-p130 complex negatively regulates the activity of key target genes. This complex is present exclusively in the G₀ phase of the cell cycle and is degraded as the cell enters the G₁ phase in a G₁ cyclin-CDK complex-dependent manner (Smith *et al.*, 1996).

Recently, a hypothesis has been put forth that E2F1 is capable of acting as an oncogene in certain situations, due to its close interaction with Rb and its regulation of the growth suppressive properties of Rb (Johnson *et al.*, 1993; Johnson *et al.*, 1994; Qin *et al.* 1995). Deregulated expression of the E2F1-DP1 complex is capable of transforming rat embryo fibroblasts in the presence of an activated *ras* oncogene (Johnson *et al.*, 1994). Overexpression of E2F1 is additionally potentially able to induce the entry of quiescent cells into the S phase of the cell cycle (Johnson *et al.*, 1993).

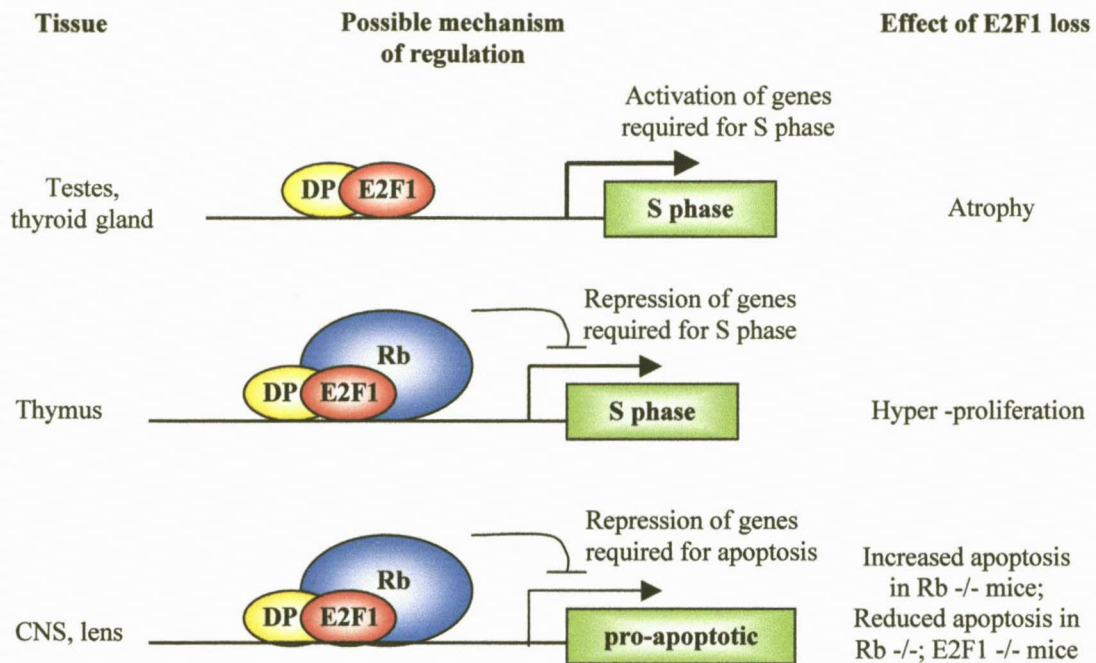


Figure 7. Different effects of E2F1 on gene regulation. E2F1 is capable of activating or repressing transcription of genes, depending on the level and tissue of expression, and the presence of the pocket protein Rb. S-phase genes are positively or negatively controlled by E2F1, while pro-apoptotic genes are negatively regulated by E2F1, explaining how E2F1 is capable of acting as both a tumour suppressor and oncogene, under the appropriate conditions (Macleod, 1999).

9. Ubiquitin-Mediated Degradation of E2F

E2F1 levels increase until late G_1 , after which E2F1 is degraded in G_2/S (Marti *et al.*, 1999). E2F1 is removed from the cell by ubiquitin-proteasome-dependent degradation elicited by a sequence in the C-terminal region of the protein. This region overlaps with a region required for binding of Rb. Binding of Rb to E2F1 results in a blockage of the ubiquitination region and subsequent stabilization of the E2F1 protein, preventing proteasome-dependent protein turnover (Campanero and Flemington, 1997).

The process of ubiquitin-mediated degradation is performed by a complex system of proteins. Three components are required for the ubiquitination of specific target proteins. The ubiquitin activating enzyme E1 activates ubiquitin, which is subsequently transferred to the second component, E2, a ubiquitin-conjugating enzyme. E3 is a ubiquitin-protein ligase that targets specific proteins through interaction with F-box proteins, and is responsible for mediating the interaction of E2 and the target protein. The multisubunit E3 enzyme is comprised of the core ubiquitin ligase SKP1-CDC53/Cul-1 and an F-box protein

that provides the specificity required for targeted degradation. Numerous F-box proteins are present that each target a specific protein for ubiquitination (Harper and Elledge, 1999). The E2F1 protein is targeted by p45^{SKP2}, a cell cycle regulated component of the SCF-type ubiquitin-protein ligase SCF^{SKP2}. This interaction is essential for degradation of E2F1, and thus for proper maintenance of E2F levels (Magae *et al.*, 1999). Polyubiquitinated proteins are subsequently recognized and degraded by the 26S proteasome (Harper and Elledge, 1999). The DP1 dimerization partner of E2F1 is also subject to ubiquitin-mediated degradation. E2F1 regulates the cellular localization of DP1 through formation of the E2F1-DP1 complex. Once E2F1, and thus the DP1 in complex with E2F1, has migrated to the nucleus, any remaining DP1, in addition to a host of other proteins, is degraded in a ubiquitin-dependent manner. It appears that this degradation process is essential for entry into S, even in the presence of activated E2F1 (Magae *et al.*, 1999).

10. Regulation of the *RB1* Gene by E2F

E2F1 is capable of interacting with Rb at the DNA level. The *RB1* gene contains a specific binding site for E2F1 through which E2F1 transactivates expression of Rb. The Rb protein is capable of suppressing the transcriptional activation by E2F1 through overexpression. Timing of E2F1 expression is synchronized with that of Rb. Taken together this implies that E2F1 is responsible for negative autoregulation of Rb (Shan *et al.*, 1994). The *RB1* promoter has a so-called RB-E2F site in its promoter to which E2F is able to bind *in vitro*. Mutation and deletion of this site has the effect of increasing transcription of *RB1*, indicating that the RB-E2F site acts as a silencer element. The effect is observed in Rb negative cell lines, suggesting that the effect is not dependent on the presence of Rb (Ohtani-Fujita *et al.*, 1994).

11. Additional Rb Interactions Independent of E2F

11.1 Rb, TAFs and the Basal Transcription Machinery

Up until this point it would seem that Rb interacts with cellular proteins exclusively in the presence of E2F, but this is certainly not the case. Rb has far-reaching effects on the cell that are independent of E2F, and is capable of affecting the cell cycle and cellular differentiation. Rb is able to inhibit transcription by direct interaction with the cellular transcriptional machinery (see figure 7). Mammalian promoters contain a region referred to

as the TATA box, which is important for assembly of the basal transcription complex. This TATA-box is subsequently the binding target of a cellular protein involved in transcription, consequently referred to as TBP (TATA-binding protein), which in turn is associated with numerous TAFs, or TBP-associated factors.

TAF_{II}250 is the largest of these TAFs and contains kinase activities in both its amino and carboxyl termini, in addition to a histone acetylase activity. TAF_{II}250 forms part of the TFIID complex, a subunit of the basal-transcription complex, which is thought to promote activated transcription of cell cycle related genes (Brehm and Kouzarides, 1999, and references therein). TAF_{II}250 N-terminal kinase activity is regulated by the Rb protein, via the Rb protein's large binding pocket, amino acids 379-928, in a direct protein-protein interaction. This TAF_{II}250 kinase activity is required for the activated transcription of certain genes, including the genes coding for cyclin A and Cdc2. Inhibition of the N-terminal kinase activity precludes phosphorylation of RAP74, a component of TFIIF, which is itself a component of the basal-transcription complex, as well as autophosphorylation of TAF_{II}250 and phosphorylation of additional target proteins (Siegert and Robbins, 1999, and references therein). Rb is thus capable of directly interacting with the cellular transcription machinery to inhibit gene expression.

In addition to acetylation of histone tails, histones are also subject to control by phosphorylation. As the histone tails of histone protein H1 are phosphorylated, the chromatin assumes an open structure. This effect is more prominent in Rb^{-/-} cells, which are unable to constrain the phosphorylation action of cyclin E-CDK2. This suggests a secondary role for Rb in chromatin structure modulation; control of phosphorylation by specific cyclin-CDK complexes (Brehm and Kouzarides, 1999; Herrera *et al.*, 1996).

11.2 Rb and Cellular Transcription Factors

The product of the *c-fos* gene associates with members of the Jun family of transcription factors to bind and activate transcription from AP-1-binding sites. AP-1 transcription factors are required for mitogenic signaling and differentiation of certain cell types. The *c-fos* gene product is itself a transcription factor that is required for entrance of quiescent cells into the cell cycle, making it a target for disruption in the development of neoplastic growth. The promoter of *c-fos* contains a binding site for the retinoblastoma protein, termed a retinoblastoma control element (RCE). Rb is capable of binding this RCE to

repress transcription of the *c-fos* gene directly, thereby inhibiting access to G₁ and passage through the cell cycle (Robbins *et al.*, 1990). The c-Jun protein is capable of directly interacting with the Rb protein. In the presence of Rb, c-Jun activates transcription from an AP-1 consensus sequence. The interaction involves the B domain of the Rb pocket and its C-terminal domain and the leucine zipper region of c-Jun. This interaction can be abrogated by the HPV E6 oncoprotein (Nead *et al.*, 1998).

The *c-myc* promoter is subject to complex control by Rb. In the presence of E2F, the Rb-E2F complex is responsible for repression of the promoter, while in the absence of E2F, Rb is capable of directly binding the promoter to activate transcription. These two mechanisms work antagonistically to regulate the *c-myc* promoter. Both E2F-dependent repression and E2F-independent activation are abrogated in the presence of the SV40 large T antigen, which specifically targets the Rb protein (Batshé *et al.*, 1994). The *neu* oncogene is also subject to bimodal control by Rb, although in this case, both mechanisms have a repressive effect on transcription. Promoter analysis of the *neu* gene led to identification of two regions in the promoter that are important for transcriptional regulation. The first sequence, upstream of the transcription initiation site, is regulated by Rb in a mechanism independent of Rb pocket integrity. The second region, further upstream, is referred to as a GTG enhancer, and is regulated in a binding pocket-dependent mechanism (Martin and Hung, 1994). Consequently only repression via the GTG site is negated in the presence of transforming oncoproteins.

11.3 Contribution of the Rb C Pocket

The small and large binding pockets of Rb are responsible for mediating a wealth of interactions with cellular transcription factors. The large A/B pocket binds E2F and the small A/B pocket recognizes the LXCXE motif found in numerous RB-binding proteins. These are, however, not the only domains of importance in the Rb protein. The C terminal region of Rb is capable of binding the c-Abl tyrosine kinase. This C pocket also contains the DNA binding activity of Rb, which, as described above, is important for the direct regulation of several oncogenes. Mutation of Trp661 results in abrogation of E2F and LXCXE binding. This mutant is, however, still capable of suppressing growth, resulting in a G₁/S arrest. This C-terminal arresting activity suppresses growth less efficiently than wildtype Rb, and is abrogated by mutations in the C pocket. Trp661 mutant Rb has been found in cases where retinoblastoma developed with low penetrance, indicative of the

lower efficiency of the mutant Rb protein in arresting growth (Whitaker *et al.*, 1998, and references therein).

12. The Role of the Retinoblastoma Protein in Cancer

12.1 Retinoblastoma

Loss of Rb is associated with the majority of cases of retinoblastoma, a childhood cancer of retinal tissue. It is possible to distinguish between hereditary and sporadic cases of the malignancy by virtue of the pattern of tumour formation. Hereditary retinoblastoma is associated with multiple, bilateral tumours that develop at an early age, while sporadic cases are characterized by the formation of fewer, unilateral foci later in life. The difference in occurrence can be explained by noting that hereditary cases have a germline abnormality, and consequently all cells have a defective allele, thus requiring only a single additional mutation for tumour formation. Sporadic retinoblastoma on the other hand is the result of loss of function of both wild type alleles in the same cell, a statistically more unlikely event. There have been reports that the retinoblastoma gene, which is ordinarily inherited with high penetrance, is inherited with lower frequency in some families. This appears to be the result of mutations in distinct exons (Greger *et al.*, 1994; Lohmann *et al.*, 1994; Munier *et al.*, 1994; Onadim *et al.*, 1992).

The presence of bilateral retinoblastoma is a poor prognostic indicator as the patient often has an increased propensity towards the development of subsequent malignancies indicating the requirement for life-long follow-up examinations (Ceha *et al.*, 1998). The presence of unilateral retinoblastoma is possibly also indicative of poor prognosis. A recent report showed that a sporadic case of unilateral retinoblastoma was associated with procurement of monosomy 7 and secondary acute myelomonocytic leukaemia (Bayar *et al.*, 1998).

12.2 Breast Cancer

Breast cancer is the most prevalent carcinoma affecting Western women. This complex neoplasia is related to numerous mutations and other genetic aberrations, and tumour suppressor proteins, including p53, Rb and p16 appear to play important roles in its genesis and progression (Cox *et al.*, 1994; Evans and Prosser, 1992; Frebourg *et al.*, 1991; Porter-Jordan and Lippman, 1994; Steeg, 1992). The murine retinoblastoma homologue is

associated with the development of mouse mammary tumours (Hanania *et al.*, 1995). The p16 protein is responsible for the inhibition of CDK4 activity and consequently inhibits the phosphorylation of Rb, one of the primary targets of CDK 4. p16 is aberrantly expressed in a significant proportion of breast tumour samples, alluding to the prevalence of this abnormality in breast carcinomas (Geradts and Wilson, 1996).

Several studies have demonstrated that loss of heterozygosity at the *RBI* locus is a relatively common event in breast cancer. Using various methods such as single-strand conformation polymorphisms (SSCP) (Tamura *et al.*, 1994), immunohistochemical detection (Bianchi *et al.*, 1994; Pietilainen *et al.*, 1995), and restriction mapping (Bookstein *et al.*, 1989; Lee *et al.*, 1988), loss of heterozygosity (LOH) at the retinoblastoma locus was found in approximately 35-50% of all cases implicating disturbance of Rb as a crucial event in breast carcinoma formation.

A study by Borg *et al.* (1992) however casts doubts as to the relevance of LOH studies where LOH at the *RBI* locus and actual decrease in Rb levels could not be faithfully correlated. Interestingly, Schott *et al.* (1994) found evidence for a second tumour suppressor protein that localizes to the area adjacent to the locus of the retinoblastoma gene. The gene, designated *Brush-1*, is located at 13q12-13, which places it proximal to the *RBI* gene. Of note is that the mRNA encoded by *Brush-1* is also 4.7 kb in length. In a study of 13 breast cancer cell lines and four primary tumour samples, they found that decreased levels of *Brush-1* mRNA were more common than a decrease in the *RBI* mRNA levels.

The *BRCA2* gene encodes a 460 kDa nuclear phosphoprotein that acts as a tumour suppressor. The gene has been associated with hereditary breast cancer. Recent evidence suggests a link between BRCA2, the p53 tumour suppressor protein, and the RAD51 double strand break DNA repair protein. This is significant in that it provides a link between a cell cycle regulator (the BRCA2 protein), an apoptosis inducer (p53) and a DNA repair protein (RAD51) and implies a link between proteins responsible for genome stability (Marmorstein *et al.*, 1998).

12.3 Osteosarcoma

The retinoblastoma protein is one of the most important genes involved in the human bone cancer osteosarcoma. Numerous techniques, including SSCP, cytogenetic analysis, DNA

and RNA analysis, and PCR have implicated Rb in approximately 60-70% of all cases of the disease (Araki *et al.*, 1991; Miller *et al.*, 1996; Wadayama *et al.*, 1994; Wunder *et al.*, 1991; Yamaguchi *et al.*, 1992). LOH studies have demonstrated the instability of the *RB1* locus. The retinoblastoma gene is however obviously not the only tumour suppressor gene participant of osteosarcoma pathogenesis. The p53 and cyclin dependent kinase CDK4 encoding genes are also abnormal in this malignancy (Kanoë *et al.*, 1998; Miller *et al.*, 1996).

Contrary to the idea that CDK4 and Rb act in the same pathway and thus are not simultaneously inactivated, Kanoë *et al.* (1998) found that in three of four cases displaying amplification of the CDK4 gene, aberrant Rb protein expression could also be detected. This implies that CDK4 is important not only for the inactivation of Rb through phosphorylation but also in an additional mechanism/s important for the development of osteosarcoma. Loss of heterozygosity at the *RB1* locus additionally serves as a prognostic indicator capable of being applied to the early diagnosis of osteosarcoma and identification of high risk patients (Feugeas *et al.*, 1996). Loss of heterozygosity is not an absolute indicator though as an intensive mutation spectrum analysis by Wadayama *et al.* (1994) showed that in 50% of cases negative for LOH there was still a loss of Rb protein expression.

12.4 Retinoblastoma and Leukemia

The retinoblastoma gene and protein have been implicated in the formation of a wide range of carcinomas, sarcomas, and leukemias, supporting its role as a tumor suppressor (Horowitz *et al.*, 1990; Zhu *et al.*, 1995). Over the years cytogenetic analysis has yielded to the rapidly advancing field of molecular biology. In a study of 215 cases of leukemias and lymphomas, Ahuja *et al.* (1991) found structural alterations in RB and low or absent protein levels to be relatively common in all types of acute leukemia.

12.4.1 Lymphoid Leukemia

Studies on chronic lymphoblastic leukemia have shown that karyotypic abnormalities are present in approximately 50% of cases. A study of 70 patients with B cell chronic lymphoblastic leukemia (B-CLL) showed that 20 patients had cytogenetic alterations in the long arm of chromosome 13 at band 14 (i.e. 13q14), the locus of the retinoblastoma susceptibility gene. These alterations included deletions and translocations, and the

corresponding clones were subjected to further analysis. Reduced mRNA levels, internal deletions, allele loss, and presence of unphosphorylated Rb were observed. Clones displaying chromosomal and DNA/RNA abnormalities showed dominance of B cells with Rb defects (Dierlamm *et al.*, 1997; Kay *et al.*, 1993). Differential PCR analysis of RB in 56 patients with early or late stage CLL indicated that only a fraction displayed abnormal levels of the gene (Neubauer *et al.*, 1993).

In the case of acute lymphoblastic leukemia (ALL), mutation of the multiple tumour suppressor gene *MTS1* has been found to be the most prevalent genetic alteration (Cayuela *et al.*, 1996; Guidal-Giroux *et al.*, 1996). The level of Rb is a prognostic indicator where low levels are associated with poor outcome in initial and relapsed childhood ALL (Sauerbrey *et al.*, 1996). Analysis of 34 cases of different types of leukemia without chromosomal abnormalities at band 13q14 resulted in the observation of a deletion of exons 18 to 27 of the RB gene in a case of Philadelphia positive-ALL (Hillion *et al.*, 1991). Abnormal expression of Rb was also observed at low frequency in ALL (1 of 12) by Furukawa *et al.* (Furukawa *et al.*, 1991). It appears that abnormalities of the retinoblastoma gene and protein are infrequent in lymphoid leukemia (Copelan and McGuire, 1995).

12.4.2 Myeloid Leukemia

Tumour suppressors are involved in the genesis of chronic myeloid leukemia (CML), but it is p53 that is most prevalent in this haematological malignancy. Several reports have shown that functional loss of this gene is responsible for progression of the disease (Aguiar *et al.*, 1995; Ahuja *et al.*, 1989; Hernández *et al.*, 1993; Nakai *et al.*, 1992; Nakai *et al.*, 1994a; Nakai *et al.*, 1994b). The Rb protein has also been implicated in the progression of CML, specifically in megakaryoblastic crisis. A study of 22 patients with CML showed that 100% (5 of 5) of cases in megakaryocytic blast crisis lacked observable expression of Rb. This is in contrast with the presence of Rb in myeloblastic and lymphoblastic crisis cases of CML determined in the same study. It is thus possible that the loss of Rb may be associated in a lineage-specific manner with the progression of CML (Towatari *et al.*, 1991). Rb abnormalities were found to be more prevalent in another study where 4 of 9 cases of B cell CML were shown to have lower levels of the protein (Furukawa *et al.*, 1991).

The retinoblastoma protein plays a more substantial role in the progression of acute myelogenous leukemia (AML). Using immunoprecipitation and Western blot analysis, 28% (5 of 18) of cases of AML were shown to contain low or zero levels of Rb. None of these cases displayed karyotypic abnormalities at the retinoblastoma locus, 13q14. Further analysis showed that the DNA and mRNA derived from it were normal, suggesting a post-translational regulation mechanism (Tang *et al.*, 1992). Using a different antibody directed against Rb, Zhu *et al.* (1994) showed that 28% (11 of 39) of patients with AML were deficient for the production of Rb. Further analysis revealed that 4 of 7 Rb negative cases were without mRNA transcripts. This lack of Rb was correlated with the ability of the clonogenic blasts to proliferate autonomously. A second group separately observed reduced levels of Rb in 8 of 32 cases of AML. Of these eight only two were absent for the corresponding mRNA (Furukawa *et al.*, 1991).

The retinoblastoma gene is not the only tumour suppressor involved in the tumorigenesis of leukemia. p53 has also been shown to contribute to the formation of these malignancies, albeit at a lower frequency than the retinoblastoma gene (Kurosawa *et al.*, 1995; Trecca *et al.*, 1994). p53 abnormalities have been observed in AML, CML, ALL, and CLL, suggesting that the protein plays a potentially significant role in leukemogenesis (Paydas, 1995).

There is also preliminary evidence for the involvement of Rb in primary leukemias. Of 130 patients two were found to contain homozygous deletion of the retinoblastoma susceptibility gene (Chen *et al.*, 1990). In addition, RB was found to be homozygously inactivated in a case of preleukemia (Hansen *et al.*, 1990). In a study primary myelomonocytic and monoblastic leukemias the Rb protein was absent or present in barely detectable levels in 55% (11 of 20) of cases (Wiede *et al.*, 1993). This is in accord with the findings of Ahuja *et al.* (1991) who found a high incidence of RB abnormalities in M₄ and M₅ myeloid leukemia with monocytic differentiation. Monoallelic deletion of RB was observed in 81% (17/21) of human myeloma cell lines. This deletion did not however influence the activation of the gene or the expression of Rb (Juge-Morineau *et al.*, 1995).

In contrast to its apparent prevalence in myeloid leukemia with monocytic differentiation, RB appears to have little influence on the development of myelodysplastic syndromes (MDS). Point mutational analysis by single strand conformation polymorphism (SSCP) detected only 2 cases out of 90 with mutation in RB (Preudhomme *et al.*, 1994).

12.5 The Retinoblastoma Protein and Additional Cancers

The Rb and cyclin D1 proteins are purportedly involved in the genesis of oesophageal cancer. In a study of oesophageal carcinoma cell lines and primary tumours from across the globe, the cyclin D1 and Rb proteins were found at abnormal levels. Interestingly, in cases where the cyclin D1 gene was amplified, the Rb gene was essentially normal, while cases involving a decrease in Rb levels did not show cyclin D1 amplification, thus suggesting two mechanisms of overcoming the G₁/S boundary imposed by the Rb protein (Jiang *et al.*, 1993). In addition, oral carcinomas related to the use of tobacco were shown to exhibit a similar relationship between Rb and cyclin D1 levels. In a study of patients that smoke or make use of smoke-free tobacco, a low frequency (9%) of Rb alterations was found, in contrast with the high frequency of cyclin D1 amplification and p53 mutation (Xu *et al.*, 1998). The results are however not completely consistent with those of the oesophageal carcinomas.

The E2F1 and Rb proteins are involved in the onset of hyperplasia associated with skin tumour development. Expression of E2F1 in the epidermis results in hyperplasia without the abrogation of terminal differentiation. Overexpression of E2F1 results in aberrant apoptosis, which is subsequently augmented through the cyclin D1-dependent phosphorylation of Rb (Pierce *et al.*, 1998).

13. *Saccharomyces cerevisiae* as a Model Organism

13.1 The *S. cerevisiae* Cell Cycle

Saccharomyces cerevisiae, also referred to as baker's yeast, is a perfect example of a model organism. This organism has been extensively studied, which has resulted in a wealth of knowledge as to its genetics and development (Lew *et al.*, 1997; Struhl, 1995). It is a budding yeast, which, as the name suggests, implies that the daughter cells bud off from the mother cell following mitosis and cytokinesis. The yeast has a cell cycle remarkably similar to that of humans, albeit a simplified version thereof. As in higher eukaryotes, the *S. cerevisiae* cell cycle consists of S and M phases, separated by G₁ and G₂ gap phases. A checkpoint is found in late G₁, the so-called Start point, which serves as a key regulatory point for determination of cell fate. Sufficiently-nourished cells commit themselves to completion of the cell cycle, while under-nourished cells are able to either withdraw from the cell cycle to enter quiescence, or sporulate. Additionally, cells subjected

to pheromones are able to begin the mating program. Transition of Start is accompanied by DNA duplication and bud formation, allowing easy identification of G₁/S transition. Transition of G₂/M, however, is less clear-cut, but can be determined by the assembly of a bipolar microtubule spindle (Lew *et al.*, 1997, and references therein).

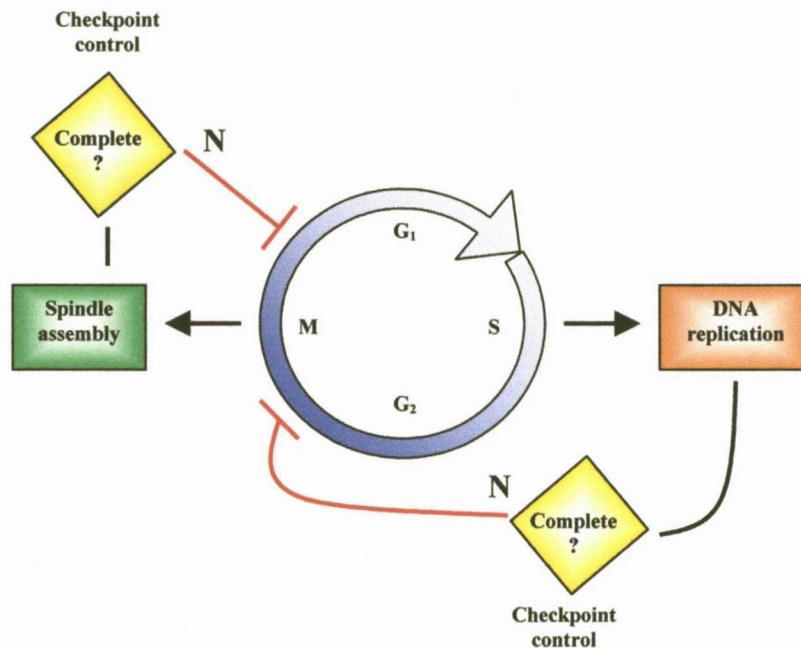


Figure 8. The *Saccharomyces cerevisiae* cell cycle is related to that of higher eukaryotes. The cell cycle is regulated by the cell cycle clock, which is itself regulated by Cdc28 and various cyclins, and several checkpoint controls, which are responsible for surveillance of, amongst others, DNA replication and spindle assembly (Lew *et al.*, 1997).

The principal effector of the yeast cell cycle is the cyclin-dependent kinase Cdc28. Unlike higher eukaryotes, where several CDKs are required, Cdc28 is almost solely responsible for passage through the cell cycle (Lew *et al.*, 1997). Pho85p, Kin28p, Srb10p and Ctk1p play important roles as well but will not be discussed here. The interested reader is referred to the review by Andrews and Measday (1998) for a more detailed discussion. Two groups of cyclins, namely Cln1-3 and Clb1-6 are necessary for activation of this kinase at appropriate times in the cell cycle. Cln1 and Cln2 are active at the G₁/S transition, while Cln3 appears to be constitutively present throughout the cell cycle, with an activity peak at the M/G₂ transition (Andrews and Measday, 1998; Lew *et al.*, 1997). Clb5 and Clb6 are active in S, Clb3 and Clb4 in G₂/M, and Clb1 and Clb2 also in G₂/M, albeit with a activity peak later than Clb5 and Clb6. The various cyclins appear to be functionally paired, except for Cln3, and it has been suggested that this is due to distinct activities in meiosis and mitosis of paired cyclins. These groups of cyclins are individually responsible for specific

cell cycle events. Additionally, the presence of functionally distinct groups of cyclins relegates each cell cycle event to a maximum frequency of once per cycle. Cdc28 is regulated through interactions with a variety of effectors, both positive and negative regulators, in addition to covalent modification by phosphorylation (Lew *et al.*, 1997; Struhl, 1995).

In addition to the cell cycle clock, several checkpoints are present that ensure the completion of specific cell cycle events. Checkpoint controls are signaling pathways that act to delay cell cycle progression when perturbations delay completion of certain cell cycle events. Checkpoints exist that monitor the completion of DNA replication and spindle assembly, in addition to the DNA damage checkpoints, which, as the name suggests, monitors DNA integrity. Each of these checkpoint is regulated by a key protein(s), which, when mutated, leads to aberrant progression of the cell cycle past the checkpoint (Lew *et al.*, 1997, and references therein).

13.2 Duplication of the *S. cerevisiae* Genome

DNA replication is initiated during late G₁, and involves numerous proteins and complexes. The ORC, or origin replication complex, is a complex of six proteins that occupies the replication origin throughout the cell cycle, while additional proteins complex with the ORC late in G₁ to initiate replication. Initiation additionally involves Cdc6p and the MCM group of proteins. These MCM proteins are excluded from the nucleus following initiation, and only re-enter after mitosis. Cdc7p and Dbf4p form a regulatory complex that is essential for replication initiation. The genes required for duplication of the yeast genome are cell cycle regulated, although it appears that this regulation is not essential (Lew *et al.*, 1997; Struhl, 1995).

13.3 Keeping the Cell Cycle on Track

The cell cycle is regulated by the so-called cell cycle clock, which comprises an array of proteins, termed CDKs. As in humans (and other eukaryotes), these CDKs are themselves regulated through complex mechanisms involving covalent modification and association with positive and negative regulators. CDKs serve to regulate target proteins through phosphorylation at appropriate times. The effect of this mechanism of cell cycle regulation is that the essential events that make up the cell cycle take place under controlled conditions, and in the correct sequence. Consequently, timing of essential events, such as

DNA replication, chromosome segregation and cytokinesis, is controlled. Additionally, several checkpoints are present in the yeast (and all other eukaryotes) cell cycle that ensure proper cycling. These checkpoint controls ensure that a specific event is complete before the next event is begun. Together, the cell cycle clock and the checkpoint controls are responsible for the efficient passage of the cell through the cell cycle (Lew *et al.*, 1997, and references therein).

13.4 Expression of Human Cell Cycle Proteins in Yeast

S. cerevisiae has been used as a host organism for analysis of proteins important to the cell cycle. The interaction of E2F1 and DP1 has been reproduced in yeast, using a two-hybrid system, to evaluate regions necessary for this interaction (Vidal *et al.*, 1996). This type of assay has the advantage of being based upon functional attributes of the proteins, thereby giving a direct measure of the efficacy of binding of various mutants to one another. p53 is one of the principal proteins in the development and pathogenesis of cancer, and it too has been examined in yeast-based models. Mutations induced by exposure to UV radiation were studied in yeast via a simple, colour-dependent method of mutation selection and identification (Moshinsky and Wogan, 1997). Furthermore, yeast has been used as the host organism for studies intent on identifying mutation hot spots in the *p53* gene (Brachmann *et al.*, 1996).

The retinoblastoma protein has been described in detail above, and its importance in regulation of the cell cycle is unquestionable. The retinoblastoma protein, once expressed in *S. cerevisiae*, can be phosphorylated by the yeast's own cyclin/CDK complexes. In *cln* negative mutants, this process can be completed by the ectopic expression of mammalian cyclins. The presence of the Rb pocket region is required for this yeast-based hyperphosphorylation, and can be abrogated by the presence of a negative growth factor (Hatakeyama *et al.*, 1994). A novel protein, termed p180 in *S. cerevisiae* and p200 in *Schizosaccharomyces pombe*, is present in these yeasts, that binds retinoblastoma control elements, the DNA sequence recognized by Rb (Cuevo *et al.*, 1997). p180 requires zinc for DNA binding, but does not require MCB, SCB or E2F sites. Furthermore, p180 is regulated in *SWI6*-dependent manner and the peak of transcription mediated by RCEs is in early- to mid-S phase. p180 could possibly, similarly to Rb, mediate the onset or progression of DNA replication (Cuevo *et al.*, 1997).

CHAPTER THREE

AIMS AND EXPERIMENTAL STRATEGY

Rb and E2F1 are clearly two of the most important proteins in the human cell, with respect to the cell cycle. Rb is of particular importance; it is the prototype tumour suppressor protein, and supporting this capacity, has been associated in mutated form with the genesis and progression of numerous neoplastic malignancies (refer to chapter one). Recent research has demonstrated that the interaction of Rb and E2F (E2F is the generic term associated with the E2F family of proteins) is of paramount importance for efficient passage through the cell cycle, and that it is this functional interaction that forms the basis for the suppressive properties of Rb (Brehm and Kouzarides, 1999). Disruption of this interaction through mutation of Rb leads to aberrations in the organized flow through the cell cycle.

The need for a simple functional assay of Rb activity is raised with each new report of cancerous malignancy, and it is this need that is addressed in this body of work. Yeast has served as a host organism for expression of numerous human proteins. Indeed, *S. cerevisiae* has previously been used to develop a functional assay for p53, the most prolific tumour suppressor (Flaman *et al.*, 1995). Manipulation of yeast is relatively simple, negating laborious procedures associated with mammalian studies. In this work, the ability of yeast to express mammalian proteins is taken advantage of to develop an assay for Rb functional activity, utilizing the interaction of Rb with the transcription factor E2F1.

To achieve this, strains of *S. cerevisiae* W303-1A will be constructed that contain a reporter gene, the *lac Z* gene of *E. coli*, which is regulated by E2F1 (figure 1). Regulation is facilitated by inclusion of an E2F recognition element (RE) in the *CyC1* promoter. The E2F RE is composed of synthetic oligonucleotides based on the known RE sequence, TTTTCGCGC. This will be incorporated into pJS205BXX, upstream of the complete *lac Z* gene. The E2F RE-*lac Z* construct will subsequently be cut from pJS205BXX and cloned into pRS405. This plasmid is void of yeast episomal replication origins, and is thus incapable of free replication. pRS405 is an integrating plasmid that contains the *S. cerevisiae* *LEU2* gene, which allows reconstitution of the genomic *LEU2* gene, for which

W303-1A is a mutant, by recombination with the plasmid, simultaneously integrating the E2F RE-*lac Z* construct into the yeast genome.

Expression vectors for both Rb and E2F1, the E2F protein studied in this work, are required, and the strategy for construction of these plasmids is identical (figure 1). Complete cDNAs of both *RBI* and *E2F1* are to be amplified from a commercially-available cDNA library. Restriction enzyme recognition sites incorporated into the PCR primers will facilitate cloning of the *RBI* and *E2F1* cDNAs into pYES2 and pYATAG200, respectively. These expression vectors will allow regulated expression of the Rb and E2F1 proteins, as pYES2 contains a *GALI* promoter downstream of the cloning site and pYATAG200 contains a *CUPI* promoter, similarly located.

Using the chromogenic substrate X-gal it will be possible to determine whether E2F1 is capable of binding the E2F RE incorporated into the yeast genome to activate transcription of the reporter gene, and whether the induced Rb protein is able to abrogate the interaction of E2F1 and the E2F RE (see figure 2). The *lac Z* gene encodes the bacterial enzyme β -galactosidase, which is capable of cleaving the colourless X-gal substrate to produce a blue-coloured product. This allows for a simple assay of E2F1 binding, characterized by blue colonies of transformed yeast cells present on solid medium containing X-gal. Rb functional activity can be analyzed identically, since abrogation of the interaction of E2F1 and the E2F RE by the binding of Rb to E2F1 will be characterized by an inability of E2F1 to up-regulate *lac Z* from the E2F RE binding site, and hence prevent the degradation of X-gal, leaving the transformed cells with their natural off-white colour.

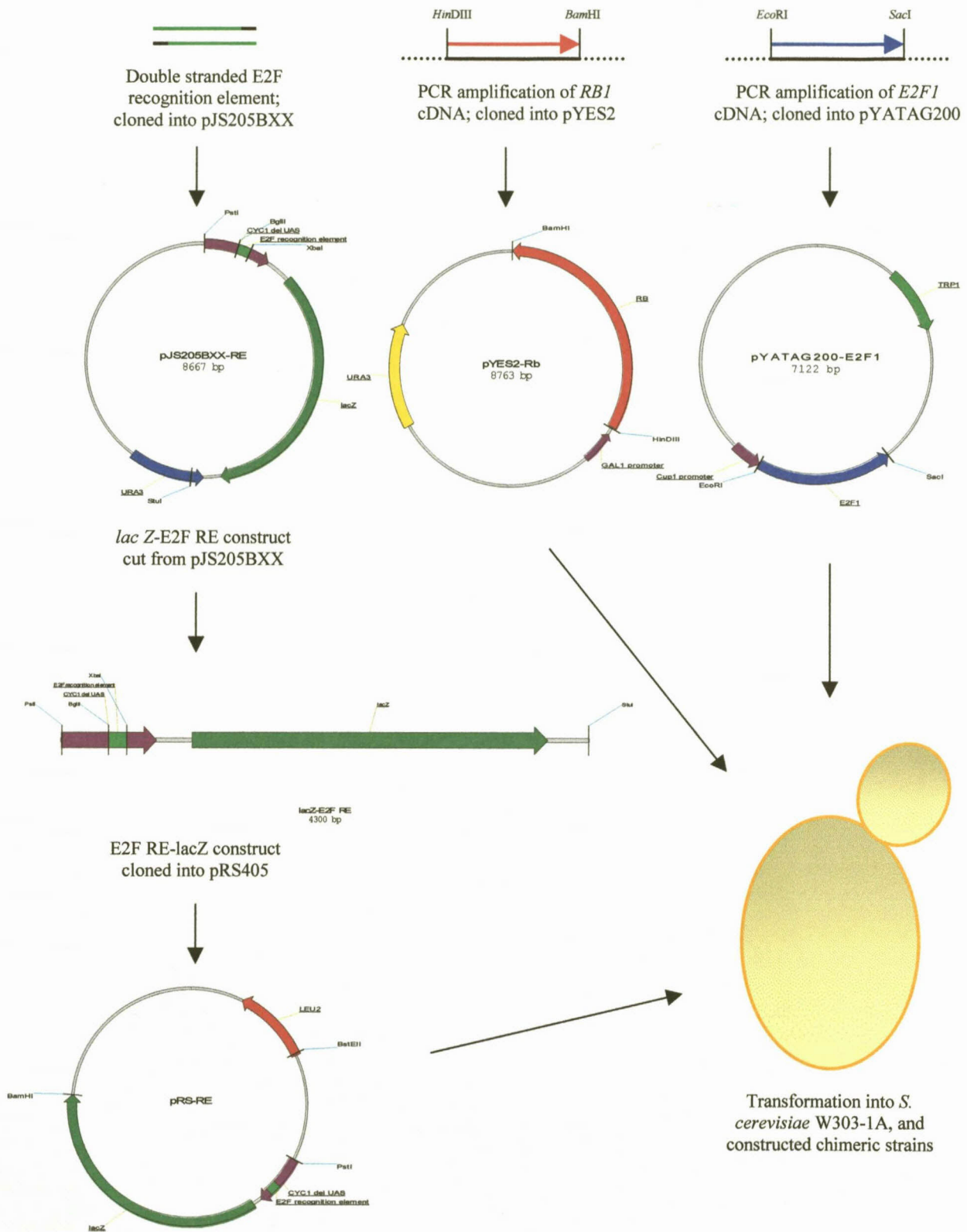


Figure 1. Strategy for development of a functional assay for Rb activity. Yeast expression vectors of Rb and E2F1 will be constructed using a PCR-based cloning procedure. A yeast strain containing an integrated *lacZ* gene regulated by an upstream E2F RE will be constructed, and consequently serve as the host for ectopic protein expression.

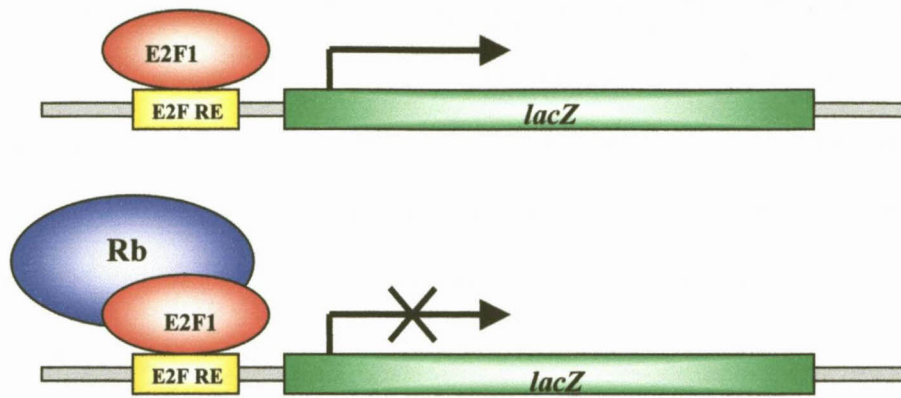


Figure 2. Functional assay for Rb activity. In the absence of Rb, E2F1 binds the genome-borne E2F RE, activating transcription of the downstream *lac Z* gene, leading to β -galactosidase production, X-gal cleavage, and ultimately blue-coloured colonies. In the presence of Rb, binding of E2F1 abrogates the activation of *lac Z*, and the colonies retain their natural colour. (Refer to text for further details).

CHAPTER FOUR

MATERIALS AND METHODS

1. Reagents

Reagents were purchased from Merck, Difco and Sigma. Molecular grade reagents were used where possible. Alternatively, the highest grade available was used. Deionized H₂O was used exclusively.

2. Media and Growth Conditions

E. coli cultures were grown in either Luria-Bertani (LB) medium or Terrific Broth (TB) at 37°C (Sambrook *et al.*, 1989). *S. cerevisiae* cultures were grown in YPD medium (1% (w/v) (unless noted, all percentages are expressed as w/v) yeast extract, 2% peptone 2% glucose) at 30°C. For yeast selection, synthetic complete (SC) medium (0.67% yeast nitrogen base, 10 µg/ml adenine, 20 µg/ml each arginine, histidine, lysine, methionine, tryptophan, uracil, 30 µg/ml each isoleucine, leucine, serine, threonine, tyrosine, valine, and 50 µg/ml phenylalanine), supplemented with 2% glucose (Glc or D), and lacking the appropriate component for selection, was used (referred to as SCD drop-out medium). 2% galactose (Gal) was used as carbon source for pYES2 induction. For E2F1 induction, CuSO₄ was included in the drop-out medium at final concentrations of 1 mM and 2.5 mM (Hottiger *et al.*, 1995). Blue-white colony selection was facilitated by the inclusion of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactose) to a final concentration of 0.2 mg per ml. Where X-gal was included the medium was buffered with 17 mM KH₂PO₄ 72 mM K₂HPO₄. All media were supplemented with 1.5% agar (2% agar for yeast media) where solid media was required.

3. Microorganisms

All plasmid construction was performed in *E. coli* strain JM105 (F' *traD36 lacI^q Δ(lacZ)M15 proA⁺ B⁺/thi rpsL (Str^r) endA sbcB15 sbcC? hsdR4 (r_K⁻m_K⁺) Δ(lac-proAB)*). *S. cerevisiae* strain W303-1A (*MATa leu2-3 /112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2*) served as host organism for protein expression and functional analysis.

4. Plasmids

pYES2 is a yeast expression plasmid used in this study for expressing Rb. The plasmid contains a *URA3* marker for yeast selection, and an ampicillin resistance (Amp^{R}) marker for bacterial selection. Protein expression is induced by galactose from the *GAL4* promoter situated upstream of the polylinker cloning site. pYATAG200 is a yeast expression plasmid used in this study for the production of E2F1 in yeast. It contains a *TRP1* marker for yeast selection, as well as an Amp^{R} marker for bacterial selection. Upstream of the polylinker cloning site is a *CUP-1* promoter, allowing for *CUP-1* promoter-controlled expression of the cloned gene. Protein expression is consequently induced by the addition of copper to the medium in the form of CuSO_4 (cupric sulphate)(Hottiger *et al.*, 1995). pJS205BXX contains the complete *E.coli lacZ* gene with an upstream polylinker site, and an Amp^{R} marker for bacterial selection. This plasmid was used to create an E2F1-regulated *lacZ* gene. pRS405 is a yeast integrating plasmid, used in this study to incorporate the E2F1-regulated *lacZ* gene into the yeast genome (Christianson *et al.*, 1992; Sikorski and Hieter, 1989). The plasmid contains a *LEU2* marker for yeast selection and a downstream polylinker cloning site. (Please refer to figure 1 for vector maps of plasmids used).

5. General Laboratory Procedures

Unless described, all standard techniques and procedures used were performed as put forth in such general molecular biology texts as Ausubel *et al.* (1995), Sambrook *et al.* (1989), and Spector *et al.* (1998), and the respective manufacturers' protocols. Klenow fragment (Roche) was used for all blunting reactions. All ligations were performed using either a Fast-Link DNA ligation kit (Epicentre) or T4 DNA ligase (Roche). Restriction enzymes were purchased from Promega, Roche, New England Labs, and Gibco BRL. All restriction digests were performed in the manufacturer's suggested buffers, with or without the addition of bovine serum albumin (BSA), and at the manufacturer's recommended temperature. Restriction digests were analyzed by electrophoresis in TBE-buffered agarose gels containing 0.5 μg ethidium bromide per ml.

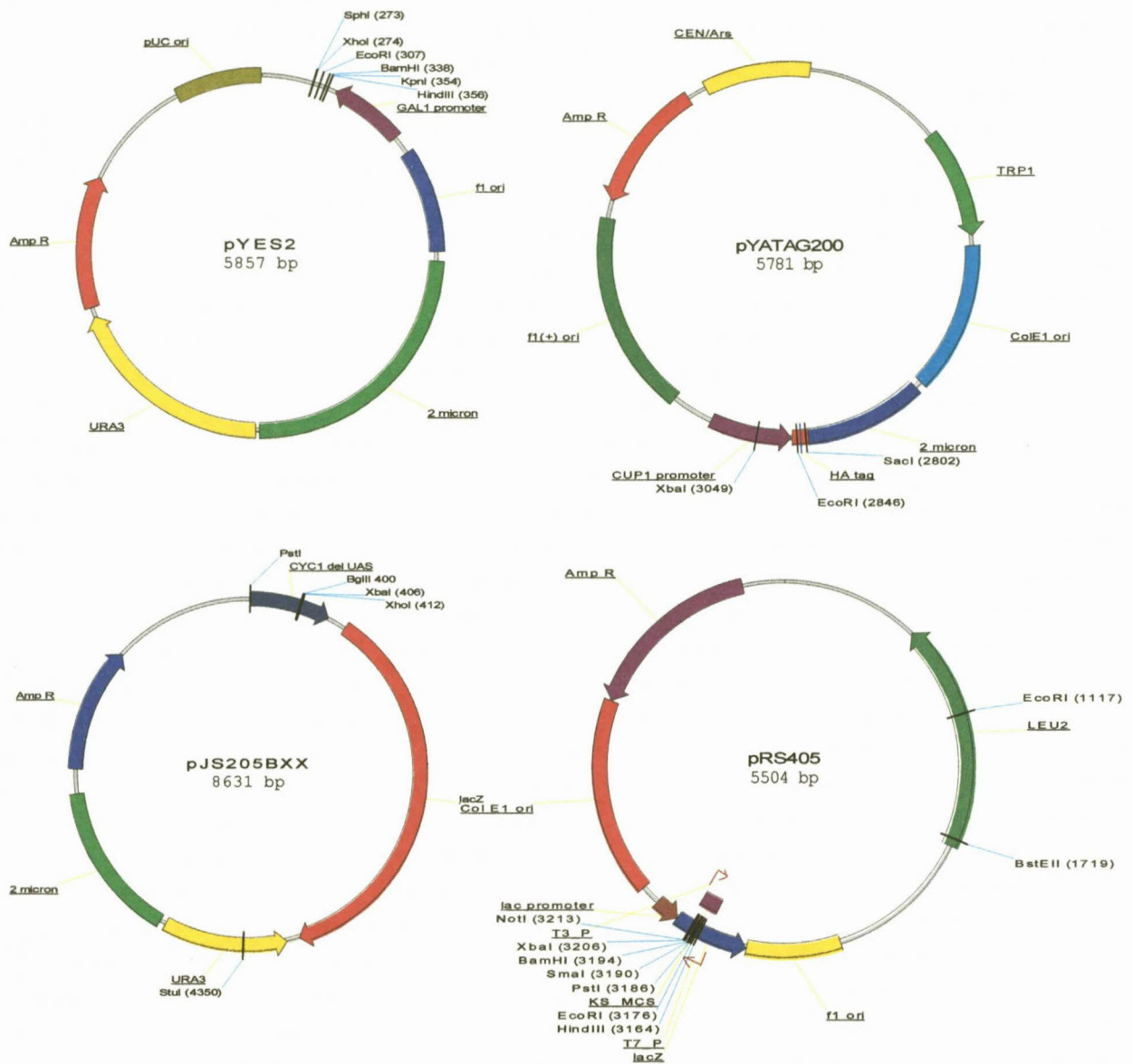


Figure 1. Vector maps of plasmids pYES2, pYATAG200, pJS205BXX, and pRS405. Please refer to text for more details.

6. Polymerase Chain Reaction

PCR was performed on a GeneAmp 2400 thermal cycler (Perkin Elmer). Reaction conditions for each experiment were optimized using appropriate reagent and/or temperature gradients, or through the addition of additives. Primers used for PCR are listed in Table 2. *Taq* polymerase (Roche), Expand Hifi (Roche), AmpliTaq[®] Gold (Perkin Elmer) and RTS Polymerase (Gibco) were used for PCR amplifications together with the manufacturers' recommended PCR buffers. All PCR products were separated by electrophoresis in TBE-buffered agarose gels containing 3.5 μ g ethidium bromide per ml.

The complete *RBI* cDNA was amplified from Superscript cDNA (Gibco), a leukocyte cDNA library. The reaction contained 400 ng Superscript cDNA, 10 pmol each of primers Rb1p and Rb1m, 3 mM MgCl₂, 300 μM dNTP, 6% (v/v) dimethyl sulphoxide (DMSO), and 1.25 units (U) RTS polymerase. An initial denaturation step of 3 min at 95°C, was followed by 35 cycles of 95°C for 30 sec, 66°C for 30 sec and 70°C for 3 min. A final elongation step of 10 min at 70°C was included. Cloned *RBI* was amplified from approximately 100 ng plasmid DNA in a reaction containing 10 pmol each of primers Rb1p and Rb1m, 1.5 mM MgCl₂, 200 μM dNTP, 6% (v/v) DMSO and 1.25 U RTS polymerase, using identical temperature cycling conditions.

The complete *E2F1* cDNA was amplified from 125 ng Superscript cDNA using 10 pmol each of primers E2FpEco and E2FmSac in a reaction containing 1.5 mM MgCl₂, 200 μM dNTP, 10% DMSO and 2.5 U AmpliTaq Gold. An initial 10 min denaturation step at 96°C was followed by 35 cycles of 96°C for 45 sec, 62°C for 15 sec and 72°C for 2 min, and finally a single elongation step of 10 min at 72°C. Cloned *E2F1* was amplified from approximately 100 ng plasmid using 1.25 U AmpliTaq Gold and identical reagent and temperature cycling conditions.

The *CyC1* promoter on pJS205BXX was amplified using primers pJSUp and pJSDwn. The reaction contained 125 ng pJS, 10 pmol each of pJSUp and pJSDwn, 1.5 mM MgCl₂, 200 μM dNTP, and 1.25 U *Taq* polymerase. An initial denaturation step of 3 min at 95°C was followed by 30 cycles of 95°C for 30 sec, 65°C for 15 sec, and 72°C for 30 sec, and finally an elongation step of 7 min at 72°C.

7. *E. coli* Transformation

Heat shock-competent *E. coli* strain JM105 cells were prepared and transformed according to a standard heat shock protocol (Sambrook *et al.*, 1989). A frozen master stock of *E. coli* strain JM105 was used to inoculate 5 ml LB, which was incubated overnight at 37°C. This culture was used to inoculate 100 ml LB, and grown at 37°C until O.D.₆₀₀ reached 0.95. The cells were pelleted by centrifugation at 2 500 rpm for 10 min (4°C). The pellet was resuspended in 10 ml 50 mM MgCl₂ 80 mM CaCl₂ and incubated on ice for 10 min. The cells were pelleted and this step was repeated. The pellet was resuspended in 5 ml 100 mM CaCl₂ and 1.5 ml 50% Glycerol was added. The heat shock-competent cells were frozen in liquid N₂ in 200 μl aliquots and stored at -70°C.

Table 1. Primers and oligonucleotides used in this study.

Name	Sequence (5'-3')	Target/Application
Rb1p*	GCG AAG CTT AAC GGG AGT CGG GAG AGG AC	<i>RB1</i> PCR, cloning
Rb1m*	CGC GGA TCC CTC ATT TCT CTT CCT TGT TTG AG	<i>RB1</i> PCR, cloning
Rb450 [#]	AAA GAT ACA GAT TCC CCA CAG TTC C	<i>RB1</i> sequencing
Rb1068 [#]	TCC ATT AGA TGT TAC AAG TCC AAG AG	<i>RB1</i> sequencing
Rb1502 [#]	CGG TAA TAC AAG CGA ACT CCA AG	<i>RB1</i> sequencing
Rb2021 [#]	GCA TTT GCA GTA GAA TTT ACA CGC	<i>RB1</i> sequencing
Rb2500 [#]	TTC GAG GAA TGT GAG GTA TTG	<i>RB1</i> sequencing
E2F1pEco [#]	CGG AAT TCC GTG AGC GTC ATG GCC TTG GC	<i>E2F1</i> PCR, cloning, sequencing
E2F1mSac [#]	GGC GAG CTC GCC CTG TCA GAA ATC CAG GG	<i>E2F1</i> PCR, cloning, sequencing
E2Ft1 [#]	GAT CTT TCG CGC	E2F RE1
E2Ft3 [#]	GAT CTT TCG CGC TTT CGC GCT TTC GCG C	E2F RE3
E2Fb1 [#]	CTA GGC GCG AAA	E2F RE1
E2Fb3 [#]	CTA GGC GCG AAA GCG CGA AAG CGC GAA A	E2F RE3
418F [#]	GAA ACT GAC CAT CAG TAC C	<i>E2F1</i> sequencing
681F [#]	GAA GTC CAA GAA CCA CAT CC	<i>E2F1</i> sequencing
1129F [#]	TCA CCA CCA CCA TCA TCT CC	<i>E2F1</i> sequencing
523R [#]	TCT CAT AGC GTG ACT TCT CC	<i>E2F1</i> sequencing
901R [#]	CTG CAA TGC TAC GAA GGT CC	<i>E2F1</i> sequencing
1170R [#]	ATC TGT GGT GAG GGA TGA GG	<i>E2F1</i> sequencing
pJSUp [#]	CCG ACA ACA ATG AGT TTA CCG	pJS205BXX promoter PCR
pJSDn [#]	TCA GCA CTA AAG TTG CCT GGC	pJS205BXX promoter PCR

*: supplied by Roche

#: supplied by Gibco BRL

E2F RE: E2F recognition element (TTTCGCGC)

RB1, *E2F1*, and *DP1* refer to the respective cDNAs

Frozen competent cells were thawed on ice before the addition of plasmid DNA. The cells were incubated on ice for 30 min, followed by a heat shock of 5 min at 37°C. 800 µl LB was added and the cells were allowed to recover at 37°C for 35 min. An appropriate volume of recovered cells was plated on LB plates supplemented with 10 µg ampicillin per ml and incubated at 37°C for 16 hr.

8. *S. cerevisiae* Transformation

The examined strains of *S. cerevisiae* were transformed according to the method outlined by Gietz and Schiestl (1998). Transformed cells were plated on the appropriate SC drop-out medium, and incubated at 30°C for 2-5 days.

9. Plasmid Isolation from *E. coli*

Plasmids were isolated from *E. coli* transformants via an alkaline lysis procedure (Ausubel *et al.*, 1995). RNA was degraded by the addition of 10 µg RNase A (Sigma) per ml and incubation at 37°C for 25 min. Plasmids required for sequencing analysis were additionally purified by incorporating an additional PEG precipitation step. 0.1 Vol 4 M NaCl and 1 vol PEG₆₀₀₀ were added to the resuspended plasmid prep. This mixture was incubated on ice for 20 min, and the plasmid DNA was pelleted by centrifugation at 14 000 rpm (4°C) for 15 min. The plasmid DNA was washed with 70% EtOH and resuspended in H₂O.

10. Sequencing Analysis

Plasmids were sequenced using either Thermosequenase II (Amersham) or DYEnamic ET Terminator (Amersham) sequencing kits according to the manufacturer's protocols. 600-800 ng plasmid DNA and 5 pmol primer were used in all reactions. In the case of pYES2-Rb and pYATAG200-E2F1, DMSO was included in the reactions at concentrations of 6 and 10%, respectively. PCR was performed on a GeneAmp 2400 thermal cycler. Identical cycling conditions were used throughout. An initial denaturation step of 3 min at 95°C was followed by 25 cycles of 95°C for 45 sec and 60°C for 1 min. Samples were analyzed on an ABI Prism 377 automated sequencer (Perkin Elmer). Specific primers used in the sequencing reactions are listed in Table 2. Sequencing data was analyzed using Sequencing Analysis, Factura and Sequence Navigator programs (Perkin Elmer).

11. Rb Expression Vector Construction

The complete *RBI* cDNA was amplified and cloned into the pYES yeast expression vector. *RBI* cDNA was amplified as outlined above. The PCR product was cleaned with a Qiaex II DNA purification kit (Qiagen). Rb1p and Rb1m contain *Hind*III and *Bam*HI sites, respectively. The *RBI* PCR product and the pYES vector were cut overnight with both 20 U *Hind*III (Roche) and 20 U *Bam*HI (Roche), simultaneously, at 37°C. The digests were purified with a Qiaex II DNA purification kit (Qiagen). 1 µg digested *RBI* and 100 ng digested pYES were ligated with 2 U T4 DNA ligase (Roche) at 4°C overnight. 1/10th of the ligase reaction was used to transform 200 µl competent JM105 *E. coli*. Positive transformants were identified by amplifying *RBI* from 100 ng of the resultant plasmid isolates as described above. Positive transformants were sequenced to confirm the presence

of the complete *Rb1* cDNA. Primers Rb1p, Rb1m, Rb450, Rb1068, Rb1502, Rb2021 and Rb2500 were used to sequence the entire cDNA as outlined above.

12. E2F1 Expression Vector Construction

The complete *E2F1* cDNA was amplified from a Superscript human leukocyte cDNA library (Gibco) and cloned into pYATAG200, a yeast expression plasmid. The *E2F1* cDNA was amplified as described above. The PCR product was cleaned using a High Pure PCR product purification kit (Roche). (Unless noted, all post-amplification and post-digestion purification was performed using this method). The primers, E2F1pEco and E2F1mSac, contain recognition sites for *EcoRI* and *SacI*, respectively. Both the *E2F1* PCR product and pYATAG200 were cut with 30 U *EcoRI* (Gibco) for 16 hr. The reactions were cleaned, then cut with 30 U *SacI* (Roche) for 16 hr. The reactions were cleaned, and 100 ng cut pYATAG200 and 50 ng *E2F1* were ligated using a Fast-Link DNA ligation kit (Epicentre). 1/5th of the ligation reaction was used to transform 200 μ l competent JM105 *E. coli*. Positive transformants were identified by amplifying *E2F1* from 100 ng of the resultant plasmid isolates as described above. Positive transformants were sequenced to confirm the presence of the complete *E2F1* cDNA. Primers E2F1pEco, E2F1mSac, 418F, 681F, 1129F, 523R, 901R and 1170R were used to sequence the entire *E2F1* cDNA as described above.

13. Construction of E2F1-Reporter Strains

The *lacZ* gene, plasmid-borne on pJS205BXX, was placed under the control of E2F1 by incorporating the E2F recognition element (RE), TTTCGCGC, into the upstream *CyC1* promoter region. The E2F-regulated *lacZ* construct (RE-*lacZ*) was then inserted into pRS405, a yeast integrating vector. The E2F-regulated *lacZ* construct was finally integrated into the genome of *S. cerevisiae* strain W303-1A. Oligonucleotides E2Ft1, E2Ft3, E2Fb1 and E2Fb3, containing the E2F recognition element, were designed. E2Ft1 and E2Fb1, and E2Ft3 and E2Fb3 were annealed to give double stranded E2F RE1 and E2F RE3, respectively. E2F RE1 contains one copy of the RE, while E2F RE3 contains 3 tandem repeats of the E2F RE. 1 μ g E2Ft1 and 1 μ g E2Fb1 were boiled for 5 min in 10 mM Tris HCl 5 mM MgCl₂ 100 mM NaCl 1 mM mercaptoethanol pH 8 (SuRE Cut buffer B, Roche) and then allowed to cool slowly to room temperature. This was repeated for E2Ft3 and E2Fb3 to yield E2F RE3. These constructs both contain 5'-overhangs, which

were subsequently blunted with Klenow enzyme. 200 ng E2F RE1 and 200 ng E2F RE3 were incubated at room temperature in the presence of 1 U Klenow enzyme (Roche), 20 μ M dNTP, buffered with 50 mM Tris HCl 10 mM MgCl₂ 100 mM NaCl 1 mM dithiothreitol pH 7.5 (SuRE Cut buffer H, Roche). pJS205BXX was cut with 24 U *Xba*I (Promega) for 8 hr at 37°C, followed by heat inactivation of the enzyme for 20 min at 65°C. Digested pJS205BXX was redigested with 20 U *Bgl*II (Roche) for 12 hr at 37 °C. The reaction was cleaned, and 1 μ g double-digested pJS205BXX was blunted at room temperature with 1 U Klenow enzyme as described above. 100 ng blunted pJS205BXX and 2 ng blunted E2F RE1, and 100 ng blunted pJS205BXX and 3 ng blunted E2F RE3 were ligated with using a Fast-Link DNA ligation kit as described above. 1/5th of each ligation reaction was used to transform 200 μ l competent JM105 *E. coli*. Positive transformants were identified by PCR amplification of the *CyC1* promoter. 100 ng plasmid DNA was used to amplify the *CyC1* promoter as described above. The sequence and orientation of each insert was confirmed by sequencing of the promoter region. Primer pJSUp was used to sequence the *CyC1* promoter as described above.

The complete *lacZ* gene and *CyC1* promoter region, containing E2F RE, was cut out of pJS205BXX-RE and inserted into pRS405. (pJS205BXX-RE refers to the E2F RE-containing plasmid constructs created above). 10 μ g pJS205BXX-RE was digested with 10 U *Stu*I (Amersham) for 16 hr at 25°C. The reaction was cleaned, redigested for 16 hr with 10 U *Pst*I (Roche) at 37°C, and cleaned again. 10 μ g pRS405 was digested with 10 U *Bam*HI (Roche) at 37°C for 16 hr, followed by heat inactivation of the restriction enzyme at 65°C for 20 min. The 5'-overhangs produced were blunted with 1 U Klenow enzyme in buffer B as described above. The blunted pRS405 was then cleaned, and redigested with 10 U *Pst*I (Roche) for 16 hr at 37°C. The reaction was heat inactivated at 65°C for 20 min and then cleaned. 100 ng pJS205BXX-RE and 100 ng pRS405 were ligated using a Fast-Link DNA ligation kit as described above. 1/5th of the ligation reaction was used to transform competent *E. coli*. Positive transformants were identified by PCR amplification of the *CyC1* promoter from 100 ng plasmid DNA, as described above, and restriction analysis of plasmid minipreps; 1 μ g of pRS405-RE-lacZ (pRS-RE) was digested with 5 U *Not*I (Promega) in a reaction containing 0.1 mg/ml BSA (bovine serum albumin).

The E2F1-regulated *lacZ* was integrated into the genome of W303-1A by transformation of the yeast with the linearized pRS-RE plasmid. 10 μ g pRS-RE was digested with 5 U *Bst*EII

for 6 hr at 60°C. The restriction product was cleaned and used to transform W303-1A. 1 µg linear and 1 µg circular pRS-RE were used, separately, to transform *S. cerevisiae* W303-1A as described above. Transformants were plated on -leucine SCD plates. Integration of the E2F RE-lacZ construct into transformants was confirmed by PCR amplification of the *CyC1* promoter from a crude DNA extract. A crude DNA extract was prepared by resuspending a single yeast colony in 10 µl H₂O. This was boiled for 10 min, cellular debris was pelleted by centrifugation, and 9 µl was used to amplify the *CyC1* promoter, as described previously.

14. E2F1 Expression in W-RE strains

Strains of W303-1A containing the integrated RE-lacZ construct, referred to as W-RE, were transformed with pYATAG200-E2F1. 4 µg pYATAG200-E2F1 was used to transform W303-1A and the constructed W-RE strains as described above. Transformed W-RE strains were split and plated on four media; 1) -tryptophan SCD, 2) -leucine -tryptophan X-gal SCD, 3) -leucine -tryptophan X-gal 1 mM CuSO₄ SCD, 4) -leucine -tryptophan X-gal 2.5 mM CuSO₄ SCD. Alternatively, transformants obtained on -tryptophan SCD plates were streaked out on: 1) -tryptophan SCD, 2) -leucine -tryptophan X-gal SCD, 3) -leucine -tryptophan X-gal 1 mM CuSO₄ SCD, 4) -leucine -tryptophan X-gal 2.5 mM CuSO₄ SCD.

15. Rb Expression in W-RE Strains

The constructed W-RE strains were used as host organisms for Rb expression, facilitated by pYES2-Rb. 4 µg pYES2-Rb was used to transform W303-1A and the constructed W-RE strains as described above. Transformed strains were plated on -uracil SCD. Transformants were subsequently picked and streaked out on: 1) -uracil SCD, 2) -uracil SCGal, 3) -uracil SCD + X-gal, 4) -uracil SCGal + X-Gal.

16. Flow Cytometric Analysis of the Effect of Rb Expression on the Yeast Cell Cycle

Various strains containing the pYES2-Rb plasmid were analyzed by flow cytometry following induction of Rb expression. W303-1A, and the W-RE strains, all transformed with pYES2-Rb, were used to inoculate 12 ml -uracil SCD media. Cultures were grown

overnight to mid-log phase. 1 ml samples ($\sim 10^7$ cells) were obtained; these would serve as time zero samples. The remaining cell cultures were split, pelleted and resuspended in -uracil SCD and -uracil SCGal respectively. Further samples were procured at 3 hr, 6 hr, and 22 hr. All samples were pelleted immediately, resuspended in 70% EtOH, and incubated at room temperature for 30 min.

Samples were subsequently stained with propidium iodide. The cells were washed with 50 mM Na-cit (pH 7.0) (sodium citrate), pelleted and resuspended in 50 mM Na-cit again. RNase A was added to a final concentration of 20 $\mu\text{g/ml}$ and incubated at 37°C for 2 hr. The cells were washed with 50 mM Na-cit, and resuspended in 50 mM Na-cit 10 mM NaCl 0.1% Nonidet P-40. Propidium iodide was added to a final concentration of 100 $\mu\text{g/ml}$ and the cells were incubated at room temperature for 30 min. The cells were pelleted and resuspended in 50 mM Na-cit. Propidium iodide was added to a final concentration of 50 $\mu\text{g/ml}$ and the cells were kept on ice until analysis. Flow cytometry was performed on a FACSCalibur flow cytometer (Becton Dickinson).

CHAPTER FIVE

RESULTS AND DISCUSSION

1. Introduction

The interaction of the tumour suppressor protein Rb and the transcription factor E2F1 is well characterized. This interaction is particularly important for the cessation of growth that precedes transition of the G₁/S boundary of the cell cycle, and aberrant Rb has been found concomitant with the deregulated transition of this checkpoint associated with neoplastic disorders (refer to chapter one for more details). There exists a need for a simple assay that permits determination of the functional activity of Rb, as Rb integrity can be correlated with diagnosis and prognosis of disease. This study is primarily focused on development of such an assay, using the yeast *Saccharomyces cerevisiae* as host organism.

2. Construction of pAW1, an Rb Expression Vector

The *RBI* gene occupies 180 kb of DNA on the long arm of chromosome 13. The resultant mRNA produced from this gene is a more-manageable 4.7 kb, but this number is further reduced due to the presence of a long non-coding 3' sequence. Rb is a protein of 928 amino acid residues, which implies a complete cDNA of 2784 bp (Lee *et al.*, 1987). The sequence of the cDNA has been reported previously (GenBank accession no. M15400), and was used to design primers, both for cloning of this complete cDNA, and subsequent sequencing of the product. Only the open reading frame was cloned as pYES2 contains a terminator containing a poly-A tail. Primers Rb1p and Rb1m were designed, including terminal *Hind*III and *Bam*HI sites, respectively, which would facilitate cloning. The complete cDNA was amplified from a Superscript human cDNA library. This library is derived from freshly harvested leukocytes from more than 2 individuals (Gibco-BRL, personal communication).

Optimization of the PCR required the inclusion of DMSO. This organic reagent assists in strand separation during PCR cycling, and is essential for amplification of the gene (Bookstein *et al.*, 1990; Hung *et al.*, 1990). No amplification of *RBI* (this implies the *RBI* cDNA, not the complete gene) was found in the absence of DMSO (figure 1A). Additionally, not all commercially available polymerases were equally effective in

amplification of *RBI* (results not shown). Optimized PCR conditions resulted in a product of 2906 bp, identical to the expected product size calculated from the known sequence (figure 1A).

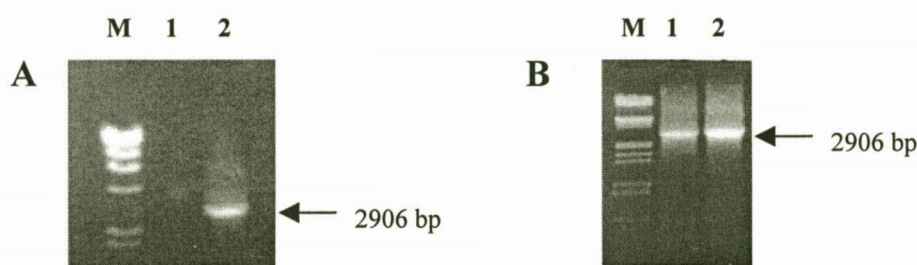


Figure 1. PCR amplification of *RBI*. **A.** The complete *RBI* cDNA was amplified from 400 ng Superscript cDNA library, in the absence (lane 1) or presence (lane 2) of 6% DMSO as described. Lane M, Marker II (Roche). **B.** *RBI* was amplified from 125 ng plasmid DNA as described. Lanes 1 and 2 are two distinct isolates; lane M, Marker III (Roche).

The *RBI* PCR product and plasmid pYES2 were digested with *Hind*III and *Bam*HI, ligated together, and subsequently used to transform competent JM105 *E. coli* cells. Positive transformants were identified by PCR amplification of *RBI* from plasmid isolates (figure 1B).

Primers Rb450, Rb1068, Rb1502, Rb2021, and Rb2500 were designed, based on the known *RBI* sequence, and, together with Rb1p and Rb1m, were used to completely sequence the cloned *RBI* cDNA (figure 2). A base insertion mutation was found upstream of the start codon, which consequently has no effect on the Rb protein sequence (figure 3). Several base change mutations were encountered throughout the sequence of the cDNA. In fact, the frequency of mutated bases leads to the speculation that perhaps these are not all derived from polymerase dysfunction. The error frequency of modern polymerase enzymes is significantly lower than is suggested by the frequency of mutations in the cloned *RBI* cDNA. It is possible that the cDNA library contains aberrant copies of the *RBI* cDNA, as the library is derived from fresh leukocytes harvested from several individuals (Gibco-BRL, personal communication), thereby making PCR amplification of the wildtype cDNA increasingly difficult. Direct sequencing of *RBI* amplification products could possibly have been attempted, but since this would give an average of the possible *RBI* variants present it was not attempted. The effect of the base changes on the protein sequence is indicated in figure 3. Interestingly, 43% (6/14) of the mutations did not result in an amino acid change. A second clone was sequenced, but this was abandoned after a deletion mutation of 128 bp between bases 275 and 403 was discovered. This precise mutation was

additionally found in a third clone sequenced (result not shown), lending weight to the theory of multiple versions of the cDNA in the library.

The cloned *RB1* is polymorphic with respect to the published sequence, possibly due to either mutations or naturally occurring innocuous polymorphisms. The resultant protein is full length and contains 8 amino acid changes, the effects of which are as yet undetermined.

*Hind*III

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1 AGCTGTAATA CGACTCACTA TAGGGAATAT TAAGCTTAAC GGGAGTCGGG AGAGGACTGG
61 GCGTGCCCGG ACGTGCGCGC GCGTCGTCTT CCCC GGCGCT CCTCCACAGC TCGCTGGCTC
121 CCGCCGCGGA AAGGCGTCAT GCCGCCAAA ACCCCCGGAA AAACGGCCGC CACCCGCCGC
181 GCTGCCGCCG CGGAACCCCC GGCACCCCGC CCGCCGCCCC CTCCTGAGGA GGACCCAGAG
241 CAGACAGCGG GCCCGGAGGA CCTGCCTCTC GTCAGGCTTG AGTTTGAAGA AACAGAAGAA
301 CCTGATTTTA CTGCATTATG TCAGAAATTA AAGATACCAG ATCATGTCTG AGAGAGAGCT
361 TGGTTAACTT GGGAGAAAGT TTCATCTGTG GATGGAGTAT TGGGAGGTTA TATTCAAAAG
421 AAAAAGGAAC TGTGGGGAAT CTGTATCTTT ATTGCAGCAG TTGACCTAGA TGAGATGTCTG
481 TTCACCTTAC TGAGCTACAG AAAAACATAC GAAATCAGTG TCCATAAATT CTTTAACTTA
541 CTA AAAAGAAA TTGATACCAG TACCAAAGTT GATAATGCTA TGTCAGACT GTTGAAGAAG
601 TATGATGTAT TGTTTGCAC TCTCAGCAA TTGGAAAGGA CATGTGAACT TATATATTTG
661 ACACAACCCA GCAGTTTCGAT ATCTACTGAA ATAAATTCTA CATTGGTGCT AAAAGTTTCT
721 TGGATCACAT TTTTATTAGC TAAAGGGGAA GTATTACAAA TGGAAGATGA TCTGGTGATT
781 TCATTTCACT TAATGCTATG TGTCCTTGAC TATTTTATTA AACTCTCACC TCCCATGTTG
841 CTCAAAGAAC CATATAAAAC AGCTGTTATA CCCATTAATG GTTCACCTCG AACACCCAGG
901 CGAGGTCAGA ACAGGAGTGC ACGGATAGCA AAACAAC TAG AAAATGATAC AAGAATTATT
961 GAAGTTCTCT GTAAAGAACA TGAATGTAAT ATAGATGAGG TGAAAAATGT TTATTTCAA
1021 AATTTTATAC CTTTATGAA TTCTCTTGGG CTTGTAAACAT CTAATGGACT CCCAGAGGTT
1081 GAAAATCTTT CTAACGATA CGAAGAAATT TATCTTAAAA ATAAAGATTT AGATGCAAGA
1141 TTATTTTGGG ATCATGATAA AACTCTTCAG ACTGATTCTA TAGACAGTTT TGAGACACAG
1201 AGAACACCAC GAAAAAGTAA CCTTGATGAA GGGGTGAATG TAATTCCTCC ACACACTCCA
1261 GTTAGGACTG TTATGAACAC TATCCAACAA TTAATGATGA TTTTAAATTC AGCAAGTGAT
1321 CAACCTTCAG AAAATCTGAT TTCCTATTTT AACAACTGCA CAGTGAATCC AAAAGAAAGT
1381 ATACTGAAAA GAGTGAAGGA TATAGGATAC ATCTTTAAAG AGAAATTTGC TAAAGCTGTG
1441 GGACAGTGTG GTGTGCGAAT TGGATCACAG CGATACAAAC TTGGAGTTCG CTTGTATTAC
1501 CGAGTAATGG AATCCATGCT TAAATCAGAA GAAGAACGAT TATCCATTCA AAATTTTAGC
1561 AAACCTCTGA ATGACAACAT TTTTCATATG TCTTTATTGG CGTGCCTCTC TGAGGTTGTA
1621 ATGCCACAT ATAGCAGAAG TACATCTCAG AATCTTGATT CTGGAACAGA TTTGTCTTTC
1681 CCATGGATTC TGAATGTGCT TAATTTAAAA GCCTTTGATT TTTACAAAGT GATCGAAAGT
1741 TTTATCAAAG CAGAAGGCAA CTTGACAAGA GAAATGATA AACATTTAGA ACGATGTGAA
1801 CATCGAATCA TGGAAATCCCT TGCATGGCTC TCAGATTCC CTTTATTGTA CTTTATTA
1861 CAATCAAAGG ACCGAGAAGG ACCAACTGAT CACCTTGAAT CTGCTTGTC TCTTAATCTT
1921 CCTCTCCAGA ATAATCACAC TGCAGCAGAT ATGTATCTTT CTCCTGTAAG ATCTCAAAG
1981 AAAAAAGGTT CAACTACGCG TGTAATTTCT ACTGCAAATG CAGAGACACA AGCAACCTCA
2041 GCCTTCAGG CCCAGAAGCC ATTGAAATCT ACCTCTCTTT CACTGTTTTA TAAAAAAGTG
2101 TATCGGCTAG CCTATCTCCG GCTAAATACA CTTTGTGAAC GCCTTCTGTC TGAGCACCCA
2161 GAATTAGAAC ATATCATCTG GACCTTTTTC CAGCACACCC TGCAGAATGA GTATGAACCT
2221 ATGAGAGACA GGCATTTGGA CCAAATTATG ATGTGTCCA TGTATGGCAT ATGCAATGTG
2281 AAGAATATAG ACCTTAAATT CAAAATCATT GTAACAGCAT ACAAGGATCT TCCTCATGCT
2341 GTTCAGGAGA CATTCAAACG TGTTTTAATC AAAGAAGAGG AGTATGATTC TATTATAGTA
2401 TTCTATAACT CGGTCTTCAT GCAGAGACTG AAAACAAATA TTTTGCAGTA TGCTCCACC
2461 AGGCCCCCTA CCTTGTCCAC AATACCTCAC ATTCTCTGAA GCCCTTACAA GTTTCTAGT
2521 TCACCCCTAC GGATTCCTGG AGGGAACATC TATATTTAC CCCTGAAGAG TCCATATAAA
2581 ATTTCAGAAG GTCTGCCAAC ACCAACAAAA ATGACTCCA GATCAAGAT CTTAGTATCA
2641 ATTGGTGAAT CTTTCGGGAC TTCTGAGAAG TTCCAGAAAA TAAATCAGAT GGATGTAAAC
2701 AGCGACCGTG TGCTCAAAG AAGTGCTGAA GGAAGCAACC CTCCTAAACC ACTGAAAAAA
2761 CTACGCTTTG ATATTGAAG ATCAGATGAA GCAGATGGAA GTAAACATCT CCCAGGAGAG
2821 TCCAACTTTC AGCAGAAACT GGCAGAAATG ACTTCTACTC GAACACGAAT GCAAAGCAG
2881 AAAATGAATG ATAGCATGGA TACCTCAAAC AAGGAAGAGA AATGAGGATC CGCG

```

*Bam*HI

Figure 2. Complete sequence of cloned *RB1* cDNA. The start codon is indicated in **pink**, the stop codon in **red**, a single base insertion in **green**, and base changes are in **blue**. The position of the primer-borne *Hind*III and *Bam*HI restriction sites are indicated in **bold**. Since the base insertion is encountered before the start codon it has no effect on the sequence of the resultant Rb protein.

WT	1	MPPKTPRKTA	ATAAAAAAEP	PAPPPPPPPE	EDPEQDSGPE	DLPLVRLEFE	ETEEDPFTAL
pAW1	1	MPPKTPRKTA	ATAAAAAAEP	PAPPPPPPPE	EDPEQDSGPE	DLPLVRLEFE	ETEEDPFTAL
WT	61	CQKLIKPDHV	RERAWLTWEK	VSSVDGVLGG	YIQKKKELWG	ICIFIARVDL	DEMSFTLLSY
pAW1	61	CQKLIKPDHV	RERAWLTWEK	VSSVDGVLGG	YIQKKKELWG	ICIFIA X VDL	DEMSFTLLSY
WT	121	RKTYEISVHK	FFNLLKEIDT	STKVDNAMS	LLKKYDVLFA	LFSKLERTCE	LIYLTQPSSS
pAW1	121	RKTYEISVHK	FFNLLKEIDT	STKVDNAMS	LLKKYDVLFA	LFSKLERTCE	LIYLTQPSSS
WT	181	ISTEINSALV	LKVSWITFLL	AKGEVLQMED	DLVISFQLML	CVLDYFIKLS	PPMLLKEPYK
pAW1	181	ISTEINS X TLV	LKVSWITFLL	AKGEVLQMED	DLVISFQLML	CVLDYFIKLS	PPMLLKEPYK
WT	241	TAVIPINGS	RTPRRGQNR	ARIAKQLEND	TRIEVLCKE	HECNIDEVKN	VYFKNFIPFM
pAW1	241	TAVIPINGS	RTPRRGQNR	ARIAKQLEND	TRIEVLCKE	HECNIDEVKN	VYFKNFIPFM
WT	301	NSLGLVTSNG	LPEVENLSKR	YEEIYLKNKD	LDRRLFLDHD	KTLQTDSIDS	FETQRTPRKS
pAW1	301	NSLGLVTSNG	LPEVENLSKR	YEEIYLKNKD	X LDARLFLDHD	KTLQTDSIDS	FETQRTPRKS
WT	361	NLDEEVNIIP	PHTPVRTVMN	TIQQLMMILN	SASDQPSENL	ISYFNNCTVN	PKESILKRVK
pAW1	361	NLDE X GVNVIP	PHTPVRTVMN	TIQQLMMILN	SASDQPSENL	ISYFNNCTVN	PKESILKRVK
WT	421	DIGYIFKEKF	AKAVGQCVE	IGSQRYKLG	RLYYRVMESM	LKSEEERLSI	QNFSKLLNDN
pAW1	421	DIGYIFKEKF	AKAVG X CCVE	IGSQRYKLG	RLYYRVMESM	LKSEEERLSI	QNFSKLLNDN
WT	481	IFHMSLLACA	LEVVMATYSR	STSQNLDSGT	DLSFPWILNV	LNLKAFDFYK	VIESFIKAEG
pAW1	481	IFHMSLLACA	LEVVMATYSR	STSQNLDSGT	DLSFPWILNV	LNLKAFDFYK	VIESFIKAEG
WT	541	NLTREMIKHL	ERCEHRIMES	LAWLSDSPLF	DLIKQSKDRE	GPTDHLESAC	PLNLPLQNNH
pAW1	541	NLTREMIKHL	ERCEHRIMES	LAWLSD X SPLF	DLIKQSKDRE	GPTDHLESAC	PLNLPLQNNH
WT	601	TAADMYLSPV	RSPKKKGSTT	RVNSTANAET	QATSAFQTQK	PLKSTSLSLF	YKKVYRLAYL
pAW1	601	TAADMYLSPV	RSPKKKGSTT	RVNSTANAET	QATSAFQTQK	PLKSTSLSLF	YKKVYRLAYL
WT	661	RLNTLCERLL	SEHPELEHII	WTLFQHTLQN	EYELMRDRHL	DQIMMCSMYG	ICKVKNIDLK
pAW1	661	RLNTLCERLL	SEHPELEHII	WTLFQHTLQN	EYELMRDRHL	DQIMMCSMYG	IC X NVKNIDLK
WT	721	FKIIVTAYKD	LPHAVQETFK	RVLIKEEEYD	SIIVFYNSVF	MQRLKTNILQ	YASTRPPTLS
pAW1	721	FKIIVTAYKD	LPHAVQETFK	RV X LIKEEEYD	SIIVFYNSVF	MQRLKTNILQ	YASTRPPTLS
WT	781	PIPHIPRSPY	KFPSSPLRIP	GGNIYISPLK	SPYKISEGLP	TPTKMTPRSR	ILVSIGESFG
pAW1	781	PIPHIPRSPY	KFPSSPLRIP	GGNIYISPLK	SPYKISEGLP	TPTKMTPRSR	ILVSIGES X FG
WT	841	TSEKFQKINQ	MVCNSDRVLK	RSAEGSNPPK	PLKKLRFDIE	GSDEADGSKH	LPGESKFQQK
pAW1	841	TSEKFQKINQ	MVCNSDRVLK	RSAEGSNPPK	PLKKLRFDIE	GSDEADGSKH	LPGES X NFQQK
WT	901	LAEMTSTRTR	MQKQKMNDSM	DTSNKEEK			
pAW1	901	LAEMTSTRTR	MQKQKMNDSM	DTSNKEEK			

Figure 3. Alignment of wildtype (WT) Rb and the predicted Rb protein sequence derived from cloned *Rb1* borne on pAW1. Aberrations in the cDNA that result in changes in the polypeptide sequence are indicated in **red**, while those that maintain the predicted amino acid residue are **blue**. Regions of the cloned Rb that correspond to the wildtype are indicated with a '-' and those that do not are indicated with an 'X'.

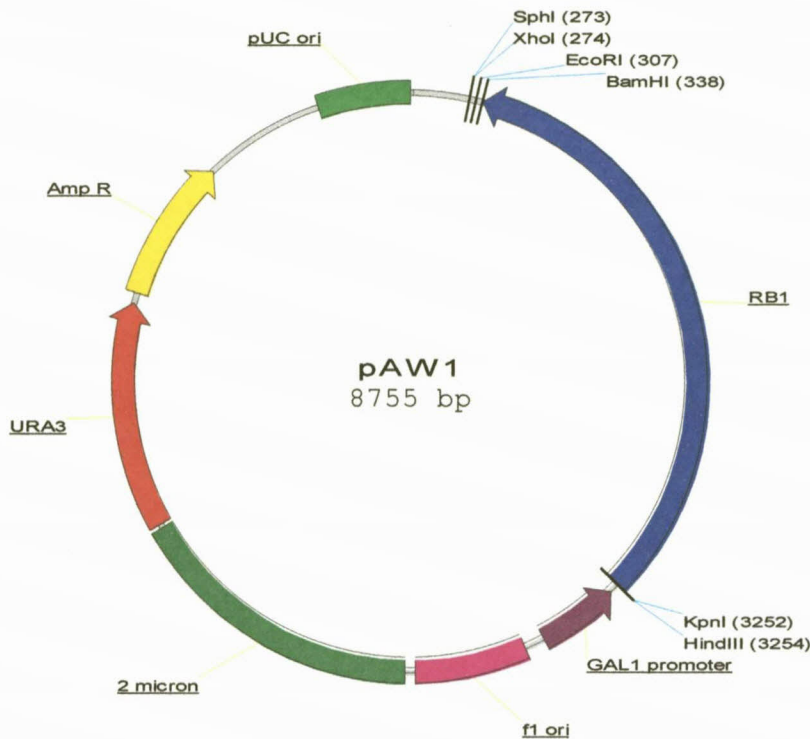


Figure 4. Vector map of pAW1, a yeast expression vector for the human Rb protein. The complete *RB1* cDNA was cloned into pYES2 and is under the control of the yeast *GAL1* promoter, permitting selective induction of the Rb protein.

3. Construction of pAW2, an E2F1 Expression Vector

The strategy for cloning of *E2F1* was almost identical to that described for *RB1*. *E2F1* is a protein of 437 amino acids, which is subsequently encoded by a cDNA with a minimum length of 1311 bp (Slansky and Farnham, 1996). The sequence of this cDNA has been determined previously (GenBank accession no. M96577), and this known sequence was used to derive primers for cloning and sequencing. Primers E2F1mSac and E2F1pEco were designed with 5' *SacI* and *EcoRI* restriction enzyme recognition sites, respectively. pYATAG200 is a yeast expression vector with a polylinker cloning site downstream of a *CUP1* promoter. This promoter is induced by copper, resulting in transcription of the downstream cDNA, in this case *E2F1*.

Optimization of the PCR conditions for amplification of *E2F1* proved to be laborious. Once again (see PCR amplification of *RB1*) DMSO was absolutely required for amplification of the cDNA. 10% DMSO proved to be optimal for amplification of *E2F1* from the Superscript leukocyte cDNA library. The expected PCR product size (derived

from the known sequence) was 1347 bp, precisely the same as that obtained by amplification of *E2F1* (figure 5A).

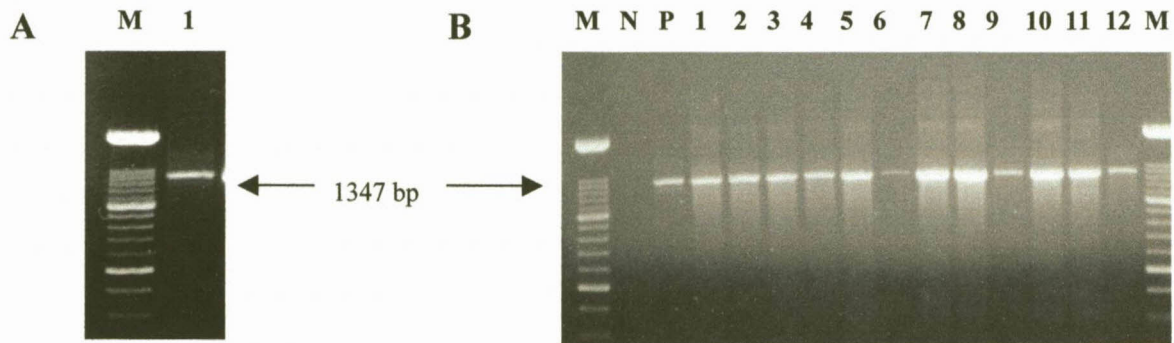


Figure 5. PCR amplification of *E2F1*. **A.** The complete *E2F1* cDNA (1347 bp) was amplified from 125 ng of a Superscript cDNA library. Lane M, Marker XIV (Roche); lane 1, *E2F1* PCR product. **B.** *E2F1* was cloned into pYATAG200, and cloning was confirmed by PCR amplification of the complete cDNA. Lanes M, Marker XIV (Roche); lane N, negative control (no DNA); lane P, positive control (amplification from cDNA library); lanes 1-12, individual clones (amplified from minipreps).

The amplified *E2F1* cDNA and plasmid pYATAG200 were cleaved with restriction enzymes *EcoRI* and *SacI* to generate sticky ends. These were ligated and the resultant plasmids were used to transform competent *E. coli* JM105. Plasmids were isolated and used for PCR amplification of *E2F1* (figure 5B).

```

1  GTTAGTGCAA  TATCATATAG  AAGTCATCGA  AATAGATATT  AAGAAAAACA  AACTGTAACG
61  AATTC CGTGA  GCGTCATGGC  CTTGGCCGGG  GCCCTGCGG  GCGGCCCATG  CGCGCCGGCG
121 CTGGAGGCC  TGCTCGGGGC  CGGCGCGCTG  CGGCTGCTCG  ACTCCTCGCA  GATCGTCATC
181 ATCTCCGCC  CGCAGGACGC  CAGCGCCCCG  CCGGCTCCCA  CCGGCCCCGC  GGCGCCCGCC
241 GCCGGCCCT  GCGACCCTGA  CCTGCTGCTC  TTCGCCACAC  CGCAGGCGCC  CCGGCCACAA
301 CCCAGTGCG  CGCGGCCCGC  GCTCGGCCGC  CCGCCGGTGA  AGCGGAGGCT  GGACCTGGAA
361 ACTGACCATC  AGTACCTGGC  CGAGAGCAGT  GGGCCAGCTC  GGGGCAGAGG  CCGCCATCCA
421 GGAAAAGGTG  TGAATCCCC  GGGGGAGAAG  TCACGCTATG  AGACCTCACT  GAATCTGACC
481 ACCAAGCGCT  TCCTGGAGCT  GCTGAGCCAC  TCGGCTGACG  GTGTCGTCGA  CCTGAACCTG
541 GCTGCCGAG  TGCTGAAGGT  GCAGAAGCGG  CGCATCTATG  ACATCACCAA  CGTCCTTGAG
601 GGCATCCAG  TCATTGCCAA  GAAGTCCAAG  AACCACATCC  AGTGGCTGGG  CAGCCACACC
661 ACAGTGGGG  TCGGCGGACG  GCTTGAGGGG  TTGACCCAGG  ACCTCCGACA  GCTGCAGGAG
721 AGCGAGCAG  AGCTGGACCA  CCTGATGAAT  ATCTGTAATA  CGCAGCTGCG  CCTGCTCTCC
781 GAGGACTCT  ACAGCCAGCG  CCTGGCCTAC  GTGACGTGTC  AGGACCTTCG  TAGCATTGCA
841 GACCCTGCAG  AGCAGATGGT  TATGGTGATC  AAAGCCCCTC  CTGAGACCCA  GCTCCAAGCC
901 GTGGACTCT  CGGAGAACTT  TCAGATCTCC  CTTAAGAGCA  AACAAGGCC  GATCGATGTT
961 TTCCTGTGCC  CTGAGGAGAC  CGTAGGTGGG  ATCAGCCCTG  GGAAGACCCC  ATCCCAGGAG
1021 GTCATTCTG  AGGAGGAGAA  CAGGGCCACT  GACTCTGCCA  CCATAGTGTC  ACCACCACCA
1081 TCATCTCCC  CCTCATCCCT  CACCACAGAT  CCCAGCCAGT  CTCTACTCAG  CTTGGAGCAA
1141 GAACCGCTGT  TGTCCCGGAT  GGGCAGCCTG  CCGGCTCCCG  TGGACGAGGA  CCGCCTGTCC
1201 CCGCTGGTGG  CGGCCGACTC  GCTCCTGGAG  CATGTGCGGG  AGGACTTCTC  CGGCCTCCTC
1261 CCTGAGGAGT  TCATCAGCCT  TTCCCACCC  CACGAGGCC  TCGACTACCA  CTTGCGCCTC
1321 GAGGAGGGCG  AGGGCATCAG  AGACCTCTTC  GACTGTGACT  TTGGGGACCT  CACCCCTGTC
1381 GATTTCTGAC  AGGGCCGAGC  TCGCC

```

Figure 6. Sequence of cloned *E2F1* cDNA. The positions of the restriction sites for *EcoRI* and *SacI* are indicated in red and blue respectively. The start codon is indicated in green and the stop codon in pink.

Primers 523R, 681F, 901R, 1129F were designed, and, together with the PCR primers E2F1pEco and E2F1mSac, were used to completely sequence the cloned *E2F1*. Sequencing revealed that the entire cDNA was present and intact, with no mutations or polymorphisms present (figures 6 and 7).

```

1 MALAGAPAGG PCAPALEALL GAGALRLLDS SQIVIISAAQ DASAPPAPTG
51 PAAPAAGPCD PDLLLFATPQ APRPTPSAPR PALGRPPVKR RLDLETDHQY
101 LAESSGPARG RGRHPGKGVK SPGEKSRYET SLNLTTKRFL ELLSHSADGV
151 VDLNWAAEVL KVQKRRIYDI TNVLEGIQLI AKKSKNHIQW LGSHTTVGVG
201 GRLEGLTQDL RQLQESEQQL DHLMNICTTQ LRLLEDTDS QRLAYVTCQD
251 LRSIADPAEQ MVMVIKAPPE TQLQAVDSSE NFQISLKSQK GPIDVFLCPE
301 ETVGGISP GK TPSQEV TSEE ENRATDSATI VSPPPSSPPS SLTTDPSQSL
351 LSLEQEPLLS RMGSLRAPVD EDRLSPLVAA DSLLEHVRED FSGLLP EEFI
401 SLSPPHEALD YHFGLEEGEG IRDLFDCDFG DLTPLDF

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Figure 7. Protein sequence of E2F1. Comparison with the known sequence indicates that the protein is complete and wildtype.

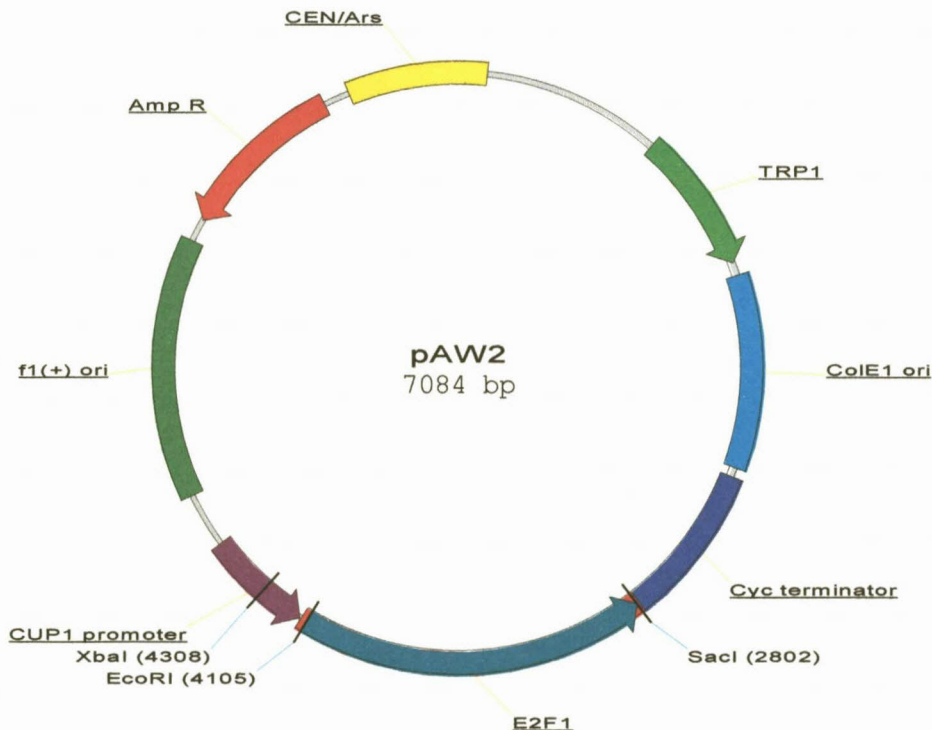


Figure 8. Vector map of pAW2, a yeast expression vector for expression of human E2F1 in *S. cerevisiae*. The complete cDNA was cloned into pYATAG200 downstream of the *CUP1* promoter, allowing induction of E2F1 protein expression.

4. Construction of W303-1A-Derived Strains Containing an E2F1 Reporter Construct

The transcription factor E2F1 recognizes and binds the E2F recognition element (RE) TTTCGCGC, and so doing is able to activate transcription of downstream target genes.

The first step in construction of E2F reporter strains was to construct a reporter gene susceptible to regulation by E2F. This was accomplished through incorporation of an E2F RE upstream of the *E. coli lacZ* gene borne on pJS205BXX. This gene encodes β -galactosidase, an enzyme that targets and cleaves the β -linkages of galactose. The plasmid pJS205BXX contains the complete *E. coli lacZ* gene downstream of a multiple cloning site, and was used for construction of an E2F-regulated *lacZ* gene. Oligonucleotides E2F b1, b3, t1, and t3 (see table 3.1) were designed using the known E2F RE sequence. Double stranded fragments were generated through annealing of E2F b1 and t1, yielding RE1 (RE1 implies a single copy of the RE), and E2F b3 and t3, yielding RE3 (similarly, RE3 implies three copies of the RE).

RE1 and RE3 were designed to contain sticky ends compatible with *Bgl*III and *Xba*I, but complications in restriction digestion of pJS205BXX led to the decision to blunt both ends of the double stranded fragment (figure 9).

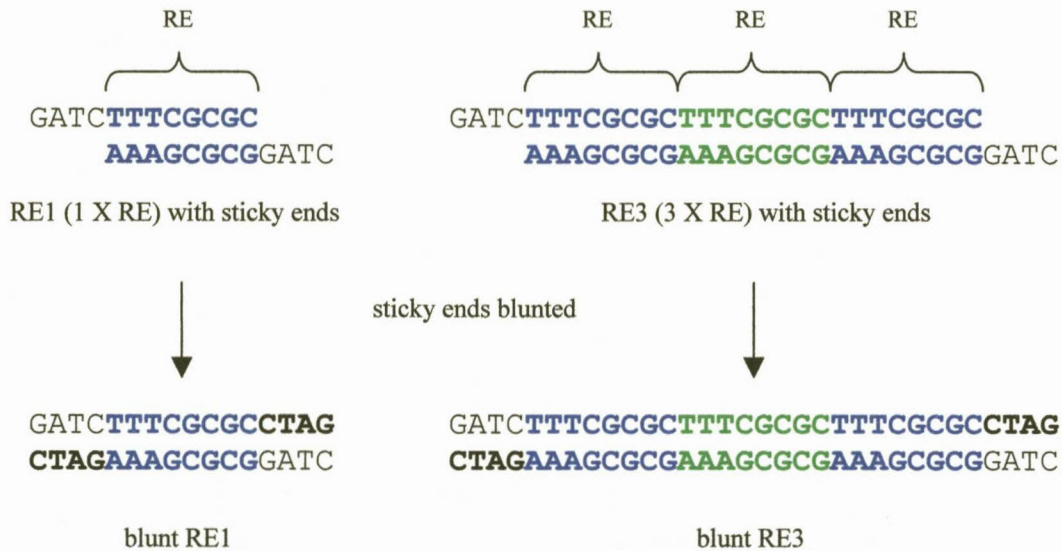


Figure 9. Double stranded RE-containing fragments were constructed, then blunted to facilitate cloning into the vector pJS205BXX.

The original cloning strategy required that pJS205BXX be cut with both *Bgl*III and *Xba*I, but this proved to be unsuccessful (results not shown). This is due to the substrate requirements of *Bgl*III. Cleavage of pJS205BXX with *Xba*I resulted in only a single base flanking the *Bgl*III site on the 3' side. This leads to inefficient cleavage by *Bgl*III, and it was consequently decided to negate the problem by blunting the overhangs resultant of *Xba*I

digestion. The RE1 and RE3 constructs, originally to be inserted by sticky end ligation, were instead blunt ligated to pJS205BXX.

Blunt ligation of RE1 and RE3 to pJS205BXX was confirmed by PCR amplification of the *CyCl1* promoter region of pJS205BXX, the region containing the multiple cloning site. Primers pJSup and pJSdwn were designed based on the known sequence of pJS205BXX, and subsequently used to amplify the *CyCl1* promoter region from plasmid isolates from positive transformants (figure 10). The amplified region encompasses 166 bp in the absence of any insert, thus the sizes from plasmids containing inserts of RE1 (16 bp) and RE3 (32 bp) would be 182 bp and 198 bp, respectively.

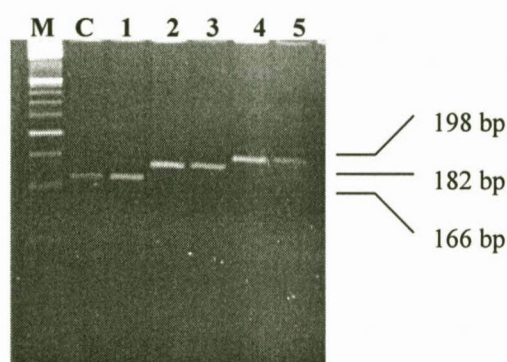


Figure 10. PCR amplification of the plasmid-borne *CyCl1* promoter from plasmid isolates following blunt ligation of pJS205BXX to RE1 and RE3. Lane M, Marker XIII (Roche); lane C, Control reaction, no insert; lane 1, no insert; lane 2, RE1 insert; lane 3, RE1 insert; lane 4, RE3 insert; lane 5, RE3 insert.

The original ligation strategy used sticky ends for a reason: to preclude insertion of the RE in the reverse orientation. With the substitution of blunt ligation this ability was lost, and the plasmids were subsequently sequenced to unequivocally confirm insertion of the RE fragments and to determine their orientation. The PCR primer pJSup was used to sequence the plasmid-borne *CyCl1* promoters from isolates predicted, by PCR analysis, to contain a single (RE1) or triple (RE3) RE construct (figure 11).

Sequencing of the plasmid isolates revealed that in the case of RE1, the construct had been inserted in both the correct (forward) and reverse orientations, yielding plasmids dubbed pJS-RE1f (forward) and pJS-RE1r (reverse), respectively. This is interesting as further experiments could determine the influence that the orientation of the RE has on the

transcriptional activation of target genes mediated by E2F1. In the case of RE3, the insert was present exclusively in the correct orientation.

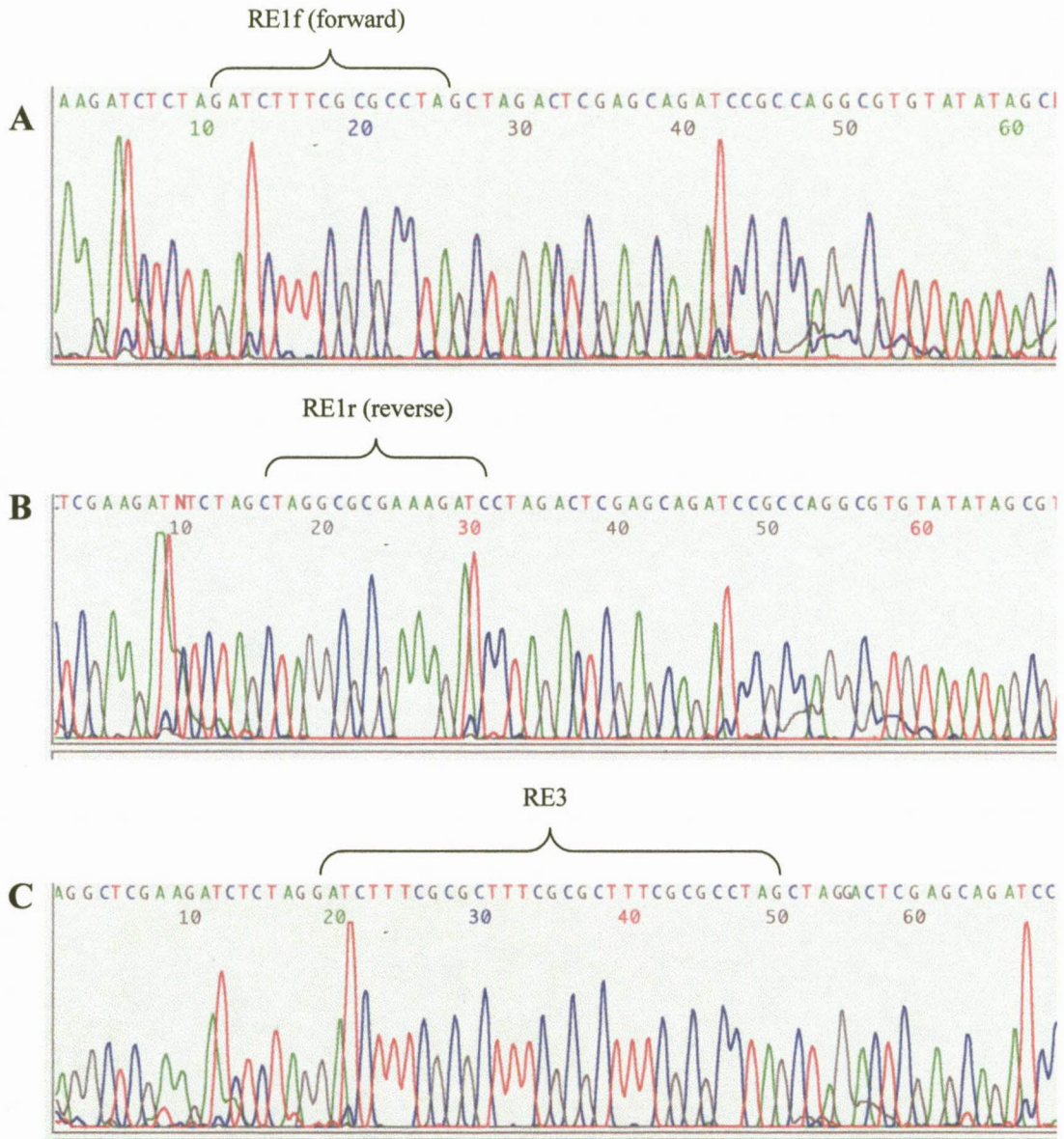


Figure 11. Sequencing of constructed plasmids containing the inserted E2F recognition element (TTCGCGC). **A.** pJS-RE1f contains a single copy of RE in the correct (forward) orientation. **B.** pJS-RE1r contains a single copy of RE in the reverse orientation. **C.** pJS-RE3 contains 3 copies of RE in the correct orientation.

The next phase in the generation of the E2F1 reporter strains was the construction of integrating plasmids containing the three E2F RE-lacZ reporter constructs. pJS-RE1f, pJS-RE1r and pJS-RE3 were digested with *Pst*I and *Stu*I, releasing the three RE-lacZ constructs. Due to the size similarity of the excised RE-lacZ fragments and the vector, further purification was impossible and the restriction products were used directly for subsequent ligations.

The yeast integrating plasmid pRS405 was used as vector for introduction of the RE-lacZ constructs into the genome of W303-1A. Ligation of pRS405 and the RE-lacZ constructs in the correct orientation required that the vector be digested with *Pst*I and *Sma*I to generate compatible sticky ends, but this was hampered by the proximity of the recognition sites to one another. Once the first enzyme was used, the portion remaining was insufficient to facilitate efficient digestion by the second enzyme. This observation was independent of the order of the restriction enzymes used (results not shown).

The problem was negated by taking advantage of the fact that both *Stu*I and *Sma*I leave blunt ends following digestion. pRS405 was initially cut with *Bam*HI, which recognizes a site 4 bp upstream of the *Sma*I site. *Bam*HI generates a 4 base 5' overhang, which was subsequently blunted to yield a blunt end compatible with that generated by *Stu*I in digestion of the pJS-RE plasmids. Only after the generation of the blunt end at the *Bam*HI restriction site was *Pst*I able to cut pRS405 effectively. The RE-lacZ constructs and pRS405 were ligated via sticky end-facilitated ligation to yield pRS-RE1f-lacZ, pRS-RE1r-lacZ and pRS-RE3-lacZ. This was confirmed by PCR amplification of the *CyC1* promoter region from plasmid isolates obtained from positive transformants (figure 12). As a control, pRS405 was additionally ligated to the *lacZ* fragment containing no RE inserts to yield pRS-lacZ.

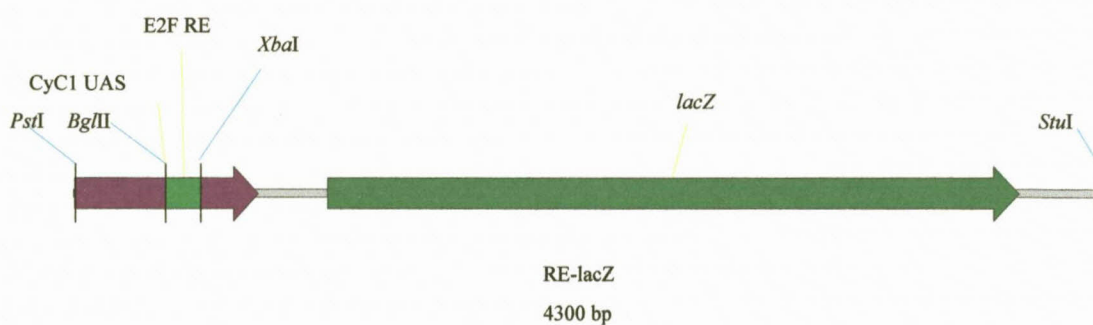


Figure 12. The RE-lacZ constructs contain the various E2F REs within the *CyC1* UAS upstream of the *lacZ* gene. The RE-lacZ constructs were cut from the pJS-RE plasmids for subsequent cloning into the yeast integrating plasmid pRS405.

The final phase in the construction process was the integration of the RE-lacZ constructs into the genome of *S. cerevisiae* strain W303-1A, a commonly used laboratory strain. Integration was facilitated by a simple transformation reaction, but due to the lack of

episomal replication origins, selection favoured transformants where the construct had been integrated. The pRS405 plasmid contains a yeast *LEU1* gene, which, when integrated, would render the integrated strain prototrophic for leucine. Prototrophic strains would then additionally contain the integrating fragment, in this case the various RE-lacZ constructs. Following transformation of W303-1A with pRS-lacZ and the various pRS-RE-lacZ plasmids, prototrophic strains were tested for the presence of the RE-lacZ construct. This was accomplished through PCR amplification of the *CyC1* promoter region from crude DNA extracts isolated from putative prototrophs (figure 14). The strains created are summarized in table 1.

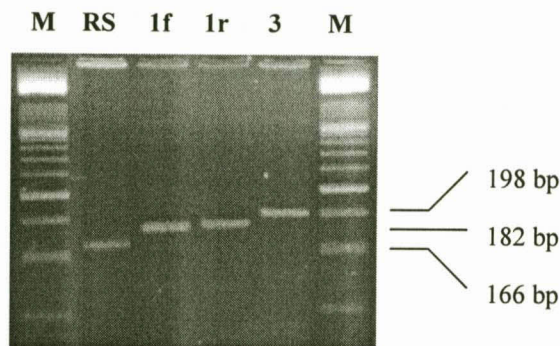


Figure 13. PCR amplification of the RE-containing *CyC1* promoter from pRS-RE-lacZ plasmids. Plasmids were isolated from cells transformed with ligations of pRS405 to lacZ (lane RS), RE1f-lacZ (lane 1f), RE1r-lacZ (lane 1r), RE3-lacZ (lane 3). Lanes M, Marker XIV (Roche).

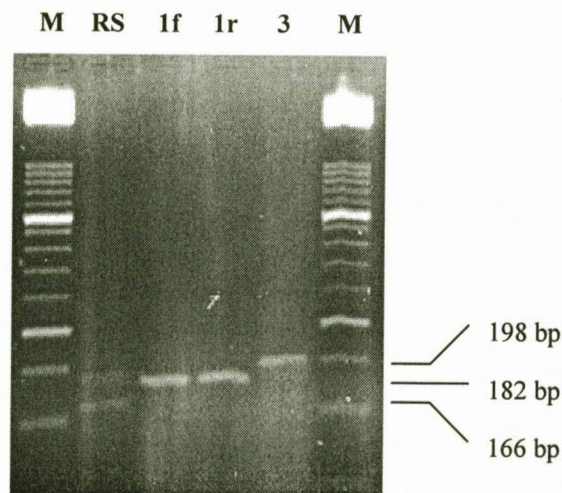


Figure 14. PCR amplification of the *CyC1* promoter from crude DNA extracts isolated from putative prototrophs following integration of pRS-lacZ and the three pRS-RE-lacZ plasmids. Lane M, Marker XIII (Roche); lane RS, pRS-lacZ; lane 1f, pRS-RE1f-lacZ; lane 1r, pRS-RE1r-lacZ; lane 3, pRS-RE3-lacZ.

Table 1. Strains created through integration of pRS-lacZ and pRS-RE-lacZ reporter constructs into W303-1A

Strain designation	Integrating vector	Genotype
W303-1A wildtype	none	<i>MATa leu2-3 /112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2</i>
W-RS	pRS-lacZ	<i>MATa leu2-3 /112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2 TRP1::CyC1_{TATA}-lacZ</i>
W-1f	pRS-RE1f-lacZ	<i>MATa leu2-3 /112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2 TRP1::E2FRE1f_{16-mer}- CyC1_{TATA}-lacZ</i>
W-1r	pRS-RE1r-lacZ	<i>MATa leu2-3 /112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2 TRP1::E2FRE1r_{16-mer}- CyC1_{TATA}-lacZ</i>
W-3	pRS-RE3-lacZ	<i>MATa leu2-3 /112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2 TRP1::E2FRE3_{32-mer}- CyC1_{TATA}-lacZ</i>

5. Ectopic Expression of E2F1 in Wildtype and Chimeric Yeast Strains

Expression of E2F1 was facilitated by the constructed pAW2 plasmid containing the complete *E2F1* cDNA. *E2F1* was introduced into strains W303-1A (wildtype), W-RS (no RE), W-1f (single RE construct in forward orientation), W-1r (single RE construct in reverse orientation), and W-3 (triple RE construct) by transformation with pAW2. The cDNA is under the regulation of the *CUP1* promoter, a strong promoter induced in the presence of copper. Copper was included in the media where induction was desired as cupric sulphate (CuSO₄), at concentrations of 1 mM and 2.5 mM, in accordance with published results (Hottiger *et al.*, 1995). However, these concentrations proved to be toxic to the four constructed chimeric strains derived from *S. cerevisiae* W303-1A (results not shown).

It is possible that at these elevated concentrations, the presence of abnormally high concentrations of the ectopic E2F1 protein were the cause of the toxicity, but this was not examined. The endogenous copper present in synthetic complete medium is adequate to induce expression of the downstream *E2F1* gene from the *CUP1* promoter (H. Patterson, personal communication). Since this negated the need for the inclusion of additional copper

in the growth media, and copper-free medium was not available, further induction experiments were performed in the absence of supplementary copper.

E2F1 was induced in the various strains in a two-step manner. Firstly, pAW2 was introduced into W303-1A, W-RS, W-1f, W-1r, and W-3 by transformation. The transformation reactions were plated out on -leu SCD to select for the presence of pAW2. Following this, positive transformants were picked and streaked out on -leu SCD + X-gal. The reason for this is that growth of transformants was significantly impeded when the initial transformation reaction was plated directly onto X-gal-containing plates (results not shown). The chromogen appears to have a growth-retarding effect on the strains examined, which was less evident when growing cells were streaked out on plates containing the colour-producing agent.

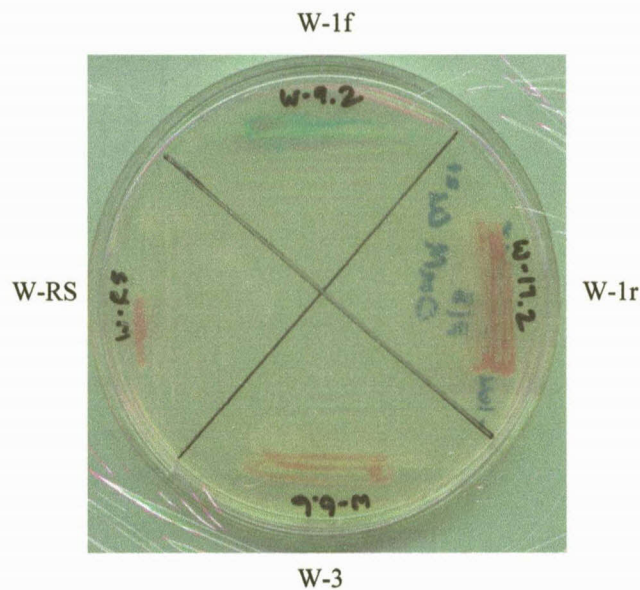


Figure 15. Induction of human E2F1 in W-RS, W-1f, W-1r, and W-3. E2F1 was introduced into the strains by transformation with pAW2, and subsequently induced from the *CUP1* promoter borne on E2F1 expression plasmid. A blue-coloured appearance, visible in W-1f, is indicative of *lacZ*-mediated cleavage of X-gal, and, by inference, of activation of transcription from the E2F RE-regulated *lacZ* promoter.

Induction of E2F1 expression was monitored as the appearance of a blue X-gal cleavage product. This blue product was observed in W-1f, the chimeric strain containing the single RE in the forward orientation, but not in W-RS (no RE), W-1r (single RE, reverse orientation), or W-3 (triple RE). This appeared to be in accordance with the hypothesis that the human E2F1 protein would be able to induce expression of a downstream reporter gene from a promoter containing an E2F recognition element.

Despite numerous literature searches during the course of this work, papers published by Mai and Lipp (1993), and Vemu and Reichel (1995) were found only in the closing stages of the study. These papers described preliminary evidence for the presence of a *S. cerevisiae* homologue of E2F1, dubbed YE2F, that recognizes the same recognition element as its human counterpart. Mai and Lipp additionally examined the effect of the orientation of the RE and found that the arrangement and number of REs had a noticeable effect on reporter activation (Mai and Lipp, 1993). W-RS, W-1f, W-1r, and W-3 were streaked out on -trp SCD plates containing X-gal. The E2F1-expression plasmid pAW2 was omitted, yet W-1f, W-1r, and W-3 were capable of producing a blue-coloured product. This is evidence for the ability of the endogenous *S. cerevisiae* E2F1-like protein to activate transcription from the reporter construct borne on the genomes of the chimeric strains. The inability of W-RS to present X-gal cleavage can be ascribed to the absence of E2F REs in the incorporated *CyC1* promoter.

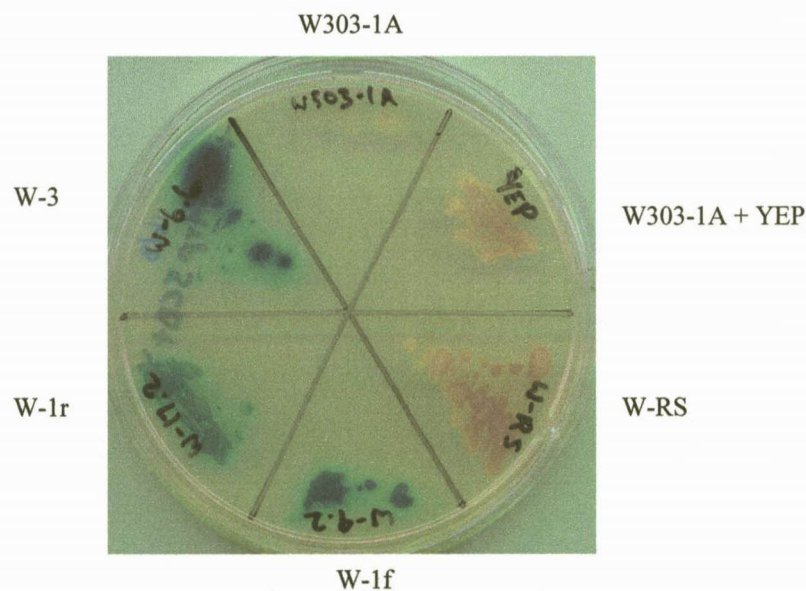


Figure 16. *S. cerevisiae* contains an endogenous E2F1-like protein capable of binding the integrated E2F RE and consequently activating transcription of the *lacZ* reporter construct. The four chimeric strains (W-RS, W-1f, W-1r, W-3), W303-1A, and W303-1A transformed with a yeast episomal plasmid (YEP) were streaked out on -trp SCD plates containing X-gal. Only the strains containing the E2F RE, namely W-1f, W-1r and W-3, were able to activate transcription of the *lacZ* reporter gene, and consequently yield the blue-coloured X-gal cleavage product.

Since YE2F is capable of activating transcription of the reporter it is impossible to discern whether the reporter gene promoter is bound by the endogenous E2F-like yeast protein or the ectopically expressed human E2F1 protein. But, the fact that the reporter was activated demonstrates that the constructed strains do indeed contain an E2F-regulated reporter

construct. These strains may be useful if it is possible to knock out the yeast E2F-like protein encoding gene. Hampering this approach is the fact that the yeast E2F homologue has yet to be cloned. Also, the effect of knocking out the endogenous YE2F could prove to be lethal. Faced with such an eventuality, a different approach to the problem of creating a yeast-based assay for Rb functional activity is required.

Comparison of the growth patterns observed in the presence or absence of ectopic expression of human E2F1 yields a clear discrepancy. In the presence of human E2F1 only strain W-1f, containing a single E2F RE in the forward orientation, resulted in X-gal cleavage, while in the experiment where ectopic expression was omitted W-1f, W-1r, and W-3 showed utilization of the chromogen. A possible explanation for this is that in the absence of human E2F the E2F-like yeast activity is able to recognise the RE in the *CyC1* promoter in either orientation (W-1f, W-1r) or in tandem repeats (W-3) and activate transcription of the the reporter gene. Alternatively, in the presence the human E2F1, this protein binds the promoter in all three instances, but is only able to activate transcription in the single copy forward orientation, thereby implying additional specificity.

6. Ectopic Expression of Rb in Wildtype and Chimeric Yeast Strains

Faced with the dilemma of the yeast E2F-like activity being able to activate transcription of the reporter gene, it was decided to examine the effect of Rb expression on the wildtype and constructed chimeric strains in the absence of co-expressed E2F1, since the effect of E2F1 expression on the yeast growth is difficult to discernible. *RBI* was introduced into W303-1A, W-1f, W-1r, and W-3 by transformation with pAW1, a yeast expression plasmid containing the complete *RBI* cDNA regulated by the *GALI* promoter. The transformed strains were initially plated on -ura SCD to select for pAW1, which contains a *URA1* marker. Transformants were subsequently streaked out on -ura SCD and -ura SCGal plates, all containing X-gal (figure 17).

As expected from previous results (see above), W-1f, W-1r, and W-3 were able to activate expression of β -galactosidase from their respective endogenous RE-containing promoters when grown on glucose-containing medium. This was not observed for W303-1A as the yeast does not contain the reporter construct. Induction of Rb expression was facilitated by the inclusion of galactose as carbon source, which consequently activates transcription of the *GALI*-regulated *RBI*. A noticeable effect was observed in both W-1f and W-1r.

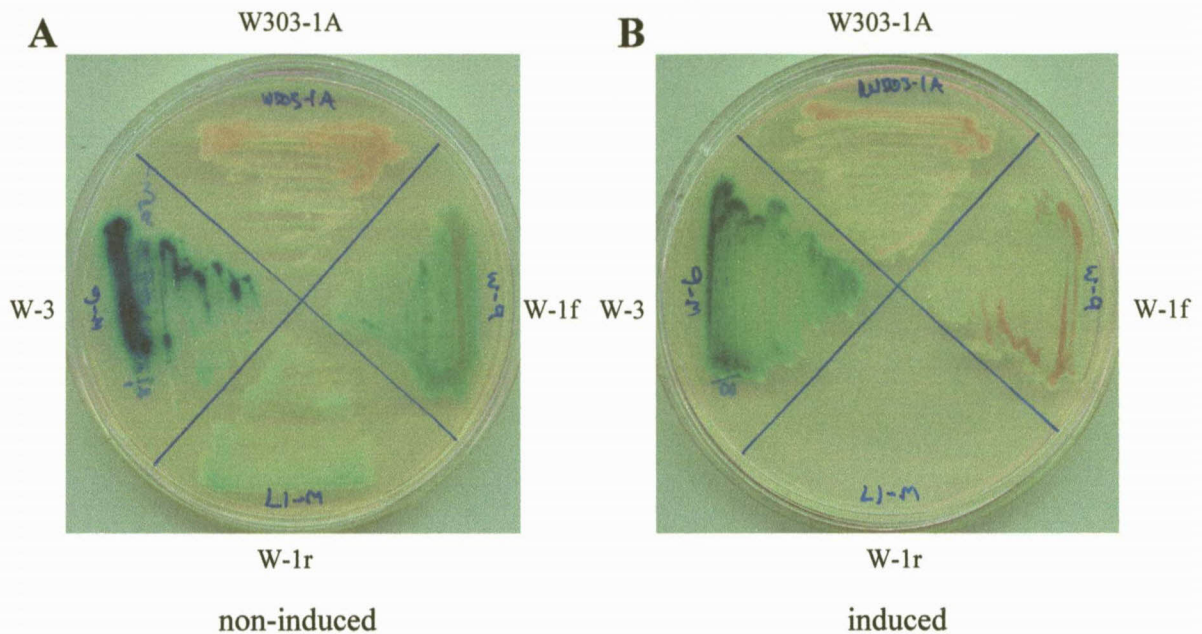


Figure 17. Induction of Rb expression in wildtype and chimeric strains. W303-1A, W-1f, W-1r, and W-3 were transformed with pAW1. Transformants were streaked out on -ura SCD + X-gal (A) and -ura SCGal + X-gal (B) resulting in non-expression and expression of Rb, respectively.

Rb expression appears to abrogate the ability of the endogenous YE2F protein to bind the RE-containing promoters found in the forward orientation in W-1f. Since one of the primary functions of Rb is to bind the E2F family of proteins, it is tempting to speculate that Rb is additionally capable of binding the yeast homologue of E2F1. Interestingly, this abrogation did not lead to complete cessation of growth, an observation which can be ascribed to the fact that binding of E2F-activity was probably not complete. This is supported by the inability of Rb to maintain this abrogation indefinitely, as further incubation for an additional three days led to the appearance of the blue-coloured X-gal cleavage product, indicative of *CyC1*-promoter binding and transcription activation (results not shown). These results were confirmed by repetition of the experimental procedure.

In contrast to the transient abrogation of YE2F activation of the reporter construct observed in W-1f, expression of Rb in W-1r appeared to completely halt growth. This can tentatively be attributed to binding of Rb to yeast E2F-like activity. Once again, the results were confirmed by repetition of the experiment. The sole genotypic difference between W-1f and W-1r is that in the case of the latter the RE is present in the reverse orientation in the *CyC1* promoter. How this can alter the response to ectopic Rb expression so drastically remains to be elucidated.

7. Cell Cycle Analysis of Wildtype and Chimeric Strains Expressing Rb

The effect of Rb on the cell cycle of the various strains was determined by flow cytometry. W303-1A, W-RS, W-1f, W-1r, and W-3, transformed with either pYES2 or pAW1, were grown in selective media containing either glucose or galactose as carbon source. These cultures were grown and samples were taken periodically. The procured data does not correlate perfectly with that obtained previously. It appears that the expression of Rb, induced during galactose utilization by strains transformed with pAW1, did not produce an observable effect within the time frame examined. Representative results are presented in figure 18.

Comparison of the results obtained during glucose and galactose utilization revealed a significant difference in the respective growth patterns. Galactose has the apparent effect of slightly retarding growth, both in the presence and absence of Rb induction, implying that it is not utilized as effectively as a carbon source as glucose is. This complicates determination of the influence of Rb on the cell cycle status of the strains examined. An observed increase in the proportion of the population in a normal growing state -containing a 2n genome- can be ascribed to either the presence of the growth suppressor Rb or the use of galactose as carbon source.

The effect of Rb expression could not be observed in strains transformed with pAW1 grown on galactose, but this is possibly due to an insufficient time frame of observation. The effect of Rb expression on growth of the examined strains on solid media was only observed after five days of incubation. It is possible that the 22 hours spent on examination of cell cycle status was not sufficient, and that prolongation could yield informative data. However, due to time and resource limitations this strategy could not be followed further.

An additional possibility is that the Rb effect seen during the streaking experiments was an artefact, and that the Rb protein induced has no effect on the growth characteristics of the examined strains. This could be attributed to the polymorphisms present in the cloned *RBI* cDNA, since the effects of each of the eight changes found in the resultant Rb protein are yet to be determined. Opposing this is the observation that all results could be duplicated. Also, the effect of expressed Rb on each of the strains examined was not identical, which is what would be expected if the protein was non-functional.

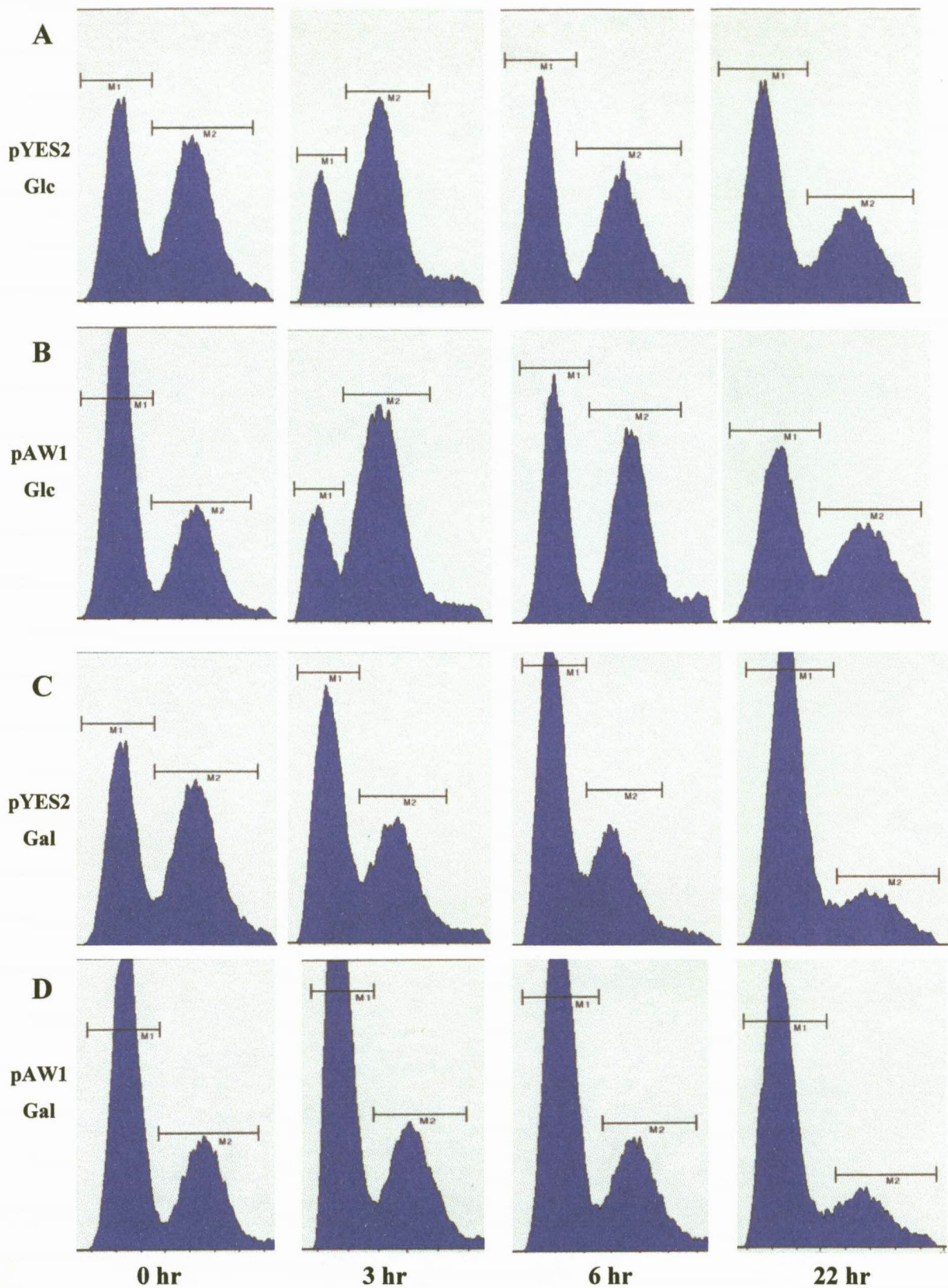


Figure 18. Cell cycle analysis of W-1f transformed with either pYES2 or pAW1, and grown in either glucose- or galactose-containing media, by flow cytometry. Samples were taken at the indicated times, stained with propidium iodide, and analyzed. **A.** Transformed with pYES2; grown in -ura SCD. **B.** Transformed with pAW1; grown in -ura SCD. **C.** Transformed with pYES2; grown in -ura SCGal. **D.** Transformed with pAW1; grown in -ura SCGal. M1 is representative of normally growing cells with an n genome (G_1/S -phase), while M2 is representative of actively dividing cells with a 2n genome ($S/G_2/M$ -phase).

CHAPTER SIX

PERSPECTIVES

The mammalian cell cycle is composed of a myriad interactions occurring in a defined sequence dictated by the flow of entropy. The decision to study the cell cycle requires entry into a world where events take place, not because they want to, but because they have to. Cells do not 'decide' to perform certain actions, but are driven by the laws of nature, the same laws that are responsible for the existence of the universe. Study of this maelstrom of reactions is truly analogous to opening Pandora's proverbial box. A peek inside and all the inner workings of the cell start to spill out. Unfortunately, this is where things start to get complicated.

Serendipitously, simpler alternatives are available. The yeast cell cycle is remarkably similar to that of higher animals. The Rb and E2F1 proteins are integral components of the mammalian cell cycle, and as such, they are found in aberrant forms in numerous malignancies. This necessitates an adequate means for the determination of the cellular status of these proteins, as prognosis and diagnosis of several neoplastic disorders are dependent thereon.

This study aimed to develop a yeast-based strategy for functional analysis of the human tumour suppressor protein Rb. This goal was impeded by one factor; the yeast cell cycle is too similar to that of humans. Several yeast strains, derived from W303-1A, were constructed that each contain a reporter gene -the *lacZ* gene of *E. coli*- regulated by E2F recognition elements introduced within the upstream *CyC1* promoter. The theory was that in the absence of Rb, ectopically expressed human E2F1 protein would be able to bind the RE and activate transcription of the reporter gene, ultimately resulting in a readily observable product. In the presence of functional Rb, E2F1 would be bound by the tumour suppressor protein, and thus be incapable of activating the reporter gene. This would provide an assay of Rb status, based not on tedious sequencing analysis of the genetic material, but on the actual functional activity of the protein.

When dealing with Mother Nature, though, we are oftentimes reminded that she has thought of everything. *S. cerevisiae* contains an E2F-like activity, capable of binding the exact RE introduced into the reporter gene promoter. This was confirmed by experiments

in this study, where the reporter gene was activated in the absence of ectopically expressed E2F1 protein. The endogenous yeast E2F-like protein is thus able to activate transcription of the reporter gene, negating the effect that would be observed by ectopic expression of human E2F1, and thus, all Rb-expression was performed in the absence of co-expressed human E2F1. Since it is impossible to distinguish between the yeast and human E2F activities, it is impossible to create a functional assay for Rb activity in the W303-1A-derived chimeras constructed.

As mentioned previously, it could be possible to overcome this problem by knocking out the yeast-borne E2F activity, but this approach is restricted by two barriers. Firstly, the yeast equivalent of the E2F-family is yet to be cloned. This problem can be approached with a transposon-based strategy. The endogenous E2F activity is capable of activating transcription of the *lacZ* reporter gene, and in so doing, provides a convenient assay for YE2F integrity. Through the use of a plasmid containing an inducible transposase it should be possible to disrupt the YE2F-encoding gene through integration of a transposon. This would be accompanied by an inability of the yeast to activate the reporter gene. The transposon, containing flanking genomic DNA, could then be retrieved and provide the basis for cloning the gene coding for YE2F. Since the reporter gene is specific for E2F-like binding, retrieval of non-specific factors should be negated.

A second problem is that, since the E2F proteins play an important role in the progression of the cell cycle in higher animals, it is possible that the yeast would not survive knocking out its homologue. Relegation of YE2F to the role of bystander could possibly wreak havoc with the delicate mechanism that is the cell cycle. Still, it would be interesting to further pursue this idea.

Ectopic expression of human Rb in the various strains used in this study provided some interesting results. Transformation of pAW1, followed by galactose-based induction of Rb expression resulted in observable differences in growth characteristics of certain strains. Those most affected were W-1f and W-1r, which each contain a single repeat of the E2F RE within the upstream promoter of the reporter gene, albeit in forward and reverse orientations, respectively. The effect was particularly evident in W-1r, where expression of Rb resulted in complete cessation of growth, probably due to binding of yeast E2F-like activity. These results could not, however, be reconciled with those obtained from cell cycle analysis with the aid of flow cytometry. These experiments did not show any

significant effect of Rb expression on the cell cycle of the examined strains. This is possibly due to an insufficient period of observation, but this could, unfortunately, neither be confirmed nor dismissed.

From the results obtained in this study it appears that construction of an apposite reporter system for the functional assay of Rb is perhaps more tricky than would be expected. The interference of endogenous proteins is a cause for concern in a development strategy such as this, and serves as a caveat for future studies in this field.

CHAPTER SEVEN

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