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Control of growth and proliferation by the retinoblastoma protein

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Summary

The retinoblastoma susceptibility gene *Rb* is an important tumour suppressor. It will inhibit both growth and proliferation when introduced into many types of cell. Furthermore, it is frequently found mutated in a range of human cancers. It is therefore of considerable importance that we should understand fully how this gene operates. The RB gene product is a 110 kDa nuclear phosphoprotein that regulates the activity of a number of key transcription factors. In turn, its activity is controlled through phosphorylation by cyclin-dependent kinases in response to the availability of growth factors. It therefore provides a mechanism for coordinating gene expression with growth factor availability. One of the principle targets of RB is a transcription factor called E2F. E2F controls the expression of a panel of genes that promote proliferation. By down-regulating these genes through its inhibitory action on E2F, RB provides a restraining influence upon cell cycle progression. It has been less clear how RB is able to suppress the growth (increase in mass) of cells. However, recent studies have suggested that it may achieve this by repressing the production of rRNA and tRNA. Loss of control over the protein synthetic apparatus may constitute an important step in tumour development.

I. Introduction

The retinoblastoma susceptibility gene *Rb* is essential for life. Its homozygous inactivation causes mouse embryos to die during the fourteenth day of gestation with defective neural and erythroid development (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). The *Rb* gene encodes a 110 kDa nuclear phosphoprotein that is expressed almost ubiquitously in normal mammalian cells (Weinberg, 1995; Whyte, 1995). It was mapped to chromosome 13q14 by virtue of its association with an inherited predisposition to retinoblastoma, a rare pediatric tumour of the retina (Friend et al., 1986). Inactivating mutations in this gene also occur in many other types of human malignancy, including small-cell lung cancers, several sarcomas and bladder carcinomas (Weinberg, 1995; Whyte, 1995). These observations suggested that *Rb* is a tumour suppressor and that loss of its function can contribute to oncogenesis. Support for this idea came from experiments in which the wild-type gene was introduced into tumour cells that lacked its function (Bookstein et al., 1990; Huang et al., 1988; Qin et al., 1992). Expression of exogenous *Rb* was found to inhibit growth, proliferation, soft agar colony formation and tumourigenicity in nude mice (Bookstein et al., 1990; Huang et al., 1988; Qin et al., 1992). Further proof of the importance of *Rb* in resisting carcinogenesis was provided by the specific mutagenesis of this gene. Although homozygous deletion of *Rb* is lethal, heterozygous mice survive and display a strong predisposition to cancer (Hu et al., 1994; Jacks et al., 1992; Lee et al., 1992; Maandag et al., 1994; Nikitin and Lee, 1996; Williams et al., 1994). These observations prove unequivocally that *Rb* is a *bona fide* tumour suppressor gene.

Having established the credentials of RB as an important tumour suppressor, it became a major priority to determine how it achieves this effect. At a cellular level, RB is involved in constraining both growth (increase in cell mass) and proliferation (increase in cell number): without it the ability of cells to shut down these functions is compromised (Weinberg, 1995; Whyte, 1995). Mammalian cells decide between proliferation and quiescence during the first two thirds of G1 phase: if growth factors are plentiful at this time they continue

through the cell cycle, but if conditions are unfavourable they withdraw from cycle and quiesce (Pardee, 1989). Before reaching the end of G1, cells become committed to complete the mitotic cycle regardless of growth conditions (Pardee, 1989). This transition to serum-independence is called the R (restriction) point (Pardee, 1989). RB serves an important function in restraining passage through the R point when growth factors are limiting (Sherr, 1994; Weinberg, 1995; Whyte, 1995). When RB function is lost, the sensitivity of cells to their normal regulatory signals is severely compromised (Sherr, 1994; Weinberg, 1995; Whyte, 1995). This constitutes a major step towards uncontrolled proliferation.

Although it is clear that RB regulates passage through the R point, many unanswered questions remain as to how this is achieved in mechanistic terms. To understand fully the complex biological effects of RB, it will be necessary to determine how it operates at the molecular level. Although some aspects of this have been characterised extensively, novel targets for RB are still being discovered (Taya, 1997). The relative contribution of each of these targets in inhibiting growth, proliferation and tumour formation will need to be established. A complete picture of how RB functions will require the careful interlinking of its various activities.

II. RB and cancer

A. Mutational inactivation of RB

People who inherit a nonfunctional allele of the *Rb* gene have an approximately 90% chance of developing retinoblastoma at an early age (Whyte, 1995). Inactivation of the remaining allele by somatic mutation seems to be a universal feature of this cancer and is probably the rate-limiting step in its initiation (Horowitz et al., 1990). Individuals who survive hereditary retinoblastoma show a strong predisposition to osteosarcomas and soft tissue sarcomas later in life: this again is associated with loss of the second *Rb* allele (Whyte, 1995). These osteosarcomas and mesenchymal tumours are less frequent than retinoblastoma in *Rb* heterozygotes, and loss of the functional copy of *Rb* may not be rate-limiting for such tumours (Whyte, 1995). Unlike humans, $Rb^{+/-}$ mice do not develop retinoblastoma: instead over 95% die 300-400 days after birth with melanotroph tumours of the intermediate pituitary lobe (Hu et al., 1994; Maandag et al., 1994; Williams et al., 1994). Sequential analyses of the initial stages of spontaneous melanotroph carcinogenesis in heterozygous mice suggest that mutation of the *Rb* gene is the initiating event of malignant transformation (Nikitin and Lee, 1996). It is not understood why murine and human *Rb* heterozygotes suffer different types of cancer. Neither is it known why melanotrophs or retinoblasts are particularly sensitive to the inactivation of RB.

Many other types of human tumour display somatic mutation of *Rb*, including osteosarcomas, small cell lung carcinomas, breast cancers, prostate and bladder carcinomas. In such cases, the patient inherits two wild-type alleles of *Rb*, but mutations arise in both copies during tumourigenesis. The most striking examples of this are the small cell lung carcinomas, where *Rb* changes are found in nearly all cases (Horowitz et al., 1990). Other types of tumour display a lower frequency of *Rb* mutation. For example, RB was found to be altered or absent in a third of bladder carcinomas that were surveyed (Horowitz et al., 1990). However, many types of tumour express apparently wild-type RB, including melanomas and colon carcinomas (Horowitz et al., 1990). Thus, mutation of *Rb* is a tumour-specific phenomenon.

B. Inactivation of RB by viral oncoproteins

A survey of human cervical carcinoma cell lines found that two out of seven bear small inactivating mutations in RB (Scheffner et al., 1991). Whereas neither of these lines were infected by human papillomavirus (HPV), each of the remaining five that expressed normal RB also contained HPV DNA (Scheffner et al., 1991). HPVs play an etiologic role in most cervical neoplasias (Vousden, 1995). The E7 oncoprotein encoded by HPV can transform established cell lines and has also been shown to bind to RB (Dyson et al., 1989; Munger et al., 1989). Some HPVs, such as HPV-16 and -18, are associated with potentially pre-cancerous genital tract lesions and a large percentage of anogenital cancers, whereas others, such as HPV-6 and -11, are associated with benign proliferative tumours with a low risk of malignant progression (e.g. condyloma acuminata) (Vousden, 1995). E7 proteins from the high risk viruses HPV-16 and -18 have higher binding affinity for RB than E7 from the lower risk types HPV-6 and -11 (Heck et al., 1992; Munger et al., 1989). Single residue substitutions in HPV-6 E7 that cause a substantial increase in affinity for RB also produce a concomitant gain in transforming activity (Heck et al., 1992; Sang and Barbosa, 1992). It is therefore likely that the ability of E7 to bind RB contributes significantly to the oncogenic capacity of HPVs. Therefore, RB function may be lost in most if not all cervical cancers; this occurs by gene mutation in the minority of HPV-negative cases and by complex formation with E7 protein in the remaining instances (Scheffner et al., 1991).

The transforming proteins of several other DNA tumour viruses can also bind RB and neutralize its function (Vousden, 1995). This property is shown by the large T antigen of simian virus 40 (SV40) (DeCaprio et al., 1988; Ewen et al., 1989; Ludlow et al., 1989; Moran, 1988) and the E1A protein of adenovirus (Whyte et al., 1988, 1989). Mutagenesis studies have shown that the regions of these oncoproteins that are necessary for binding RB are also required for their transforming properties (DeCaprio et al., 1988; Ewen et al., 1989; Moran, 1988; Whyte et al., 1989). Furthermore, the parts of RB that are needed for association with E1A and T antigen are also common sites for mutations (Hu et al., 1990). By binding to RB, these viral proteins can interfere with its normal

cellular functions and thereby mimick the effects of the *Rb* mutations that occur in many tumours.

C. Inactivation of RB by phosphorylation

RB can be switched off through phosphorylation (Hunter and Pines, 1994; Pines, 1995; Sherr, 1994; Weinberg, 1995). This constitutes a normal control mechanism that is used to regulate progress through the cell cycle (Hunter and Pines, 1994; Pines, 1995; Sherr, 1994; Weinberg, 1995). Thus, RB is underphosphorylated during the first two thirds of G1 phase and whilst in this condition it helps prevent cells from passing through the R point (Hunter and Pines, 1994; Pines, 1995; Sherr, 1994; Weinberg, 1995). Near the end of G1, if conditions are propitious, RB becomes phosphorylated at multiple sites and loses its ability to inhibit passage into S phase (Hunter and Pines, 1994; Pines, 1995; Sherr, 1994; Weinberg, 1995). Its affinity for the nuclear compartment is also diminished (Mittnacht and Weinberg, 1991). The cyclin D- and cyclin E-dependent kinases are responsible for controlling RB in this way (Hunter and Pines, 1994; Pines, 1995; Sherr, 1994; Weinberg, 1995).

The activity of cyclin D-dependent kinases is abnormally elevated in a variety of cancers and this provides another mechanism whereby RB function is lost (Bates and Peters, 1995; Hunter and Pines, 1994; Pines, 1995; Weinberg, 1995). The gene for cyclin D1 is amplified in at least 15% of primary breast cancers and an even greater proportion of squamous cell carcinomas of the neck, head, oesophagus and lung (Bates and Peters, 1995; Hunter and Pines, 1994). Furthermore, cyclin D1 RNA and protein is overexpressed in 30-40% of primary breast tumours, suggesting that gene amplification is not the only mechanism contributing to increased levels of the product (Bates and Peters, 1995). In some parathyroid adenomas and B cell lymphomas, chromosomal translocations cause overproduction of cyclin D1 (Bates and Peters, 1995; Hunter and Pines, 1994). When Epstein-Barr virus immortalizes B-lymphocytes, cyclin D2 becomes activated (Sinclair et al., 1994). The gene for cyclin-dependent kinase 4 is amplified in many glioblastomas and some gliomas (Weinberg, 1995). In addition to these diverse situations in which cyclins or their associated kinases are activated directly, many other cancers lose the function of p16 and/or p15, which are important repressors of the cyclin D-dependent kinases (Hirama and Koeffler, 1995; Hunter and Pines, 1994; Weinberg, 1995). For example, the genes for p16 and p15 are deleted in many glioblastomas, oesophageal, bladder, lung and pancreatic carcinomas, and are sometimes mutated in familial melanomas (Hirama and Koeffler, 1995; Weinberg, 1995). Thus, the cyclin D-dependent kinases become abnormally active in a broad spectrum of cancers through a variety of mechanisms. This has the effect of switching off RB.

It is therefore certain that RB function is lost in a high proportion of tumours. Indeed, it has been suggested that the control pathway involving RB may become deregulated in all human malignancies (Weinberg, 1995). This can be achieved in a variety of different ways - gene mutation, association with viral oncoproteins, or hyperphosphorylation. A good illustration of the importance of inactivating RB during tumour progression was provided by a survey of small cell lung carcinomas (Otterson et al., 1994). This study tested 55 small cell lung cancers and found that 48 lacked normal RB expression but contained wild-type p16; six out of the remaining seven lacked functional p16 (Otterson et al., 1994).

III. RB targets

A. E2F

As explained above, RB acts as a signal transducer which controls gene expression in response to the availability of growth factors. It does this by targetting a number of key transcription factors and regulating their functions. Perhaps the best characterised of these is E2F (Adams and Kaelin, 1995; La Thangue, 1994; Lam and La Thangue, 1994; Weinberg, 1996). E2F is a heterodimeric transcription factor composed of an E2F polypeptide and a DP polypeptide. In vertebrates, five E2F genes and three DP genes have been identified (Adams and Kaelin, 1995). Heterodimerization results in a synergistic increase in both the DNA-binding and transcriptional activation functions of these proteins (Bandara et al., 1993; Helin et al., 1993; Krek et al., 1993). It also enhances the ability to recognize RB (Helin et al., 1993; Krek et al., 1993). Not only does RB mask the transactivation domain of E2F, but it can exert a dominant silencing activity that represses promoters with E2F-binding sites (Weintraub et al., 1992). When growth factors are limiting RB is underphosphorylated and active; it binds to E2F and inhibits it (**Figure 1**). Following serum stimulation, RB becomes phosphorylated at multiple sites by the cyclin D-dependent kinases; this inactivates it and causes it to dissociate from E2F, thereby allowing the expression of E2F-responsive genes (Adams and Kaelin, 1995).

Table 1 lists some of the genes that contain E2F sites in their promoters. Many of these have been shown to be regulated by E2F, but it has not been proven in every case. These potential target genes can be divided into five categories. One group consists of genes encoding subunits of E2F, which suggests that autoregulation may occur. A second category contains *Rb* and the related gene *p107*, which implies further opportunities for feedback control. The next class consists of the oncogenes *B-myb* , *N-myc* and *c-myc*. Another group contains several genes that are directly involved in driving the cell cycle, including components of the cyclin-dependent kinases and the cdc25C phosphatase that activates these. The fifth and largest group consists of many genes that encode components of the DNA replication apparatus, including DNA polymerase α, the origin recognition factor HsOrc1, and several enzymes involved in nucleotide biosynthesis. A striking feature of this list is that many of the genes with E2F sites would be predicted to contribute to cellular

proliferation. This is the case for the oncogenes and for *cdc2* and the cyclins, which have a positive effect on cell

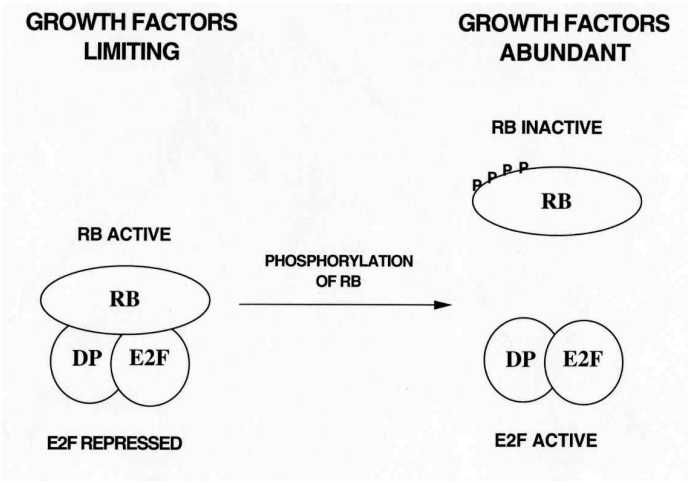

Figure 1. When growth factors are limiting, RB binds to E2F and represses its ability to activate transcription. Following serum stimulation, RB becomes hyperphosphorylated at multiple sites through the action of cyclin-dependent kinases. This inactivates RB and causes it to dissociate from E2F, which allows expression of E2F-responsive genes.

Table 1. Genes regulated by E2F

1. E2F Components
- E2F-1, -4, -5
- DP-1

2. Pocket proteins
- RB
- p107

3. Oncogenes
- B-myb
- c-myc
- N-myc

4. Genes that drive the cell cycle
- cdc2
- cyclin A
- cyclin D
- cyclin E
- cdc25C

5. Genes required for DNA replication
- DNA polymerase α
- HsOrc1
- PCNA
- topoisomerase I
- thymidylate synthase
- thymidine kinase
- ribonucleotide reductase

cycle progression. Furthermore, DNA replication is clearly a prerequisite of productive cell division. One would therefore predict that by inhibiting the expression of the batteries of genes listed in **Table 1**, through its repressive effect on E2F, RB would be able to achieve a very potent block upon proliferation.

As yet it is unclear how many of the genes with E2F sites are actually regulated by RB. The most stringent test of this is to look for changes in expression following the specific deletion of the *Rb* gene. Very few of the genes listed in **Table 1** pass this test. One study of primary mouse embryonic fibroblasts (MEFs) examined ten genes with E2F sites and found that only cyclin E and p107 synthesis were changed following homozygous inactivation of *Rb* (Hurford et al., 1997). During G0 and G1 phases, cyclin E and p107 mRNA levels were twofold higher in $Rb^{-/-}$ MEFs compared to wildtype controls (Hurford et al., 1997). The expression of *B-myb*, *cdc2*, *E2F-1*, *TS*, *RRM2*, *cyclin A2*, *DHFR*, *TK*, *DNA polymerase* α, and *Cdc25C* genes were unaffected by the RB-knockout (Hurford et al., 1997). Another study found a ten-fold increase in cyclin E protein and a two- to fourfold increase in cyclin D1 when $Rb^{-/-}$ MEFs were compared to the corresponding $Rb^{+/+}$cells (Herrera et al., 1996). The surprising lack of effect that deleting *Rb* has upon most E2F target genes is probably due to redundancy in the RB family. RB has two close relatives called p107 and p130 (**Figure 2**). These three proteins show substantial similarity in primary sequence and are thought to perform overlapping functions (Whyte, 1995). They are most highly related in a bipartite domain called the pocket, which is responsible for binding E1A and E2F (Whyte, 1995). As a consequence, they are sometimes referred to as the pocket proteins. It may be that when *Rb* is deleted, its relatives can assume many of its functions. Indeed, a double knockout of *Rb* and *p107* has a more severe phenotype than single knockouts of either (Lee et al., 1996). This is certainly consistent with a functional overlap. However, p107 and p130 are much more similar to each other than they are to RB. Indeed, whereas RB specifically targets E2F-1, -2 and -3, p107 and p130 appear to bind only E2F-4 and E2F-5 (Weinberg, 1995). A $p107^{-/-}/p130^{-/-}$ double knockout strongly derepresses *B-myb* but has no effect on *cyclin E* (Hurford et al., 1997). It therefore seems that p107 and p130 can only assume some of the functions that are performed by RB. The most striking difference between the pocket proteins is that *p107* and *p130* have never been found to be mutated in cancers.

Even if many of the genes listed in **Table 1** are not subject to control by RB, repressing the synthesis of cyclins E and D1 through its action on E2F should in itself be sufficient to provide a brake upon cell cycle progression and hence proliferation. Indeed, under certain circumstances dominant-negative mutants that abolish E2F activity can block the cell cycle (Dobrowliski et al., 1994;Wu et al., 1996). However, this is by no means the

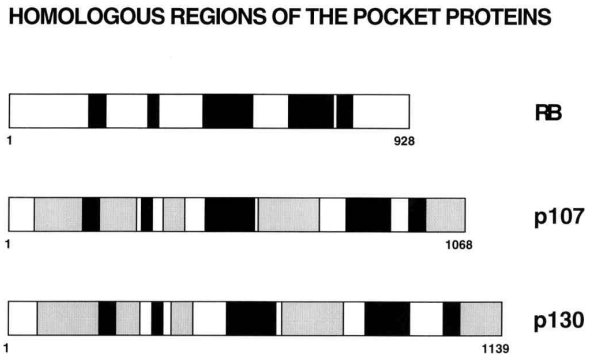

Figure 2. Regions of homology between all three pocket proteins are shown as black blocks. Regions that are homologous between p107 and p130, but are not shared by RB, are shaded.

whole story. In molar terms, RB is two orders of magnitude more abundant than E2F within the cell (Weinberg, 1995). This suggests that RB regulates additional targets besides E2F. Indeed, one study found that the proliferation rate of epithelial cells is not affected when endogenous E2F is inactivated using dominant-negative mutants (Bargou et al., 1996). It is therefore highly likely that E2F-independent pathways contribute to the physiological effects of RB. A diverse array of cellular proteins have been shown to bind RB (Taya, 1997; Whyte, 1995). Some of these are listed in **Table 2**. They include the tyrosine kinase c-Abl (Welch and Wang, 1993) and the factors BRM and BRG1 that are involved in controlling nucleosome structure (Dunaief et al., 1994; Singh et al., 1995). Several others are transcription factors. I shall concentrate on two of these, UBF and TFIIIB, which may have key roles in controlling cell growth.

Table 2. Some of the cellular proteins that interact with RB

PROTEIN	FUNCTION
E2F	Transcription factor
UBF	Transcription factor
TFIIIB	Transcription factor
Elf-1	Transcription factor
PU.1	Transcription factor
Brm	Reorganising chromatin structure
BRG1	Reorganising chromatin structure
MyoD	Transcription factor
ATF-2	Transcription factor
MDM2	Oncoprotein
c-Abl	Tyrosine kinase
D-type cyclins	Cdk activators
PP1	Protein phosphatase

B. UBF

A nucleolar transcription factor called UBF was identified as an RB-binding protein by screening a cDNA expression library with purified RB as probe (Shan et al., 1992). Subsequent studies have confirmed the ability of recombinant RB to bind specifically to UBF (Cavanaugh et al., 1995; Voit et al., 1997). Furthermore, immunoprecipitation assays with cellular extracts demonstrated that endogenous RB and UBF associate when present at physiological ratios (Cavanaugh et al., 1995). The identification of UBF as a target for RB was somewhat unexpected. All previous studies on RB had concentrated on genes that are transcribed by RNA polymerase II (pol II), which synthesizes the messenger RNA (mRNA) in cells. However, UBF is only involved in regulating transcription by pol I, the polymerase responsible for synthesizing large ribosomal RNA (rRNA). UBF binds to the promoters of rRNA genes and stimulates transcription in several ways; it helps fold the DNA and it recruits pol I and an essential factor called SL1 or TIF-IB (Reeder et al., 1995). *In vitro* experiments demonstrated that recombinant RB can indeed inhibit the synthesis of large rRNA by pol I (Cavanaugh et al., 1995; Voit et al., 1997). Whereas RB represses rRNA production in the presence of UBF, it does not affect the low level of basal transcription that occurs in a UBF-depleted system (Cavanaugh et al., 1995). RB diminishes specifically the ability of UBF to bind to DNA (Voit et al., 1997). The physiological relevance of these results was confirmed by immunofluorescence analyses of intact cells (Rogalsky et al., 1993; Cavanaugh et al., 1995). This approach allows one to visualise the nucleolus, which is the site of synthesis of large rRNA. It was found that RB accumulates in the nucleolus when cells stop growing, in parallel with a decrease in pol I activity (Rogalsky et al., 1993; Cavanaugh et al., 1995). Furthermore, immunoprecipitation experiments showed that the interaction between RB and UBF increases when cells down-regulate pol I transcription as their rate of growth decreases (Cavanaugh et al., 1995). Thus, there is a clear *in vivo* correlation between growth arrest, the association of RB with UBF, and the repression of rRNA synthesis.

C. TFIIIB

Although 5.8S, 18S and 28S rRNAs are made by pol I as a single precursor transcript, the 5S rRNA is made separately by pol III, the largest and most complex of the eukaryotic RNA polymerases (White, 1994). 5S rRNA synthesis is independent of E2F and UBF, but is nevertheless repressed by RB (White et al., 1996; Larminie et al., 1997). Indeed, RB appears to be capable of inhibiting the production of all pol III products, including transfer RNA (tRNA), the U6 small nuclear RNA (snRNA) that is required for splicing, and the adenoviral VA RNAs that are involved in subverting the host cell's

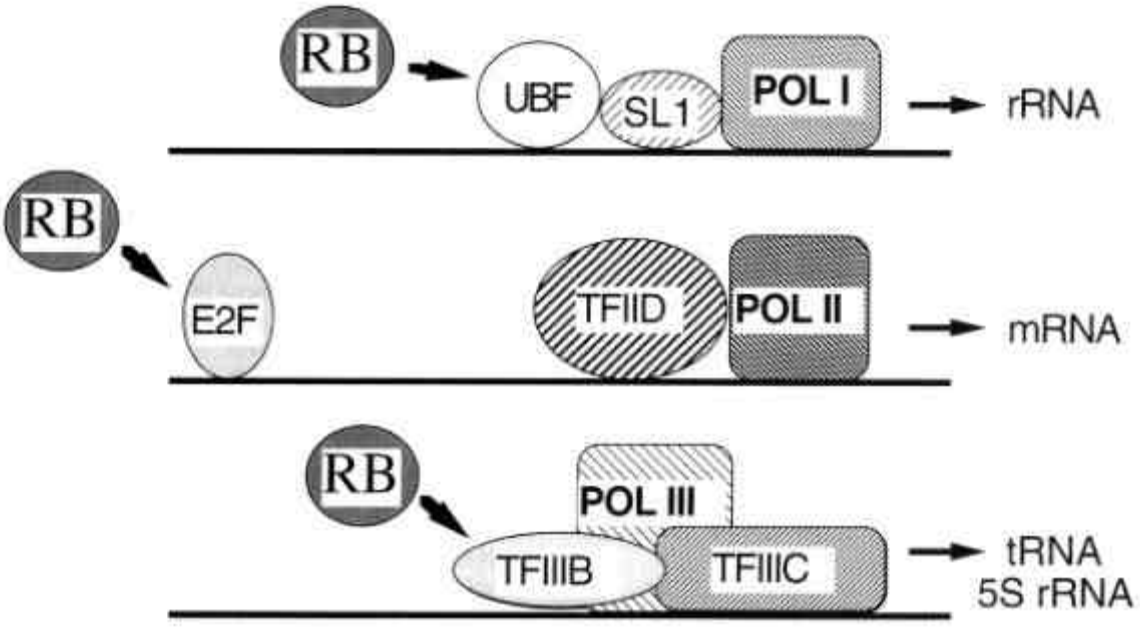

Figure 3. RB can regulate transcription by all three nuclear RNA polymerases. Pol I synthesizes large rRNA; pol II synthesizes mRNA; and pol III synthesizes tRNA and 5S rRNA. A simplified basal transcription complex is drawn for each polymerase; additional basal factors are required that are not shown in the figure. RB represses pol I via the factor UBF. RB represses a subset of pol II templates via gene-specific regulatory factors such as E2F. RB represses pol III via the general factor TFIIIB.

translational apparatus (White et al., 1996; Larminie et al., 1997). Initial evidence that this is the case came from biochemical assays which tested whether RB can regulate pol III in a cell-free system. We found that adding recombinant RB to a system reconstituted using fractionated factors repressed expression of every pol III template tested, whereas control protein had little or no effect (White et al., 1996). Support for the in vivo relevance of these observations came from transient transfection experiments, which showed that overexpressing RB can repress pol III transcription without affecting a control promoter (White et al., 1996). These results demonstrated for the first time that high levels of RB can inhibit pol III activity.

Overexpressing proteins at abnormally elevated levels can sometimes force proteins into artifactual interactions. It was therefore important to determine whether RB plays a significant role in controlling pol III when present at physiological concentrations within a cell. To begin to address this, we compared two human osteosarcoma cell lines; SAOS2, which expresses only a truncated nonfunctional form of RB, and U2OS, which contains wild-type RB. SAOS2 cells were shown to express a transfected pol III template 5-fold more actively than U2OS cells (White et al., 1996). Transcription assays carried out using extracted proteins confirmed the higher activity of the pol III factors from the RB-negative SAOS2 cells (White et al., 1996). As a more rigorous test of the function of endogenous RB, we made use of knockout mice in which the the *Rb* gene had been inactivated by site-directed mutagenesis. Nuclear run-on assays were used to measure directly the transcription of endogenous genes in intact nuclei from primary MEFs of these RB-knockout mice. We found that tRNA and 5S rRNA synthesis by pol III is 5-fold more active in the $Rb^{-/-}$ cells than in equivalent fibroblasts from wild-type mice (White et al., 1996). In contrast, the total level of pol II transcription is not increased when the *Rb* gene is deleted (White et al., 1996). *In vitro* assays with extracted factors again established that the increased production of tRNA and 5S rRNA in the RB-negative MEFs is due to a more active pol III transcription apparatus (White et al., 1996). Since the only genetic difference between the $Rb^{+/+}$ and the $Rb^{-/-}$ fibroblasts is the presence of the *Rb* gene, these results established that endogenous RB plays a very major role in suppressing the level of pol III transcription *in vivo*.

RB appears to regulate tRNA and rRNA synthesis by targetting a factor called TFIIIB (Larminie et al., 1997). TFIIIB is a multisubunit complex that contains the TATA-binding protein (TBP) and at least two additional polypeptides (Rigby, 1993; White, 1994). Its function is to recruit pol III to the appropriate promoters and position it at the transcription start site (Kassavetis et al., 1990; White, 1994). We found that recombinant RB interacts with TFIIIB and represses it specifically (Larminie et al.,

1997). Furthermore, immunoprecipitation and cofractionation experiments indicated that a population of endogenous RB molecules associates with TFIIIB at physiological concentrations (Larminie et al., 1997). This interaction is diminished or abolished in SAOS2 osteosarcoma cells, which contain only a truncated mutant form of RB (Larminie et al., 1997). The activity of TFIIIB is elevated specifically in primary fibroblasts from RB-deficient mice (Larminie et al., 1997). These results established that TFIIIB is a target for repression by RB (**Figure 3**). This conclusion fits well with previous data indicating that TFIIIB activity rises as cells progress from G1 into S phase, the time when RB is silenced through hyperphosphorylation (White et al., 1995).

A subsequent investigation by Chu et al. (1997) provided independent support for these analyses. This study confirmed that overexpressing RB represses pol III transcription in transfected cells and in vitro (Chu et al., 1997). Consistent with the earlier investigations, RB was shown to bind to TFIIIB (Chu et al., 1997). Furthermore, clustered substitutions in RB that disrupt the interaction with TFIIIB also prevent repression (Chu et al., 1997). In addition, Chu et al. (1997) reported an interaction between overexpressed RB and another pol III factor called TFIIIC2. A model was proposed in which RB utilises distinct domains to bind either TFIIIB or TFIIIC2 (Chu et al., 1997). However, there was little correlation between TFIIIC2 binding and the ability of RB mutants to repress pol III transcription (Chu et al., 1997). Furthermore, there is no evidence that TFIIIC2 and RB interact when present at physiological ratios. Chu et al. (1997) concluded that TFIIIB is the principal target for RB-mediated repression of pol III, but that a subsidiary interaction with TFIIIC2 may also contribute to the effect.

Although it has been shown that RB binds to one or more of the general pol III factors, it remains to be determined how this leads to transcriptional repression. One possibility is that RB blocks interactions with promoter DNA. Precedent for this is provided by the pol I system, where RB interferes with the DNA-binding properties of UBF (Voit et al., 1997). An alternative is that RB disrupts the structure of TFIIIB in some way. A growth suppressor called Dr1 has been shown to use this mechanism (White et al., 1994). Dr1 inhibits tRNA synthesis both in vitro and in vivo (White et al., 1994; Kim et al., 1997). It achieves this by displacing one of the essential subunits of TFIIIB from its interaction with TBP (White et al., 1994). Other possible mechanisms might involve RB disrupting the protein-protein interactions between TFIIIB and TFIIIC or pol III. Order of addition experiments showed that the pol III factors remain susceptible to RB even after they have been assembled into a stable preinitiation complex on the VA_I promoter (Larminie et al., 1997).

TFIIIB is required for all pol III transcription (White, 1994; Willis, 1993). Therefore by repressing TFIIIB, RB can provide blanket repression of all pol III templates. This contrasts strongly with the situation for pol II, where only a small proportion of promoters, such as those with E2F sites, are controlled by RB. The majority of genes that are transcribed by pol II are not affected directly by the presence of RB (White et al., 1996). Therefore, RB is a gene-specific regulator of pol II but a general regulator of pol III. This distinction is meaningless in the pol I system, since pol I only transcribes a single highly reiterated template that encodes the large rRNA.

The number of genes that are controlled by E2F is relatively small and very few of these become activated in $Rb^{-/-}$ knockouts (Herrera et al., 1996; Hurford et al., 1997). UBF probably regulates a larger number of genes, since there are ~400 copies of the large rRNA template in diploid human cells (Long and Dawid, 1980). It remains to be determined whether these are affected by knocking out RB. The number of promoters that require TFIIIB exceeds this by over three orders of magnitude. Thus, a diploid human cell contains around a million Alu genes, 2600 tRNA genes, 600 5S rRNA genes, 200 U6 snRNA genes and a range of other less abundant pol III templates (White, 1994). All of these need TFIIIB to be expressed (White, 1994). The tRNA and 5S genes have been shown to be activated in $Rb^{-/-}$ knockouts, whereas the other classes have yet to be tested in this way (White et al., 1996). Since deleting *Rb* results in a five-fold increase in tRNA and 5S rRNA production (White et al., 1996), it is highly likely that the majority of these genes are subject to repression by RB. These observations suggest that the pol III templates constitute by far the largest category of genes that are controlled directly by RB.

IV. Control of growth and proliferation by RB

Both the growth (increase in mass) and proliferation (increase in number) of cells are suppressed by RB. It is essential that these two processes are coordinated, because a significant imbalance can trigger apoptosis (Kung et al., 1993; Qin et al., 1994; Rueckert and Mueller, 1960; Shan and Lee, 1994). In order to maintain a constant size, a cell must ensure that all its components are duplicated at a similar rate. Thus, DNA content and protein levels generally increase in parallel (Stanners et al., 1979) and attempts to dissociate them with specific inhibitors can have lethal consequences (Kung et al., 1993). The control of proliferation by RB can be largely explained by its ability to regulate E2F. As described above, E2F regulates a range of pol II-transcribed genes that promote cell cycle progression (Adams and Kaelin, 1995; La Thangue, 1994; Lam and La Thangue, 1994; Weinberg, 1996). These include several genes that are required for DNA replication, such as those encoding DNA polymerase α and the replication origin-binding protein HsOrc1, as well as genes that drive the cell cycle, such as cyclin A and *cdc2* (Adams and Kaelin, 1995; Weinberg, 1996). By repressing some of these through its inhibitory effect on E2F, RB can often

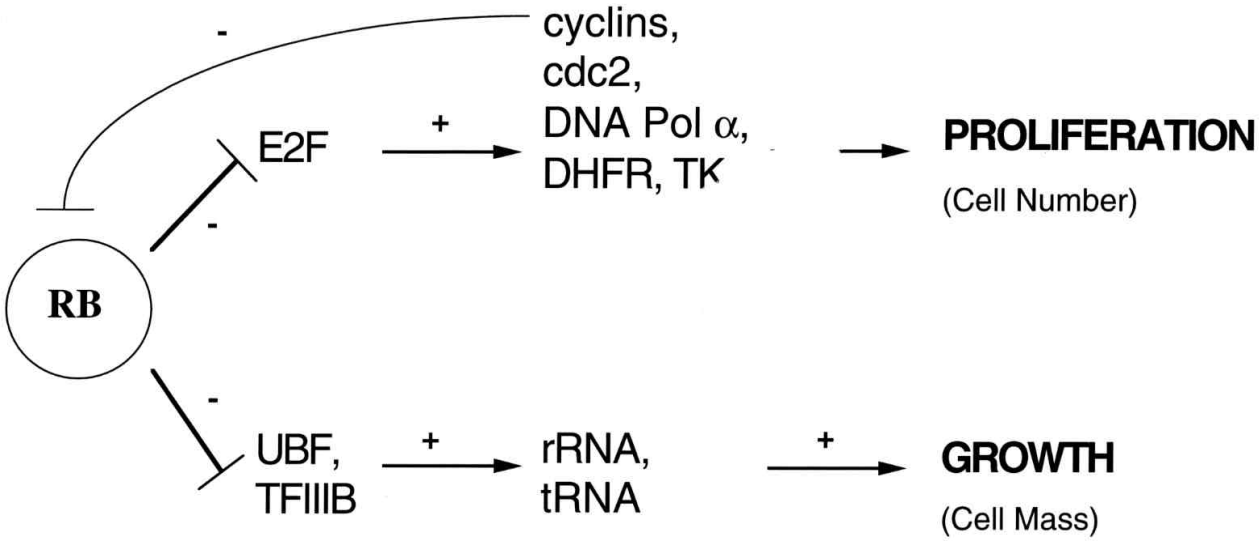

Figure 4. Mechanisms that may enable RB to restrict cell growth and proliferation. E2F promotes cell cycle progression. It appears to do this by activating the synthesis of proteins required for DNA replication, such as thymidine kinase (TK), dihydrofolate reductase (DHFR) and DNA polymerase α, as well as proteins that drive the cell cycle, such as cdc2 and cyclins D and E. By repressing E2F, RB may limit the production of these products and therefore provide a brake on proliferation. UBF and TFIIIB are required for the synthesis of rRNA and tRNA, essential raw materials for protein synthesis. By repressing these transcription factors, RB may be able to limit the rate of translation and therefore provide a brake on cellular growth.

provide a brake on DNA replication and passage through the cell cycle. However, the genes regulated by E2F are primarily involved in controlling proliferation and provide few obvious links to the control of growth. Control of E2F on its own may therefore be insufficient to achieve a balanced regulation of both growth and proliferation. One could imagine that growth is somehow tied to the cell cycle, so that regulating the latter is sufficient to achieve indirect control of the former. However, most of the available evidence argues against this (Nasmyth, 1996). In fact, in bacteria and in yeast the dependence works primarily the other way round, with cell cycle progression requiring attainment of a critical mass (Nasmyth, 1996). The basic principles observed in microrganisms are likely to be conserved in higher orders. For example, murine fibroblasts must reach a certain mass before they can initiate DNA synthesis (Killander and Zetterberg, 1965). Furthermore, a survey of mammalian cell types found that in most cases size continues to increase when DNA synthesis is inhibited using aphidicolin (Kung et al., 1993). HeLa cells provided a striking exception, and in this line biosynthesis and growth decrease in response to the cell cycle block (Kung et al., 1993). HeLa cells are highly abnormal and it is likely that their anomalous behaviour does not provide a reliable indicator of the control mechanisms that operate in most animal cells. In general, the growth of mammalian cells appears not to depend on chromosome duplication, at least in the short term. Thus, the control of proliferation seems insufficient to explain fully the ability of RB to inhibit cell growth. One possibility is that this is achieved by regulating the production of tRNA and rRNA, which are major determinants of biosynthetic capacity (Nasmyth, 1996; White, 1997). (**Figure 4**).

A substantial weight of evidence shows that the regulation of protein synthesis is an important aspect of growth control. When cells quiesce, tRNA and rRNA levels decrease, polysomes disperse into free ribosomes, and the overall rate of protein accumulation is reduced. Following mitogenic stimulation, the production of tRNA, rRNA, ribosomal proteins and translation factors accelerates and protein synthesis increases before cells reach S phase (Clarke et al., 1996; Johnson et al., 1974; Kief and Warner, 1981; Mauck and Green, 1974; Redpath and Proud, 1994; Rosenwald, 1996a,b; Stanners et al., 1979; Tatsuka et al., 1992). Ribosome content is proportional to the rate of growth (Kief and Warner, 1981). Indeed, careful measurements in animal cells have demonstrated that growth rate is directly proportional to the rate of protein accumulation (Baxter and Stanners, 1978). The main determinant of protein accumulation is translation, although turnover also makes a significant contribution (Baxter and Stanners, 1978). A 50% reduction in the rate of protein synthesis is sufficient to cause proliferating cells to withdraw from cycle and quiesce (Brooks, 1977; Ronning et al., 1981). Translation is clearly dependent on the availability of tRNA and rRNA. By limiting the production of these, RB may be able to suppress the level of protein synthesis, which could in turn provide a brake on cellular growth. As yet, this

should still be regarded as speculation, although it must be true to some extent. However, it remains to be determined to what degree the activities of pols I and III are ever limiting for growth under physiological conditions. A good indication that they may be came from a study carried out in *S. cerevisiae.* It was found that in this yeast a two-fold reduction in the level of initiator tRNA results in a three-fold increase in doubling time (Francis and Rajbhandary, 1990). If the same were true of a mammalian cell, then the 5-fold depression in tRNA synthesis that is imposed by RB must surely have a substantial impact upon the rate of growth. During tumour development, when RB function is compromised, the release of pol III from this major constraint may be an important step towards neoplasia.

V. RNA polymerase III and cancer

If RB plays an important physiological role in restraining pol III transcription, then one would expect to find that pol III activity is elevated in a broad range of cancers, where RB function is compromised. This is indeed the case. Many studies have observed that the abundance of pol III transcripts is abnormally elevated in transformed and tumour cells. This was first discovered by Kramerov et al. (1982), who examined carcinoma and plasmacytoma lines. Subsequent work extended the observation to include cells that have been transformed by DNA tumour viruses, RNA tumour viruses, or chemical carcinogens (Brickell et al., 1983; Carey and Singh, 1988; Carey et al., 1986; Kramerov et al., 1990; Lania et al., 1987; Majello et al., 1985; Ryskov et al., 1985; Scott et al., 1983; Singh et al., 1985; White et al., 1990). This activation is very general, but not universal, there being a few examples of transformed lines that do not display the characteristic increase in pol III transcript levels (Ryskov et al., 1985; Scott et al., 1983). A tight causal link between pol III activation and transformation is suggested by the fact that two fibroblast lines transformed by temperature-sensitive mutants of the SV40 large T antigen down-regulate pol III transcription at the non-permissive temperature whilst reverting to normal morphology and phenotype (Scott et al., 1983). The abundance of pol III transcripts varies substantially between different SV40-transformed lines and the highest levels correlate with progression to a more tumorigenic phenotype (Scott et al., 1983; White et al., 1990).

A recent study provided convincing evidence that a pol III product is induced in rodent tumours. This investigation examined a pol III transcript called BC1, which is unusual because it is normally only expressed in neurons (DeChiara and Brosius, 1987). The function of BC1 has yet to be determined. Northern analysis showed BC1 expression in breast carcinomas, colonic adenocarcinomas and skin fibrosarcomas, but not in the corresponding untransformed tissues (Chen et al., 1997). In situ hybridisation studies of theses tumours confirmed the presence of BC1 RNA in the neoplastic cells, whereas it was absent from the surrounding tissues (Chen et al., 1997). Although the fibrosarcomas and adenocarcinomas were induced by local inoculation with cells that had been treated with chemical carcinogens, the breast carcinoma analysed was a primary tumour induced by ras (Chen et al., 1997). Similar studies have shown that BC200 RNA, the primate analogue of BC1, is expressed in many, but not all, primary human tumours (Chen et al., 1997). Like BC1, BC200 RNA is found exclusively in the malignant cells and not in the adjacent normal tissue (Chen et al., 1997). Thus, abnormal activation of pol III expression is a frequent feature of tumours in vivo.

As already explained, RB function is lost in many human cancers through a variety of mechanisms. It will be important to determine to what extent this is responsible for activating pol III. Deletion and substitution analyses have demonstrated that the RB sequences which control pol III correspond to the domains that are mutated frequently in tumours (White et al., 1996; Chu et al., 1997). Indeed, the minimal region of RB that is necessary to regulate cell growth and proliferation is also sufficient to repress transcription by pol III (White et al., 1996). Several examples have been characterised of highly localised mutations that inactivate RB in human cancers. For example, in one small cell lung carcinoma a single base change in a splice acceptor site gave rise to an RB polypeptide that lacked the 35 amino acids encoded by exon 21 (Horowitz et al., 1990). In another small cell lung carcinoma, a point mutation created a stop codon and a novel splice donor site within exon 22, thereby eliminating 38 residues from the product (Horowitz et al., 1990). A third inactivating mutation from a small cell lung cancer resulted in a single amino acid substitution at codon 706 (Kaye et al., 1990). We tested the ability of each of these three naturally occurring mutants to regulate pol III transcription and found that repression was lost in every case (White et al., 1996). Although this is clearly a limited survey, it nevertheless demonstrates a correlation between the function of RB as a tumour suppressor and its ability to control pol III.

As described above, the E1A oncoprotein of adenovirus and the large T antigen of SV40 bind and neutralize RB, a property which is important for their transforming capabilities (DeCaprio et al., 1988; Ewen et al., 1989; Ludlow et al., 1989; Moran, 1988; Whyte et al., 1988; Whyte et al., 1989). Both E1A and T antigen can also stimulate the rate of pol III transcription (Loeken et al., 1988; Patel and Jones, 1990; White et al., 1996). We found that E1A and T antigen can release pol III from repression by RB (White et al., 1996). These viral oncoproteins are believed to regulate gene expression through multiple mechanisms, but one way in which they can stimulate pol III involves overcoming the physiological constraint that is normally provided by RB. In cells transformed by E1A or T antigen, the loss of RB function is likely to contribute substantially to an activation of pol III transcription.

VI. Components of the translation apparatus are often deregulated in cancer cells

There is a multitude of documented examples in which the translation machinery has become deregulated following transformation (Rosenwald, 1996a). This fact provides strong support for the contention that the control of protein synthesis is an important aspect of growth regulation. For example, fibroblasts transformed by polyoma virus were found to synthesize protein more rapidly than normal parental cells and have lost control over ribosome production (Stanners et al., 1979). In untransformed revertants of these fibroblasts, correct regulation is recovered (Stanners et al., 1979). One study compared levels of expression of ribosomal proteins in colorectal tumours from eight different individuals with normal colonic mucosa from the same patients (Pogue-Geile et al., 1991). In every case, the adenocarcinomas overexpressed all six ribosomal protein transcripts that were tested (Pogue-Geile et al., 1991). The levels of these mRNAs were also generally elevated in adenomatous polyps, the presumed precursors of the carcinomas (Pogue-Geile et al., 1991). This implies that increased ribosomal protein production occurs early during the development of these tumours, perhaps concomitant with the onset of neoplasia. Colorectal tumours and tumour-derived cell lines were also reported to produce higher levels of rRNAs than normal colonic mucosa, consistent with a general increase in ribosomal components (Pogue-Geile et al., 1991). Constitutive expression of EF-1α, a translation factor that catalyses the attachment of aminoacyl-tRNAs to the ribosome, makes fibroblasts highly susceptible to transformation by 3-methylcholanthrene or ultraviolet light (Tatsuka et al., 1992). These observations suggest that deregulation of the protein synthesis machinery can predispose cells to malignant transformation.

Perhaps the most striking demonstration that the translation apparatus becomes activated during tumourigenesis came from a recent study that used serial analysis of gene expression (SAGE) to document the expression profiles of 45,000 genes in gastrointestinal tumours (Zhang et al., 1997). Only 108 pol II transcripts were found to be expressed at higher levels in primary colon cancers relative to normal colonic epithelium (Zhang et al., 1997). Of these, 48 encode ribosomal proteins and 5 encode translation elongation factors (Zhang et al., 1997). Similar results were obtained with pancreatic cancers (Zhang et al., 1997). These observations provide compelling evidence that deregulation of protein synthesis is intimately linked with tumour formation.

Several studies have shown that the abnormal activation of translation factors is actually sufficient to trigger neoplastic transformation (Rosenwald, 1996a). eIF-4E, the mRNA cap-binding protein, and eIF-2, which brings initiator methionine tRNA to the 40S ribosomal subunit, appear particularly important in this regard. eIF-4E is the least abundant of the translation initiation factors and is rate limiting for protein synthesis (Duncan et al., 1987). As such, it is of key importance in controlling the rate of translation. Overexpression of eIF-4E in various types of fibroblast stimulates growth and proliferation and induces morphological transformation (Lazaris-Karatzas et al., 1990; Lazaris-Karatzas and Sonenberg, 1992). Cells with abnormally high eIF-4E levels also induce tumours in nude mice (Lazaris-Karatzas et al., 1990; Lazaris-Karatzas and Sonenberg, 1992). Similarly, overexpression of eIF-4E in HeLa cells accelerates growth and results in the formation of overcrowded multilayered foci (De Benedetti and Rhoads, 1990). Conversely, reducing the level of eIF-4E with antisense RNA inhibits the growth of HeLa cells (De Benedetti et al., 1991) and the tumorigenicity of ras-transformed fibroblasts (Rinkerr-Schaffer et al., 1993).

In serum-starved cells, the recycling of eIF-2 is inhibited by phosphorylation of its α subunit, thereby impairing translational initiation (Redpath and Proud, 1994). Mutation of eIF-2α so that it can no longer be phosphorylated causes malignant transformation of NIH 3T3 cells (Donze et al., 1995). Malignancy can also be induced by dominant negative forms of the eIF-2α kinase, whereas the wild-type kinase inhibits growth when overexpressed in mammalian fibroblasts or yeast (Chong et al., 1992; Koromilas et al., 1992; Meurs et al., 1993). The eIF-2α kinase (which is also referred to as PKR) is inducible by interferon and is likely to contribute to the action of interferons as growth inhibitors and anti-tumour agents (Clemens, 1992; Lengyel, 1993).

The cellular activity of eIF-2α and eIF-4E increases in response to various oncogenes (Rosenwald, 1996b). The levels of eIF-2α and eIF-4E mRNA and protein are elevated in fibroblasts that overexpress c-myc (Rosenwald et al., 1993a,b; Rosenwald, 1995; Jones et al., 1996; Rosenwald, 1996b). This is associated with accelerated rates of protein accumulation and cell growth (Rosenwald, 1996b). v-src and v-abl have similar effects on eIF-2α and eIF-4E, but this may reflect the ability of these oncoproteins to stimulate c-myc production (Rosenwald et al., 1993a,b; Rosenwald, 1995, 1996b). v-src and ras also increase the phosphorylation of eIF-4E, which can activate its function (Frederickson et al., 1991; Rinker-Schaeffer et al., 1992). In addition, ras can deregulate eIF-2 by inducing an inhibitor of eIF-2α kinase (Mundschau and Faller, 1992). These many examples provide abundant evidence that abnormal stimulation of the translation apparatus is a frequent characteristic of transformed cells. This supports the idea that elevated rates of protein synthesis are necessary to sustain the development of many tumours.

VII. c-Myc: a foot in both camps?

The oncogene c-myc may have a foot in both the growth and proliferation camps. The c-myc promoter contains an E2F binding site and is subject to repression by RB (Hiebert et al., 1989; Zou et al., 1997). If c-myc expression is prevented using antisense technology, cells stop growing and arrest in G1 phase (Heikkila et al., 1987; Prochownik et al., 1988). Myc has been shown to

promote cell cycle progression by stimulating production of cdc25 and the activation of cyclin D and E-dependent kinases (Galaktionov et al., 1996; Steiner et al., 1995). In addition, the activation of c-myc in rodent fibroblasts results in an increase in the abundance of the translation

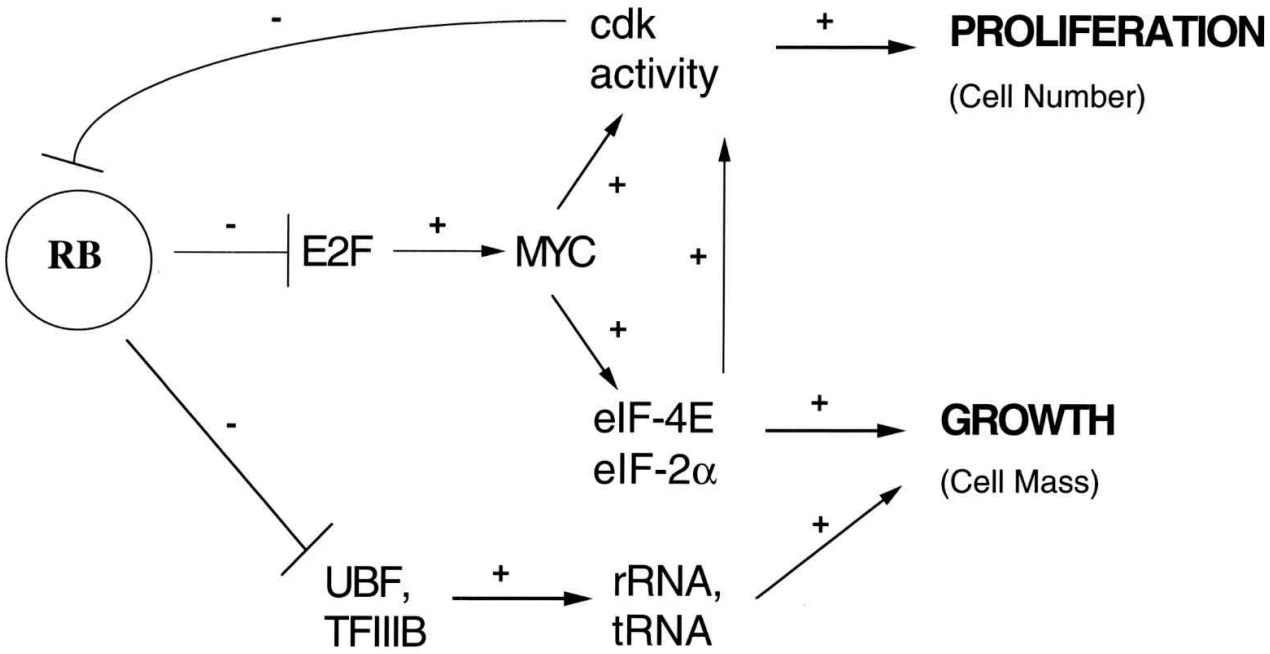

Figure 5. Control of c-myc production may provide an additional mechanism for RB to influence both growth and proliferation. Myc can stimulate cell cycle progression through the activation of cyclin-dependent kinases (cdks). Myc may promote protein synthesis and cellular growth by increasing the production of the translation initiation factors eIF-2α and eIF-4E. eIF-4E can stimulate cyclin D1 production. This, in turn, might be expected to switch off RB and thereby activate UBF and TFIIIB. The *c-myc* promoter contains a binding site for E2F and may therefore be subject to repression by RB. Inhibiting the production of myc may provide a mechanism for RB to control both growth and proliferation.

initiation factors eIF-2α and eIF-4E (Jones et al., 1996; Rosenwald, 1996a,b; Rosenwald et al., 1993a,b). The elevated concentrations of eIF-2α and eIF-4E that accompany activation of c-myc correlate with a rise in the net rate of protein synthesis and accelerated growth (Rosenwald, 1996b). Furthermore, overexpression of eIF-4E results in a selective increase in cyclin D1 production (Rosenwald et al., 1993a). This, in turn, might be expected to switch off RB and thereby activate UBF and TFIIIB. By silencing the c-myc promoter, RB may be able to suppress both proliferation and growth. (**Figure 5**).

VIII. Discussion

There is substantial evidence that the deregulation of translation is an important aspect of neoplastic transformation. Rapid growth undoubtedly requires elevated rates of protein accumulation; without it, a tumour would be unable to maintain its increase in mass. For rapidly dividing cells to sustain a high rate of translation will require efficient production of tRNA and rRNA. In addition to the clear correlation between protein accumulation and growth, constitutively elevated translation might drive a population to proliferate. This could work as follows: unbalanced growth in the absence of cell replication is likely to trigger apoptosis; such conditions may select for cells that have acquired the ability to bypass the apoptotic pathway and multiply continuously.

In this review, I have drawn a clear distinction between growth and proliferation. I have also argued that separate mechanisms appear to be involved in controlling these processes. However, the point must be emphasised that growth and proliferation are intimately linked and there is undoubtedly substantial cross-talk between the two. Many potential examples of this can be envisaged. For example, E2F is involved in regulating the genes for cyclins A, D and E. Since these cyclins control kinases that can inactivate RB, there is obvious potential for a feedback loop. Moreover, through its action on cyclins and hence RB, E2F might be expected to influence UBF and TFIIIB

activity, and hence translation and growth. As explained above, c-myc may also have direct impact upon both the cell cycle machinery and the translation apparatus. It is clearly of benefit to the cell to have growth and proliferation coordinated by unifying control mechanisms. RB may provide such a regulatory switch.

RB is a tumour suppressor of major importance, with a key role in controlling cell growth and proliferation. By regulating E2F, RB has the potential to inhibit the synthesis of gene products that are necessary for DNA synthesis, chromosomal replication and cell cycle progression. By repressing UBF and TFIIIB, RB can reduce the production of tRNA and rRNA. This may allow it to limit the rate of protein accumulation, which will provide a brake on cellular growth. Coregulating these essential processes may allow RB to achieve the necessary balance between growth and proliferation (White, 1997). Many other molecular targets have been identified for RB and these provide additional controls over cellular activity (Taya, 1997; Whyte, 1995). Regulating a range of key components may enable RB to coordinate a number of disparate processes. The loss of these controls will undoubtedly constitute a major step towards tumour development.

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