A thesis entitled

The regulation of RNA polymerase I and RNA polymerase III transcription by the pocket proteins

Presented by

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Abstract

RB has been shown to repress pol III transcription by targeting the pol III-specific general factor TFIIIB. Work presented here demonstrates that RB binds to the BRF subunit of TFIIIB. The binding interaction between these two proteins has been shown to require the RB large pocket domain and the amino-terminal repeats of BRF. In addition, the mechanism whereby RB represses pol III transcription has been established; namely, RB does not compromise the interactions within TFIIIB, but instead disrupts the association between TFIIIB and TFIIIC and between TFIIIB and pol III.

In human cancers, RB is generally inactivated by one of three means: hyperphosphorylation, mutation or viral oncoprotein sequestration. SV40-transformed cell lines displayed a diminished interaction between RB and BRF in comparison to the untransformed parental line. The association was also alleviated if RB was mutated or phosphorylated. In addition, mutant RB forms were unable to disrupt the interaction between TFIIIB and TFIIIC and between TFIIIB and pol III.

RB has also been shown to repress pol I transcription. The current work demonstrated an interaction between RB and the pol I transcription factors UBF and SL1. The human and *Xenopus* UBF-binding domain in RB was identified as the large pocket, whilst xUBF was shown to bind RB through HMG boxes 1 and 2.

The RB-related proteins p107 and p130 were investigated for their ability to undertake RB functions. Indeed, p107 and p130 were both able to interact with the pol I transcription factor UBF and the BRF subunit of the pol III transcription factor TFIIIB.

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Abbreviations

aa	amino acids
Ab	antibody
Amp	Ampicillin
APS	Ammonium persulphate
ATP	Adenosine triphosphate
bp	base pairs
BRF	TFIIB-related factor
BSA	Bovine Serum Albumin
CAT	chloramphenicol acetyltransferase
cdk/CDK	cyclin dependent kinases
CKII	caesin kinase II
CTP	Cytosine triphosphate
DEPC-H_2O	diethylpyrocarbonate water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetate
GST	Glutathione-S-transferase
rGTP	Guanine triphosphate
h	human
HDAC	histone deacetylase

Hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
His	Histidine
HMG	high mobility group
HPV-16	human papillomavirus type –16
ICR	intragenic control regions
IGS	intragenic spacer
INK4	inhibitor of cdk4
IP	Immunoprecipitation
IPTG	Isopropylthiogalactosidase
kD	kilo Dalton
LB	liquid broth
mA	milliamps
μg	micrograms
mg	milligrams
μl	microlitres
ml	millilitres
mm	millimeter
mM	millimolar
M	molar
mRNA	messenger ribonucleic acid
nm	nanometers
°C	degrees centigrade
p	plasmid

PBP	PSE binding protein
PMSF	phenylmethylsulphonyl fluoride
pol I	RNA polymerase I
pol II	RNA polymerase II
pol III	RNA polymerase III
PSE	proximal sequence element
PTF	proximal sequence element binding transcription factor
rNTPs	ribonucleotidetriphosphates
Rb	Retinoblastoma gene
RB	Retinoblastoma protein
RCE	retinoblastoma control elements
RNA	Ribonucleic acid
RNasin	RNase inhibitor
rRNA	ribosomal ribonucleic acid
RT	room temperature
SDS	Sodium dodecyl Sulphate
SDS-PAGE	Sodium dodecyl Sulphate polyacrylamide gel electrophoresis
SINEs	Short interspersed repeats
SL1	selectivity factor 1
snRNA	small nuclear ribonucleic acid
STEM	scanning transmission electron microscopy
SV40	Simian Virus 40

TAF	TBP-associated factor
TBP	TATA binding protein
TDF	Translation dependent factor
TEMED	N, N, N', N'-tetramethylethylenediamine
TFIIIA	Transcription factor IIIA
TFIIB	Transcription factor IIB
TFIIIB	Transcription factor IIIB
TFIIIC	Transcription factor IIIC
TFIID	Transcription factor IID
TFIIID	Transcription factor IIID
TFIIIE	Transcription factor IIIE
TIP-120	TBP-interacting protein-120
tis	transcription initiation site
TGF	transforming growth factor
Tris	tris(hydroxymethyl)methylamine
tRNA	transfer ribonucleic acid
Tween-20	Polyoxyethylene sorbitan monolaurate
U	units
UBF	upstream promoter element binding factor
UPE/UCE	upstream promoter element
UV	ultraviolet light
UTP	uridine triphosphate
V	Volts
v/v	volume/volume

w/v	weight/volume
y	yeast

Publications

The following publications were submitted during the course of the work presented in this thesis.

Sutcliffe, J.E., Cairns, C.A., McLees, A., Allison, S.J., Tosh, k. and White, R. J. (1999) RNA polymerase III transcription factor IIIB is a target for repression by pocket proteins p107 and p130. *Mol. Cell. Biol.*, **19**, 4255-4261.

Larminie, C.G.C., **Sutcliffe, J.E.**, Tosh, K., Winter, A.G., Felton-Edkins, Z. and White, R.J. (1999) Activation of RNA polymerase III transcription in cells transformed by simian virus 40. *Mol. Cell. Biol.*, **19**, 4927-4934.

Sutcliffe, J. E., Brown, T. R. P., Allison, S. J., Scott, P. H. and White, R. J. (2000) Retinoblastoma protein disrupts interactions required for RNA polymerase III transcription. *Mol. Cell. Biol.* In press.

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Chapter 1

Introduction

1 Introduction

The retinoblastoma tumour suppressor protein RB can repress transcription by all three eukaryotic nuclear RNA polymerases. This chapter shall discuss the cellular functions of RB, and the two proteins to which it is related p107 and p130. In addition, the properties of pol I and pol III will be described.

1.1 The retinoblastoma gene

The retinoblastoma susceptibility gene, *Rb*, encodes the retinoblastoma protein, RB. Retinoblastoma is a childhood cancer of the retina, which arises when both alleles of *Rb* are inactivated. In 1971, Knudson proposed that for retinoblastoma to arise, two mutational events must occur at the same locus (Knudson, 1971). Loss of heterozygosity at the *Rb1* locus in conjunction with the development of retinoblastomas, pointed to the existence of a retinoblastoma susceptibility gene (Cavenee et al., 1983). The human *Rb1* gene was identified and isolated (Friend et al., 1986), and found to encode a protein of 928 amino acids (Friend et al., 1987).

The *Rb1* gene has been found to be mutated in many cancers in addition to retinoblastomas, such as osteosarcomas, small-cell lung carcinomas, prostate carcinomas, breast carcinomas, some types of leukaemia and cervical carcinoma (Herwig and Strauss, 1997; Weinberg, 1995). Children who inherit a mutant *Rb1* allele often develop other cancers, such as osteosaromas, later in life when cured from

retinoblastoma (Abramson et al., 1979). In mice the situation is altered. Homozygous null *Rb1* mice do not develop retinoblastoma; instead they die at mid-gestation with abnormalities in the haematopoietic and nervous system (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Mice displaying heterozygous mutations in *Rb1* develop pituitary and thyroid tumours (Jacks et al., 1992; Mulligan and Jacks, 1998). These observations suggest a role for RB not only in cell growth, but also in development and differentiation (Herwig and Strauss, 1997).

1.2 *Rb1* is a tumour suppressor gene

The link between inactivation or loss of the *Rb1* gene and the development of tumourigenesis classified *Rb1* as a tumour suppressor gene. For example, introduction of the wild type *Rb1* gene into cells lacking a functional RB suppressed cell growth and tumourigenicity, thereby verifying *Rb1* as a tumour suppressor gene (Bookstein et al., 1990; Huang et al., 1988; Qin et al., 1992). The ability of RB to regulate cell cycle progression was also suggested by investigations demonstrating the ability of viral oncoproteins to deregulate cell cycle control. The SV40 T antigen (DeCaprio et al., 1988), adenovirus E1A (Whyte et al., 1988) and human papillomavirus type-16 (HPV-16) E7 protein (Dyson et al., 1989b) all interact with the hypophosphorylated form of RB. This has lead to a widely accepted model, in which viral oncoproteins promote growth by sequestering RB, which normally acts as a brake of the cell cycle by binding E2F and preventing the expression of products required for S-phase entry (La Thangue, 1994). The inactivation of RB by mutation

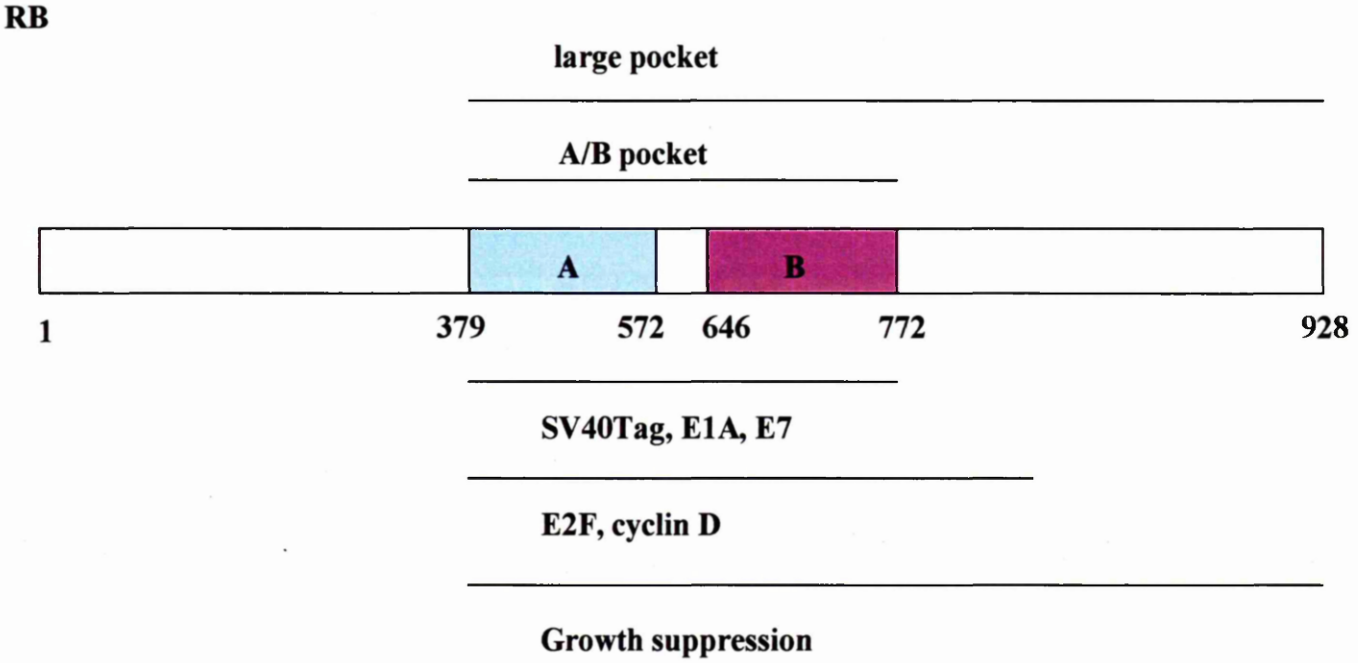
or phosphorylation was observed to have similar effects, namely the release of E2F (Herwig and Strauss, 1997).

1.3 The retinoblastoma protein

RB is a ubiquitously expressed nuclear phosphoprotein and is composed of 928 amino acids (Lee et al., 1987b; Lee et al., 1987a). Within RB are two stretches of amino acids, the A-domain (aa 379-572) and the B-domain (aa 646-772), which have been termed the A/B or small pocket (Hu et al., 1990; Kaelin et al., 1990). The addition of the carboxy-terminal region (aa 768-928) gives rise to the large pocket domain (fig. 1.1). The small pocket is responsible for binding viral oncoproteins, such as adenovirus E1A, HPV-16 E7 and SV40 T antigen (Kaelin et al., 1991). The large pocket domain region is required for growth suppression and mediates the RB/E2F interaction (Qian et al., 1992; Qin et al., 1992). This domain is also recognised by D-type cyclins and suffers the most mutations detected in human tumours (Weinberg, 1995). There are two proteins that are related in sequence and function to RB, which have been called p107 and p130. Collectively these proteins are referred to as the pocket proteins and the family will be discussed in section 1.9.

Fig. 1.1 Diagrammatic representation of the structure of RB.

RB is 928 amino acids long, with an A-domain (aa 379-572) and a B-domain (aa 646-772). Together these constitute the A/B pocket that is responsible for interacting with the viral oncoproteins SV40 Tag, E1A and E7. A slight extension of the A/B pocket is recognised by E2F and cyclin D. The region encompassing aa 379-928 is referred to as the large pocket. This is the region which is responsible for growth suppression.


RB
large pocket
A/B pocket
A
B
1
379
572
646
772
928
SV40Tag, E1A, E7
E2F, cyclin D
Growth suppression

1.4 RB and the cell cycle

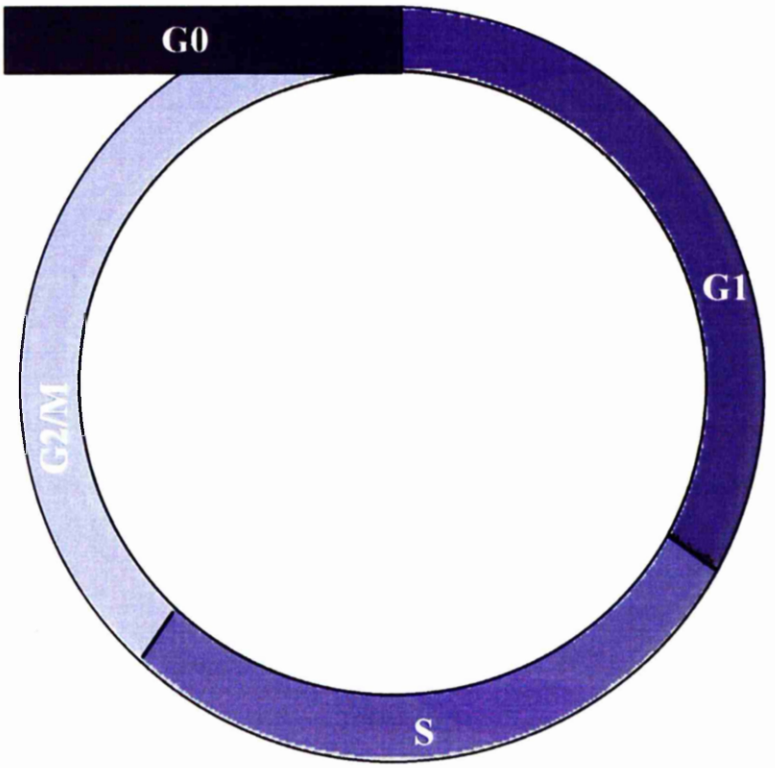
The fundamental task of the cell cycle is to faithfully replicate DNA and ensure equal distribution of chromosomes to daughter cells. It is composed of 4 phases, G1, S, G2 and M, and there is a resting G0 phase. The G phases are gap phases, the S-phase is when DNA replication occurs and the M-phase is mitosis (fig. 1.2).

The cell cycle has various check-points which function to ensure conditions are propitious for cell cycle progression. One such check-point occurs two-thirds into the G1 phase, and is called the restriction point in mammals (Pardee, 1989). The restriction point denotes the transition from serum-dependence to serum-independence; once cells pass this point, they are committed to enter S phase and no longer respond to mitogens (Pardee, 1989). There are alternative routes a cell may take, other than entry into S phase, such as differentiation, senescence, or cell death, subject to external signals (Herwig and Strauss, 1997).

RB undergoes alterations that coincide with the restriction point. Preceding G1, RB is found in a hypophosphorylated form. In contrast, RB isolated from cells in the last hours of G1 is hyperphosphorlyated, and remains so until exit from M (Buchkovich et al., 1989). There are several lines of evidence to suggest that upon phosphorylation RB is inactivated. Firstly, DNA tumour virus oncoproteins eliminate RB function by binding hypophosphorlyated but not hyperphosphorylated RB. Secondly, only the hypophosphorylated form of RB binds a variety of cellular

Fig. 1.2 Diagram of the cell cycle.

The cell cycle is composed of four phases which are in sequential order G1, S G2 and M phases. Cells can withdraw from the cell cycle upon quiescence. They re-enter the cell cycle at G1.


G0
G1
S
G2/M

proteins. Third, conditions which cause RB phosphorylation also favour cell proliferation. These observations allowed a model regarding RB and the regulation of cell cycle progression to be proposed; as a cell proceeds through G1 it encounters the restriction point, which is held shut by RB. Upon conditions becoming favourable for cell cycle progression, RB will be phosphorylated and inactivated. Hence, the arrest imposed by RB has been removed, and cells can now proceed into late G1.

1.5 Phosphorylation of RB by cyclin-dependent kinases

There is much evidence to suggest that components of the cell cycle mediate RB phosphorylation. Phosphopeptide analysis of RB identified multiple phosphorylation sites, which are typical of sites modified by cyclin-dependent kinases (-cdks). (Lees et al., 1991; Lin et al., 1991). Hence G1 cyclins, in conjunction with cdks, identify RB as a substrate for phosphorylation.

The prototype cdk, cdc2, regulates mitosis in all eukaryotic cells. It does this by complexing with its regulatory subunit cyclin B and subsequently phosphorylating mitotic proteins. Many proteins sharing sequence similarities to cdc2 have been identified, as have their regulatory cyclin subunits. D cyclins, D1, D2 and D3 complex to their catalytic partners, cdk4/cdk6, and phosphorylate RB, as do cyclin E/cdk2 complexes (Weinberg, 1995). However, these complexes act during temporally distinct phases during the cell cycle. Upon serum stimulation, a quiescent cell enters G1; this is accompanied by the synthesis of D-type cyclins. These subunits

assemble with their catalytic partners and phosphorylate RB (Sherr, 1994). Cyclin D-cdk4/6 complexes first manifest in mid-G1, and increase until the G1/S transition. Cyclin D synthesis is halted upon mitogen withdrawal; hence, the associated kinase activity diminishes (Sherr, 1996). Cyclin E-cdk2 also contributes to RB phosphorylation, but recognises different sites from cyclin D-cdk4/6 (Connell-Crowley et al., 1997), but this complex is not prevalent until late G1 (Sherr, 1996). Unlike cyclin D-cdk4/6, cyclin E-cdk2 maintains the phosphorylated status of RB in a mitogen-independent fashion. RB remains phosphorylated throughout the cell cycle until emergence from mitosis. This is maintained by cyclin A-cdk2 in S phase, then cyclin A-cdc2 in late S and G2, followed by cyclin B-cdc2 until the cell completes mitosis and re-enters G1 (or G0).

The activity of the cyclin D-cdk4/6 complex can be blocked by proteins called inhibitors of cdk4 or INK4s. The four known INK4 proteins, $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$, bind and specifically inhibit cdk4/6. Furthermore, there is a family of at least three proteins, $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$, which negatively regulate cyclin D-, E- and A-dependent kinases. Of these, KIP1 would appear to be most directly involved in restriction point control. This is underscored by the observation that in quiescent cells KIP1 levels are high; upon entry into the cell cycle they fall. Hence, there is a pathway which regulates the phosphorylation of RB; it is called the RB pathway.

1.6 Disruption of RB activity

To reprise the above introduction, a cell proceeding through G1 will encounter the restriction point, which is governed by RB. If conditions are favourable the cell will pass into late G1. However, cells lacking functional RB, as in cancer cells, will proceed blithely into late G1.

Hence, how is the activity of RB deregulated, so that it can no longer control progression through the restriction point?

The transforming proteins of DNA tumour viruses are known to compromise the activity of RB. These oncoproteins bind and inactivate RB. In cervical cancer, for example, RB is sequestered by the HPV E7 oncoprotein (Dyson et al., 1989b). Other oncoproteins, such as adenovirus E1A and SV40 large T antigen, are also capable of binding and inactivating RB (DeCaprio et al., 1988; Whyte et al., 1988). These oncoproteins inactivate RB by disrupting its interactions with cellular proteins, such as the pol II transcription factor E2F (Bandara and La Thangue, 1991). Hence, upon DNA tumour virus transformation, the viral oncoproteins bind and inactivate RB by physically associating with the site of RB, the large pocket, that is recognised by many cellular proteins (Weinberg, 1995).

RB function can also be lost through mutation of the *Rb* gene, as is observed in a variety of cancers, such as retinoblastomas, small cell lung carcinomas and many sarcomas and bladder carcinomas (Weinberg, 1995). Two RB deletion mutants have

been isolated from small cell lung carcinoma cell lines NCI-H1436 and NCI-H69C. These have been called, RBΔ21, which lacks 35 amino acids from exon 21, and RBΔ22, in which 38 amino acids from exon 22 are deleted. Studies involving these two mutants showed that neither are able to interact with adenovirus E1A or SV40 T antigen (Horowitz et al., 1990). Another RB mutant, isolated from small cell lung carcinoma cell line NCI-H209, was also investigated for its ability to mimic the functions of wild type RB. This RB mutant contains a point mutation in exon 21 of residue 706, from cysteine to phenylalanine (Kaye et al., 1990). This mutant was not only unable to bind SV40 T antigen, but it was also underphosphorylated (Kaye et al., 1990). The inability of mutant RB forms to mimic wild type RB is not a peculiarity of those isolated from small cell lung carcinomas. For example, the osteosarcoma cell line SAOS2 also contains mutant RB, in which exons 21-27 are removed (Shew et al., 1990). This form is unable to bind SV40 T antigen and is also not phosphorylated. Moreover, the introduction of wild type RB suppresses the neoplastic phenotype of the SAOS2 cell line, thereby suggesting the endogenous RB to be inactive (Shew et al., 1990).

As mentioned above, phosphorylation of RB results in its inactivation. Hence, another mechanism of inactivating RB found in human cancers is maintaining RB in a hyperphosphorylated state. This may be achieved by a variety of means, such as the amplification of cyclin D1, as observed in many esophageal, breast, and squamous cell carcinomas (Jiang et al., 1992; Lammie et al., 1991). In addition, the cdk4 gene can be overexpressed, as is found in glioblastomas and some gliomas (He et al., 1994;

Schmidt et al., 1994). Furthermore, the genes encoding p15 or p16, inhibitors of cyclin D-cdk4/6, may be deleted resulting in the constitutive phosphorylation of RB, as observed in esophageal squamous cell carcinomas (Mori et al., 1994; Schmidt et al., 1994).

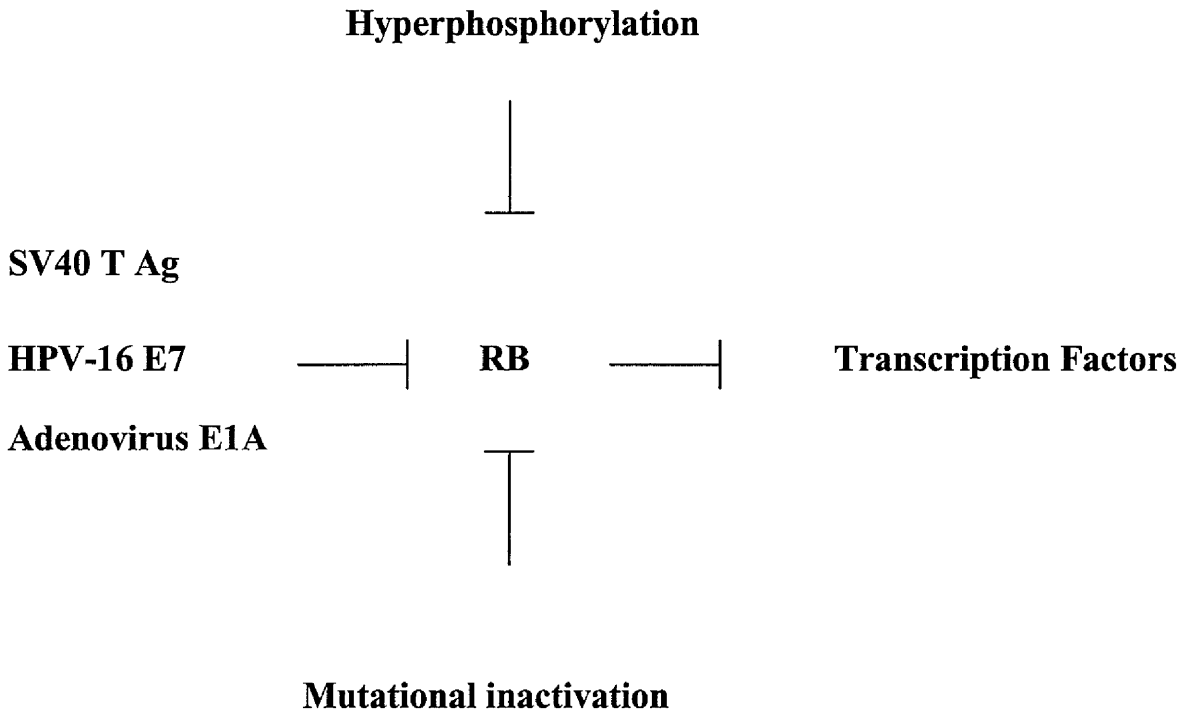
All of these mechanisms converge on the same point, that is the removal of RB activity, so that it can no longer function as a brake of the cell cycle, or bind cellular proteins (fig. 1.3).

`1.7 RB regulates transcription factors

The importance of RB in controlling gene expression was suggested when it was shown that E1A prevents RB from complexing with a cellular transcription factor (Bandara and La Thangue, 1991). It was proposed that transforming proteins inactivated the transcriptional-repression mediated by RB, and thereby indirectly activated the transcription of genes normally negatively controlled by RB. RB was subsequently shown to bind to a family of heterodimeric transcriptional regulators called E2F (Chellappan et al., 1991). Hence, RB binds E2F and disrupts its ability to promote transcription (Weintraub et al., 1995; Weintraub et al., 1992). The E2F family of transcription factors promotes the expression of genes whose products are important for S phase entry. Many are involved in cell cycle regulation, such as cyclin E (Geng et al., 1996), or DNA metabolism, such as dihydrofolate reductase (DHFR) (Slansky et al., 1993).

Fig. 1.3 Diagram of RB inactivation observed in human cancer.

RB can be inactivated by one of three means in human cancers: sequestration by viral oncoproteins, hyperphosphorylation and mutational inactivation. Upon inactivation, RB is no longer able to exert an effect on transcription factors.


Hyperphosphorylation
SV40 T Ag
HPV-16 E7
Adenovirus E1A
RB
Transcription Factors
Mutational inactivation

Through interaction with E2F, RB plays a central role in the control of cell division, by regulating cell cycle progression and DNA replication. Until recently it was believed that the ability of RB to mediate cell cycle arrest was solely via its interaction with E2F. However, recent studies suggest that there are alternative mechanisms which may contribute to this effect, such as the ability of RB to control cell growth by repressing both pol I and pol III transcription (Cavanaugh et al., 1995; White et al., 1996). These studies offer an explanation as to how tumour cells which lack RB can increase overall biosynthesis, without which an increase in the frequency of chromosome replication would be lethal (Nasmyth, 1996).

1.8 Other cellular functions of RB

Aside from interacting with E2F, and pol I and pol III transcription factors, which will discussed below, RB interacts with other cellular proteins, and is involved in variety of activities.

RB binds to a number of cellular proteins that may directly or indirectly be involved in transcriptional regulation of genes relevant for controlling the cell cycle. The promoters of growth control genes c-fos, c-myc, and transforming growth factor-β1 (TGF-β1) contain sequences termed retinoblastoma control elements (RCE). Sp1 is one of three RCE-binding proteins. RB can interact with Sp1, resulting in super-activation of Sp1-mediated transcription (Udvadia et al., 1993). RB can also activate

transcription mediated by the AP-1 family of transcription factors by binding to c-Jun (Nead et al., 1998).

RB has also been shown to repress pol II transcription by recruiting a histone deacetylase. RB binds histone deacetylase 1 (HDAC1) through the pocket domain. HDAC1 and RB cooperate to repress the E2F-regulated promoter of cyclin E (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998). Hence, by recruiting HDAC1, RB is able to halt cell cycle progression.

RB has also been shown to be required for differentiation of certain cell types. This is characterised by permanent cell cycle withdrawal, and the expression of genes required for the differentiated phenotype. The first evidence that RB was involved in differentiation came from *Rb*-/- knock-out mice, which die *in utero* due to defects in erythropoiesis and neural development (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). A role for RB in differentiation has been identified for various cell lines. In all systems studied, an early development is the dephosphorylation of RB, which correlates to cell cycle arrest in G0/G1, and provides a prerequisite to enter the differentiation pathway. For example, in differentiating muscle cells RB complexes with myogenic transcription factors, such as MyoD. This interaction prevents RB from being rephosphorlyated and locks the cell in a differentiated state (Gu et al., 1993).

RB is involved in protecting against apoptosis (programmed cell death). Apoptosis is a genetically controlled process; genes involved have been identified,

such as p53 and myc. The ectopic expression of wild type RB in SAOS2 cells increases radioresistance; that is, the ionising radiation induces less cell death in cells which express RB (Haas-Kogan et al., 1995). The mechanism whereby RB protects cells from radiation-induced apoptosis is unclear. RB appears to be involved in regulating the apoptotic function of p53, as both of these tumour suppressor proteins are able to interact with the same oncoprotein MDM2. Indeed, RB can overcome the anti-apoptotic function of MDM2 on p53-induced apoptosis (Hsieh et al., 1999a).

1.9 The pocket proteins p107 and p130

RB belongs to a family of proteins, termed the pocket proteins, of which there are two other members, p107 and p130. These pocket proteins were identified as resembling RB in being able to physically associate with viral oncoproteins such as adenovirus E1A, SV40 and JC virus Tag, and HPV-16 E7 (Dyson et al., 1989a; Ewen et al., 1991; Hannon et al., 1993; Li et al., 1993; Mayol et al., 1993). All three of these pocket proteins share sequence homologies and functional similarities.

1.9.1 Sequence homology between the pocket proteins

p107 and p130 are closely related to each other (~50% amino acid identity) and to RB (~30-35% amino acid identity) (Grana et al., 1998; Mulligan and Jacks, 1998). The structural conservation between RB, p107 and p130 is clustered into five

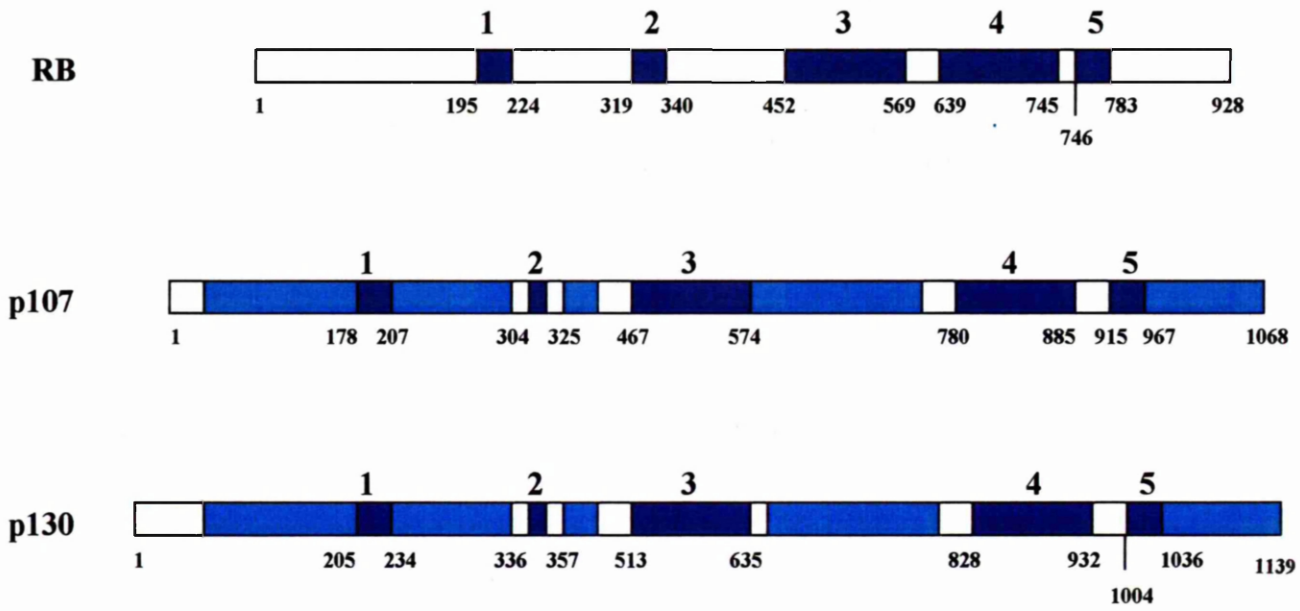
regions, the majority of which is localised to the small pocket domain (Ewen et al., 1991; Hannon et al., 1993; Li et al., 1993; Mayol et al., 1993) (fig. 1.4). The spacer region between the A- and B- domains is well conserved between p107 and p130, but not between all three pocket proteins. It provides a site for interactions with cyclin A-cdk2 and cyclin E-cdk2 (Lees et al., 1992: Cobrinik et al., 1993; Li et al., 1993). Indeed, the pocket protein/cyclin-cdk complexes have been shown to display reduced kinase activity. It has been proposed that this contributes to the ability of p107 and p130 to arrest cell growth (De Luca et al., 1997; Hauser et al., 1997; Woo et al., 1997).

1.9.2 p107 and p130 interact with E2F family members

The E2F family of transcription factors provides an example of functional similarities between the pocket proteins. E2F1 can be detected in immunoprecipitates of RB, as can E2F2, E2F3 (Lees et al., 1993) and to a lesser extent E2F4 (Morberg et al., 1996). Whilst, p107 and p130 are not detected in the immunoprecipitates of E2F1-3, they are observed in those of E2F4 (Beijersbergen et al., 1994; Dyson et al., 1993; Ginsberg et al., 1994; Vairo et al., 1995). In addition, p130 can be found in complexes with E2F5 (Hijmans et al., 1995). The coimmunoprecipitation data were substantiated by the finding that E2F4 relieves p130-mediated G1 arrest more effectively than E2F1 (Vairo et al., 1995). E2F4 and E2F5 lack the C-terminal RB-binding domain found in E2F1-3, but contain a DNA-binding and a transactivation domain (Beijersbergen et al., 1994; Ginsberg et al., 1994). All three pocket proteins

Fig. 1.4 Homologous domains between the pocket proteins.

There are five domains of homology between RB, p107 and p130, denoted as dark blue regions. Most of this homology is located within the large pocket domain. There is greater homology between p107 and p130, and these regions are coloured light blue.


RB
1
2
3
4
5
1
195
224
319
340
452
569
639
745
746
783
928
p107
1
2
3
4
5
1
178
207
304
325
467
574
780
885
915
967
1068
p130
1
2
3
4
5
1
205
234
336
357
513
635
828
932
1004
1036
1139

can interact with E2F family members. However, deregulation of E2F-target genes was observed in cells lacking RB or both p107 and p130, but not in cells lacking either p107 or p130. Interestingly, the genes deregulated under these two conditions were completely different. Hence, RB, p107 and p130 provide different functions in E2F regulation (Hurford et al., 1997).

Like RB, p107 and p130 form a complex with their E2F partner at temporally distinct points during the cell cycle (Morberg et al., 1996). RB associates with E2F mainly in G1 and S phase. p107 forms two complexes with E2F, one with cdk2 and cyclin A that predominates in the S phase; the other complex consists of cdk2 and cyclin E and is found in the G1 phase (Lees et al., 1992). p130-E2F complexes are primarily found in cells in G0 (Cobrinik et al., 1993; Smith et al., 1996); once the cell has passed into G1, cyclin A- or cyclin E- cdk 2 enters the complex (Li et al., 1993).

It is of interest to note that the major pocket protein/E2F complexes observed in the cell cycle coincide with the expression of p107 and p130, which fluctuate throughout the cell cycle. When cells enter quiescence, the level of p130 rises; as a cell passes towards S phase, the level of p130 sharply declines, then increases again as a cell exits the cell cycle (Mayol et al., 1996). The levels of p107 expression follow a near opposite trend; little p107 is detected in quiescent cells, but p107 accumulates sharply as cells progress through the cell cycle (Beijersbergen et al., 1995; Cobrinik et al., 1993). It would appear that the increase in p107 is accompanied by an increase in p107 mRNA (Hurford et al., 1997). The regulation of p130 expression is post-transcriptional, as there is little variation in p130 mRNA levels (Grana et al., 1998).

1.9.3 p107 and p130 can induce cell cycle arrest when overexpressed

As mentioned above, the overexpression of RB in cells results in an increase in the number of cells in G1 phase of the cell cycle (Goodrich et al., 1991; Qin et al., 1992). As p107 and p130 were shown to display considerable homology to RB, it was investigated if they could also arrest cell cycle progression. Upon overexpression p107 arrests progression through the cell cycle (Zhu et al., 1993), as does p130 (Claudio et al., 1994).

1.9.4 Analysis of pocket proteins in mutant mouse strains

Analyses of *Rb* family mouse mutants demonstrate an important in vivo role of these genes in regulatory functions. Homozygous *Rb* knock-out mice die between 13 and 15 days of gestation with defective neurogenesis and erythropoiesis, whilst heterozygous mice develop tumours of the pituitary and thyroid, but do not display retinoblastoma (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). In contrast, animals lacking functional p107 or p130 develop normally, with no observed predisposition to tumours. Animals lacking both p107 and p130 are born with shortened limbs and rib bones, fail to respire and die rapidly (Cobrinik et al., 1996). Interbreeding of the *Rb* and *p107* mouse strains points to species-specific susceptibility to retinoblastoma, which might be related to differential requirements for gene mutation. *Rb*+/- *p107*-/- animals display lesions characterised by severe disruption of normal retinal structure, but these are not observed in other

combinations (*Rb*+/- *p130*-/-, *p130*+/- *p107*-/- or *p107*+/- *p130*-/-). The retinal phenotype of *Rb*+/- *p107*-/- animals suggests that p107 and RB have overlapping functions in the control of cell growth in the murine retina.

1.9.5 Functional compensation by the pocket proteins

As has been mentioned, the functions of the pocket proteins may overlap, but, there also appears to be functional compensation. This is illustrated by peripheral T lymphocytes from p130-deficient mice. As mentioned above, the p130-E2F4 complex is most prevalent in quiescent cells, suggesting a role for p130 in the resting status of a cell; disruption of this complex causes the cell to enter the cell cycle. However, in *p130*-/- T lymphocytes the quiescence status and re-entry into the cell cycle are identical to *p130*+/+ and *p130*+/-. The levels of p107 in these mutant cells are significantly elevated, and mobility shift analyses indicate that p107 has replaced p130 in the E2F regulatory complex. In T lymphocytes deficient in both p130 and p107, there are elevated levels of free E2F and also a RB-E2F4 complex (Mulligan et al., 1998), which suggests functional compensation.

1.9.6 p107 and p130 have not been identified as tumour suppressor proteins

The role of p107 and p130 in tumour suppression remains unclear. RB is mutated in a variety of human cancers (Weinberg, 1995). However, no mutant forms

of p107 have been reported, and only one mutant form of p130 has been detected in a small-cell lung cancer cell line (Helin et al., 1997). It could be that one compensates for the others absence, and that both need to be inactivated to reveal their tumour suppressing functions. However, both p130+/-/p107-/- and p130-/-/p107+/- mice failed to exhibit an obvious tumour phenotype, suggesting that loss of both p130 and p107 is insufficient for tumour progression in murine tissues. In addition, p107 has been mapped to a chromosome region not altered in human cancers (Ewen et al., 1991). p130, however, mapped to a chromosome region which is deleted in a variety of cancers (Yeung et al., 1993). This suggests a possible role for p130 as a tumour suppressor protein in humans.

As discussed above, p107 and p130 are able to mimic some of the functions performed by RB; for example, they are recognised by viral oncoproteins, they bind various E2F family members and they can arrest cell growth when overexpressed. Between the three pocket proteins there appears to be some functional overlap and compensation. However, unlike RB, p107 and p130 are not regarded as tumour suppressor proteins.

1.10 Nuclear RNA polymerases

Eukaryotic organisms have three nuclear RNA polymerases, namely RNA polymerase (pol) I, pol II and pol III. Each RNA polymerase synthesises a unique set of products: pol I synthesises the 5.8S, 18S and 28S ribosomal RNAs (rRNA). pol II

synthesises messenger RNA (mRNA) and most small nuclear RNA (snRNA), whilst pol III synthesises transfer RNA (tRNA), 5S rRNA and various small nuclear RNAs and viral RNAs. Below, the properties of pol I and pol III transcription shall be discussed, as will the ability of RB to regulate them.

The three classes of RNA polymerases that occur in eukaryotes were originally separated by chromatography on DEAE Sephadex (Roeder and Rutter, 1969). They were classified according to chromatographic properties, salt requirements, template preferences and their sensitivity to the toxin α-amanitin, a cyclic octapeptide produced by the poisonous *Amanita* mushrooms (Chambon, 1975; Kedinger et al., 1970; Roeder and Rutter, 1969). In mammals, pol II is the most sensitive (50% inhibition at 25ng/ml), with pol III displaying intermediate sensitivity, (50% inhibition at 20μg/ml), whilst pol I is completely resistant (Schwartz et al., 1974).

There are five subunits which are found in all three nuclear RNA polymerases (ABC 27, 23, 14.5, 10α and 10β, in *S. cerevisiae*). Pol I and pol III share two additional subunits (AC19 and AC40 in *S. cerevisiae*) (Mann et al., 1987). These subunits are not present in pol II, but, B12.5 and B44 are functionally equivalent (Paule and White, 2000).

1.11 RNA pol III transcription

1.11.1 Promoter Structure of Class III genes

The majority of class III genes include discontinuous intragenic structures termed internal control regions (ICRs). There are three types of class III gene promoter, called type I, II, and III. Each is composed of specific sequences and is present in particular genes (as summarised in fig. 1.5).

The ICRs of 5S rRNA genes are unique and are often referred to as type I. They are composed of two functional domains located within the transcribed region; an A-block, and a second domain consisting of an intermediate element and a C-block; a prime example is the *Xenopus laevis* 5S rRNA gene. The location of the ICR lies between base pairs +50 and +97; the A-block lies between +50 and +64, the intermediate element +67 and +72 and the C-block +80 and + 97 (Pieler et al., 1987). The bases between these regions serve as spacers, but their sequence does not influence transcription efficiency (Pieler et al., 1987).

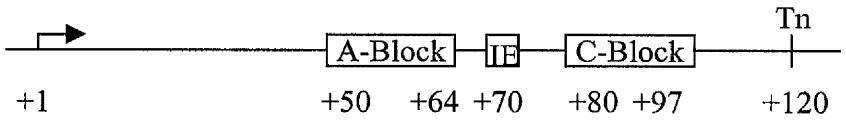
The majority of class III genes display a type II promoter, such as tRNA, VA, Alu and 7SL genes. These promoters are located downstream of +1 and are composed of two domains of 10 base pairs, termed the A- and B-blocks. The sequences of the A- and B-blocks of many eukaryotic tRNA genes have been determined, allowing a consensus sequence for each block to be derived; namely TGGCNNAGTGG for the A-block and GGTTCGANNCC for the B-block (Galli et al., 1981). The A-blocks of

Fig. 1.5 Promoter structure of class III genes.

Diagrammatic depiction of the three types of class III promoters, which are characterised by particular sequences. An example of a type I promoter is found in *Xenopus* somatic 5S rRNA genes. It is characterised by three promoter elements, an A-block (+50 to +60), an intermediate element (~ +70) and a C-block (+80 to +97). Type II promoters, such as the SUP4 tRNA promoter of *S. cerevisiae*, display two conserved sequences, an A-block (+8 to +19) and a B-block (+52 to +62). Human U6 snRNA genes have a type III promoter structure, consisting of a DSE (-244 to -214), a PSE (-66 to -47) and a TATA box (-30 to -25). The site of transcription initiation is denoted by a bent arrow.

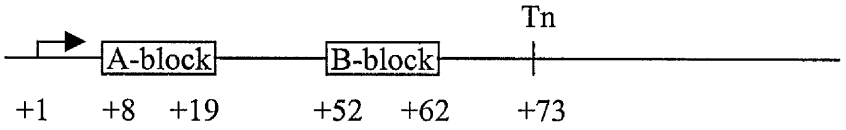
Type I Promoter

e.g. *Xenopus* somatic 5S rRNA Genes



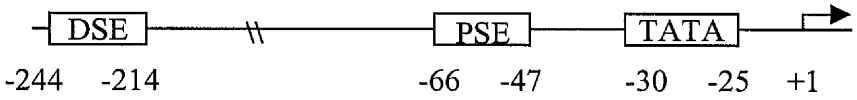
Type II Promoter

e.g. *Saccharomyces* SUP4 tRNA Genes



Type III Promoter

e.g. Human U6 snRNA Gene



type II promoters are located closer to +1 then those of type I promoters. For example, in the *S. cerevisiae* SUP4 $tRNA^{Tyr}$ promoter, the A-block is positioned from +8 to +19, whilst the B-block encompasses bp +52 to +62 (Allison et al., 1983). The location of the B-block varies. An interblock separation of 30-60 bp is optimal for transcription. However, a distance of 365 bp can be tolerated (Baker et al., 1987; Fabrizio et al., 1987). This is quite remarkable, when one considers that both of these blocks are contacted by the same transcription factor TFIIIC (Schultz et al., 1989).

The final promoter type is independent of intragenic elements and is located within 5' flanking regions; an example is provided by the human U6 gene. The human U6 promoter consists of a TATA box, between -30 and -25, a proximal sequence element (PSE) located between -60 and -47, and a distal sequence element (DSE) between -244 and -214 (Das et al., 1988; Kunkel and Pederson, 1989; Lobo and Hernandez, 1989). The U6 PSE and DSE are homologous and interchangeable with elements at similar positions in the class II U2 snRNA gene (Lobo and Hernandez, 1989). A TATA box is not found in the U2 promoter, which is surprising as TATA sequences are normally associated with class II gene promoters. Moreover, the insertion of a TATA box into the U2 promoter converts it to a pol III promoter, and removal of the TATA sequence from the U6 promoter allows it to be transcribed by pol II (Lobo and Hernandez, 1989; Mattaj et al., 1988).

There are other class III genes, whose promoter types do not fall into any of the above classifications, such as the Epstein-Barr virus EBER2 gene (Howe and Shu, 1989).

1.11.2 Transcription factors utilised by pol III

Purified pol III initiates transcription randomly (Hammond and Holland, 1983). In order for accurate and specific transcription to occur, the assistance of transcription factors is required.

1.11.2.1 TBP

TBP was originally thought to be restricted to the expression of class II genes. Upon elucidation of the U6 promoter sequence, it was proposed that a TATA-binding factor is utilised for expression of U6 snRNA (Mattaj et al., 1988). Observations by Margottin and co-workers (Margottin et al., 1991) showed that TBP was indeed able to support U6 transcription by pol III. It was subsequently shown, by competition and reconstitution analyses, to be required for the expression of pol III genes whose promoters did not contain a TATA-box (White et al., 1992a; White et al., 1992b).

TBP was originally purified (Buratowski et al., 1988; Cavallini et al., 1988; Horikoshi et al., 1989a) then cloned, (Cavallini et al., 1989; Fikes et al., 1990; Hahn et al., 1989; Hoffmann et al., 1990; Horikoshi et al., 1989b; Schmidt et al., 1989) from yeast. TBP encoding genes give rise to a protein of 27-38kD. The N-terminal region is variable in both size and sequence; in contrast, the C-terminal domain is highly conserved. For example, the C-terminal 180 residues of TBP isolated from

human, mice and frog are 100% identical (Hashimoto et al., 1992; Tamura et al., 1991), and at least 80% conserved to TBP isolated from yeast (Fikes et al., 1990).

Upon chromatography, TBP fractionates heterogeneously, suggesting its inclusion in various complexes (Timmers and Sharp, 1991). Proteins which associate with TBP are called TBP associated factors or TAFs (Hernandez, 1993; Rigby, 1993; White and Jackson, 1992c). In the pol II system the TBP-TAF complex is TFIID. In pol I it is referred to as SL1 or TIF-IB, it is composed of TBP and three TAFs, (Comai et al., 1992; Zomerdijk et al., 1994). The TBP containing complexes of pol III transcription are TFIIIB and SNAPc.

1.11.2.2 TFIIIB

TFIIIB alone is sufficient to recruit the polymerase and specify the start site of transcription (Kassavetis et al., 1990). Kassavetis *et al.*, (Kassavetis et al., 1989) split a TFIIIB fraction into two components called B' and B'' by chromatography on Mono S. Both of these fractions were shown to be necessary for the transcription of tRNA in the presence of purified TFIIIC and pol III. TFIIIB does not bind to TATA-less class III genes directly (Johnson et al., 1991), instead it is recruited into the vicinity of DNA by interacting with TFIIIC (Kassavetis et al., 1989). Bartholomew *et. al.* (Bartholomew et al., 1990) exploited this property to probe the polypeptide composition of TFIIIB by photocross-linking. This approach detected two polypeptides of 70 and 90 kD within the TFIIIB fraction which are recruited to the

promoter by TFIIIC (Bartholomew et al., 1991). The 70kD polypeptide was found in the B' Mono S fraction and the 90kD polypeptide in the B" fraction (Bartholomew et al., 1991).

The gene encoding the 70kD subunit of yeast TFIIIB was cloned. The gene has been named *BRF1* (Colbert and Hahn, 1992), *TDS4* (Buratowski and Zhou, 1992) and *PCF4* (Lopez-de-Leon et al., 1992), here it will be referred to as *BRF1* and its product yBRF. *BRF1* has been shown to be essential for growth (Buratowski and Zhou, 1992; Colbert and Hahn, 1992). Introduction of mutations induce a rapid decline in the in vivo expression of tRNA, but not of class I or class II genes (Buratowski and Zhou, 1992; Lopez-de-Leon et al., 1992). *BRF1* encodes a protein of 596 amino acids, with a predicted molecular mass of 67kD and a pI of ~ 6.9 (Buratowski and Zhou, 1992; Colbert and Hahn, 1992; Lopez-de-Leon et al., 1992). Its structure can be split down the middle. There is a zinc ribbon at the extreme amino-terminus. The remainder of the amino half displays two highly conserved imperfect repeats of 76 amino acids (Buratowski and Zhou, 1992; Colbert and Hahn, 1992; Lopez-de-Leon et al., 1992). In contrast, the carboxy-terminal half has no homology to any known protein. However, three regions of conservation have been identified between BRF cloned from evolutionarily diverse yeast species (Khoo et al., 1994).

yBRF and TBP were both identified in the B' fraction by western analysis (Buratowski and Zhou, 1992; Colbert and Hahn, 1992; Kassavetis et al., 1992). This was confirmed as antibodies raised against recombinant TBP or yBRF were

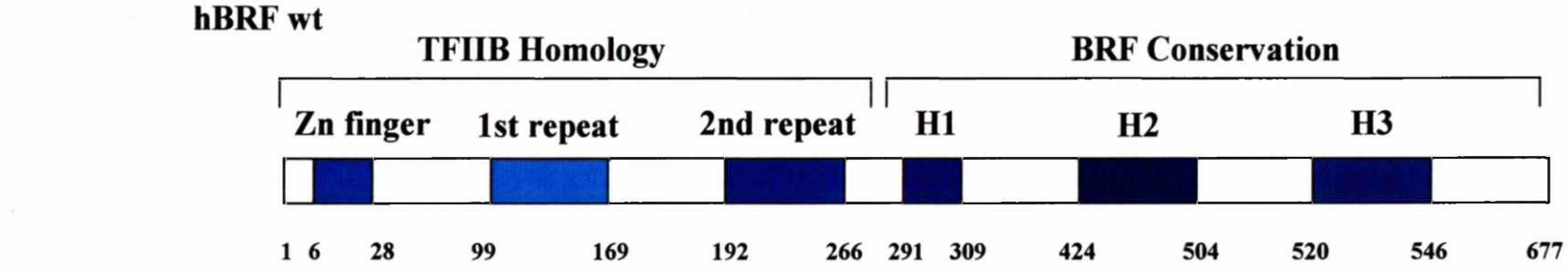
specifically able to supershift a TFIIIB/DNA complex in a gel retardation assay (Buratowski and Zhou, 1992; Kassavetis et al., 1992). Furthermore, Kassavetis *et al*, (Kassavetis et al., 1992) showed that recombinant TBP and recombinant BRF was sufficient to reconstitute all the known properties of the B' fraction, thereby providing strong evidence that TBP and BRF interact to form part of TFIIIB. GST pull down analyses showed that within BRF there are two TBP binding sites (Khoo et al., 1994). One mapped to the direct repeat region; the other, which showed the greatest affinity, was the H2 region in the carboxy terminal half (Andrau et al., 1999; Kassavetis, 1998)(see fig. 1.6 for the binding regions identified in BRF).

The other TFIIIB fraction observed by Kassavetis et al was B" (Kassavetis et al., 1991), the activity of which was found to co-migrate with the 90kD polypepetide in a SDS-polyacrylamide gel (Kassavetis et al., 1992). Definite evidence that the 90kD subunit is responsible for B" activity came with the cloning of its gene (Kassavetis et al., 1995; Roberts et al., 1996; Ruth et al., 1996). It encodes a 594 amino acid protein with a calculated mass of ~ 68 kD, but which migrates at ~ 90kD (Kassavetis et al., 1995; Roberts et al., 1996; Ruth et al., 1996). B" displays little homology to other known proteins, expect for a putative SANT domain (Aasland et al., 1996).

B" can retain its activities when extensively truncated (Kassavetis et al., 1995; Kumar et al., 1997). A core domain of 176 residues (residues 224-400) is sufficient to support U6 transcription (Kumar et al., 1997). Two distinct regions, residues 224-305 and 390-460 are both necessary for tRNA synthesis but can function on an either/or

Fig. 1.6 The structure of BRF.

The N-terminal half of BRF consists of an N-terminal Zn ribbon and two imperfect repeats. The N-terminal 320 amino acids of yBRF are 23% identical to either human or yeast TFIIB. The TFIIIC binding domain in BRF has been identified as encompassing much of the N-terminal half. TBP has two binding sites within BRF: one located in each half, the site in the C-terminal half is the strongest. The region responsible for binding pol III is the carboxy terminus of the 1st imperfect repeat.


hBRF wt
TFIIB Homology
BRF Conservation
Zn finger
1st repeat
2nd repeat
H1
H2
H3
1 6 28 99 169 192 266 291 309 424 504 520 546 677
TFIIIC binding
TBP binding
Pol III binding

basis for U6 snRNA expression (Kumar et al., 1997). Protein footprinting suggests that B" is folded such that these two domains are in close proximity when TFIIIB is assembled onto DNA (Kumar et al., 1997). The resilience of B" to deletion mutagensis may be accounted for if the multiple protein/protein and protein/DNA interactions made by this polypeptide allow it to compensate for the loss of any individual contact. Recombinant B" binds weakly to TBP, but this binding can be greatly enhanced by the presence of yBRF. In contrast, no interaction is observed between yBRF and B" in the absence of TBP (Roberts et al., 1996).

S. cerevisiae TFIIIB can be reconstituted from recombinant TBP, BRF and B" (Kassavetis et al., 1995; Roberts et al., 1996; Ruth et al., 1996). The activity observed for the recombinant complex is lower than that for native TFIIIB (Kassavetis et al., 1995; Ruth et al., 1996). This might be because the recombinant TFIIIB complex lacks some post-translational modification(s) that are present in the native complex, or perhaps an essential component is missing (Kassavetis et al., 1995).

A putative gene encoding a homologue of yBRF has been identified in *Caenorhabditis elegans* (Larminie and White, 1998). The CeBRF protein is predicted to be 26% identical and 35% similar to yBRF from *S. cerevisiae*. It is much longer, consisting of 759 residues (Larminie and White, 1998). The greatest homology lies in the N-terminal half, which contains a putative zinc ribbon and two imperfect direct repeats (Larminie and White, 1998). The C-terminal half bears two of the three regions of homology that are shared by the yBRFs (Larminie and White, 1998).

Mammalian TFIIIB is less well understood. It has been purified using various chromatography techniques. Using anti-TBP antibodies, a TAF of 88-90kD could be immunoprecipitated from fractions containing TFIIIB (Mital et al., 1996; Wang and Roeder, 1995). The cDNA encoding this TAF was isolated by peptide sequencing, and the product was found to contain 677 residues (Mital et al., 1996). The N-terminal 280 residues are 24% identical to human TFIIB and 41% identical to yBRF from *S. cerevisiae*; hence it was called BRF (Mital et al., 1996). Like the other BRF sequences, the N-terminal half of human BRF or hBRF contains a zinc ribbon and two imperfect direct repeats (Mital et al., 1996). The C-terminal half displays little homology to yBRFs, except for the residues which constitute the H2 region (Mital et al., 1996), but shows more homology with CeBRF (Larminie and White, 1998). Transcription of VA, 5S and tRNA genes is compromised following immunodepletion of hBRF, but expression can be restored by the addition of recombinant TBP and hBRF (Mital et al., 1996; Wang and Roeder, 1995). Recently another mammalian TFIIIB TAF was described. It displays homology to yeast B", and is necessary for the transcription from both TATA-less and snRNA-type promoters (Schramm et al., 2000).

The requirement of TFIIIB for type III promoters is different than that of type I and type II promoters (Lobo et al., 1992). BRF may not be involved in U6 transcription, as immunodepletion of BRF inhibits VA transcription but does not affect U6 transcription, (Mital et al., 1996). Hence, it was proposed that type III promoters utilise a modified form of BRF. Lately, various splice variants of hBRF have been cloned; all of these can complex with TBP. However, they are required for

transcription at structurally distinct promoter types; hBRF1 is required for transcription from 5S, VA1, 7SL and EBER2 promoters, whilst hBRF2 is recruited to the U6 promoter (McCulloch et al., 2000). Schramm and co-workers have also observed the presence of hBRF splice variants. They identified a factor related to BRF, hBRFU, which is required for transcription from snRNA-type TATA-containing promoters (Schramm et al., 2000).

1.11.2.3 TFIIIC

TFIIIC is another general transcription factor of pol III transcription. Like TFIIIB it was first purified from yeast, by a combination of ion-exchange and affinity chromatography on specific tDNA or B-block columns (Kassavetis et al., 1989; Parsons and Weil, 1990; Swanson et al., 1991). Polypeptides of approximately 138, 131, 95, 91, 60 and 55 kD copurify with TFIIIC transcription and DNA-binding activity (Bartholomew et al., 1990; Braun et al., 1992; Gabrielsen et al., 1989; Parsons and Weil, 1990; Swanson et al., 1991). As yeast TFIIIC is sometimes referred to as τ, its subunits have been named τ138, τ131, τ95, τ91, τ60 and τ55. Visualisation studies using scanning transmission electron microscopy (STEM) showed that TFIIIC/tDNA complexes appear dumbbell shaped, with individual protein domains bound to the A- and B- blocks, which are referred to as the τA and τB domains, respectively (Schultz et al., 1989). τB has been demonstrated to be recognised by an anti-τ138 antibody, but not an anti-τ95 antibody, indicating that

τ138 is part of the τB domain (Gabrielsen et al., 1989). In contrast, τ131, τ95 and τ55 can be crosslinked to N_3RdU residues in and around the A-block (Bartholomew et al., 1990).

Whereas yeast and human TFIIIB appear to be similar, human and yeast TFIIIC are different. Human TFIIIC was first resolved into two components, TFIIIC1 and TFIIIC2 (Dean and Berk, 1987; Yoshinaga et al., 1987). Whereas TFIIIC1 is ill-defined, TFIIIC2 is far more characterised. The binding activity of TFIIIC2 to VA is localised to fractions containing five polypeptides, of 240, 110, 100, 80 and 60 kD, referred to as α, β, γ, δ and ε respectively (Yoshinaga et al., 1989). These fractions display transcriptional activity in the presence of TFIIIB, TFIIIC1 and pol III (Yoshinaga et al., 1989). TFIIIC2 binds to the B-block regions of VA1 and tRNA genes (Boulanger et al., 1989; Wang and Roeder, 1996; Yoshinaga et al., 1987). The addition of TFIIIC1 increases the intensity of the footprint generated by TFIIIC2 and extends it to include the A-block (Wang and Roeder, 1996; Yoshinaga et al., 1987). HeLa cell TFIIIC2 was resolved into two DNA binding forms on non-denaturing gels (Hoeffler et al., 1988). The slower migrating one has been named TFIIIC2a, whereas the high mobility form is referred to as TFIIIC2b (Sinn et al., 1995). These two forms have identical footprints on VA1 and have similar DNA binding activities (Hoeffler et al., 1988; Kovelman and Roeder, 1992). Transcriptional activity, however, is only associated with TFIIIC2a (Hoeffler et al., 1988; Kovelman and Roeder, 1992). Silver staining revealed that TFIIIC2b was missing the TFIIICβ, 110 kD subunit, and instead a band of 77 kD was present (Kovelman and Roeder, 1992; Sinn et al., 1995).

Phosphatase treatment of TFIIIC2a converted it into inactive TFIIIC2b, suggesting that the transcriptional activity of TFIIIC can be modulated by phosphorylation (Hoeffler et al., 1988).

The genes for the subunits of TFIIIC2 have been cloned. Three of them, hTFIIIC220, hTFIIIC110 and hTFIIIC90 display no homology to *S. cerevisiae* TFIIIC. The other two display some weak homology to yeast TFIIIC; hTFIIIC63 shows 22% homology to τ95, whilst hTFIIIC102 is 31% identical to τ131, and both these subunits contain 11 copies of a tetratricopeptide repeat (Hsieh et al., 1999b).

TFIIIC plays a pivotal role by recruiting TFIIIB to the promoter. However, there are other transcriptional activities involving TFIIIC. For example, it relieves chromatin repression of *S. cerevisiae* U6 snRNA transcription (Burnol et al., 1993). hTFIIIC is also able to relieve chromatin-mediated repression, as it displays an intrinsic histone acetyltransferase activity (Hsieh et al., 1999b; Kundun et al., 1999).

1.11.2.4 TFIIIA

The expression of 5S genes requires an additional factor called TFIIIA (Engelke et al., 1980; Segall et al., 1980). TFIIIA was the first eukaryotic transcription factor to be purified to homogeneity (Engelke et al., 1980), and have its gene cloned (Ginsberg et al., 1984). This was probably facilitated by its abundance in

early *Xenopus* oocytes. The predicted TFIIIA protein is 344 amino acids in length, the majority of which constitutes 9 tandem repeats of 27 amino acids. These repeats are called zinc fingers (Miller et al., 1985), and are essential for DNA binding activities (Hanas et al., 1983). The DNA binding domain consists of three distinct regions; those composed of fingers 1-3 and 7-9 are compact, whereas the region composed of fingers 4-6 is extended (Hayes and Clemens, 1992; Hayes and Tullius, 1992). Fingers 1-3 bind to the C-block, finger 5 binds the intermediate element, fingers 7-9 bind the A-block, whilst fingers 4 and 6 span these promoter regions (Hayes and Clemens, 1992; Hayes and Tullius, 1992). Mutation of finger 3 has the most detrimental effect upon binding to the 5S ICR (Del Rio and Setzer, 1993), whilst mutation of finger 9 causes a severe loss in transcriptional activity (Del Rio and Setzer, 1993). In addition to the transcripton of 5S genes, TFIIIA plays an important role in the storage and transport of 5S rRNA; fingers 4-7 display the greatest importance in binding RNA (Friesen and Darby, 1997).

1.11.2.5 SNAPc

Another gene-specific factor recognises the PSE of type III promoters. This factor is known as PBP, PTF and SNAPc (Henry et al., 1995; Sadowski et al., 1993). It has a mass of ~ 200 kD (Sadowski et al., 1993), and contains polypeptides of 190, 50, 45 and 43 kD, which correlates to those previously observed (Henry et al., 1995; Sadowski et al., 1996). Recently, an additional subunit has been identified, called $SNAP_{19}$ (Henry et al., 1998). Immunodepleting against $SNAP_{50}$, $SNAP_{43}$ or $SNAP_{45}$,

specifically repressed transcription from U6 and 7SK promoters, but not transcription from VA or AdML promoters (Bai et al., 1996; Henry et al., 1996; Henry et al., 1995; Sadowski et al., 1996; Yoon and Roeder, 1996). SNAPc interacts with TBP, but the amount of TBP that copurifies with SNAPc is variable (Henry et al., 1995; Sadowski et al., 1993).

1.11.3 Transcription complex formation on class III genes

In yeast, the positions of TFIIIC subunits with respect to a tRNA gene have been deduced by photocross-linking (Bartholomew et al., 1990). τ138 is located around the B-block, whilst τ95 and τ55 are associated with the A-block region (Bartholomew et al., 1990). The τ91 subunit is located downstream of class III genes (Braun et al., 1992) and cooperates with τ138 to bind DNA (Arrebola et al., 1998). τ131 is the only subunit to be located upstream of the transcription start site (Bartholomew et al., 1994; Bartholomew et al., 1991). The footprint of τ131 is extended upon the addition of TFIIIB (Braun et al., 1989; Kassavetis et al., 1990; Kassavetis et al., 1989). As τ131 extends upstream of the transcription start site, it is likely to recruit TFIIIB to the promoter (Bartholomew et al., 1991). Indeed, recombinant yBRF has been shown to bind to τ131 via its amino-terminal half (Chaussivert et al., 1995; Khoo et al., 1994). Once yBRF is brought into the complex, TBP is then recruited, followed by B" (Kassavetis et al., 1992). Pol III is brought into the initiation complex via an interaction between its C34 subunit and the amino

terminal imperfect repeats of yBRF (Khoo et al., 1994; Werner et al., 1993). (see fig. 1.6 for schematic representation of binding sites in BRF).

In vertebrates, TFIIIC binds predominantly to the B-block of tRNA and VA genes (Carey et al., 1986a; Cromlish and Roeder, 1989; Fuhrman et al., 1984; Wang and Roeder, 1996). TFIIIC2 binds to the B-block (Dean and Berk, 1988). TFIIIC1 is then recruited, resulting in the footprint of TFIIIC2 being extended to encompass the A-block (Oettel et al., 1997; Wang and Roeder, 1996; Yoshinaga et al., 1987). TFIIIB is then brought into the complex. hBRF can bind the pol III subunit C39, the human homologue of yeast C34 (Wang and Roeder, 1997), thereby bringing pol III to the start site (fig. 1.7.A).

TFIIIA binding to the promoter is the first step in transcription complex formation on type I promoters. TFIIIA binds to the 5S ICR, with its amino-terminus pointing towards the 3' end of the gene (Bieker and Roeder, 1984; Smith et al., 1984; Taylor et al., 1986; Vrana et al., 1988). The TFIIIA/5S DNA complex is relatively unstable (Bieker et al., 1985), but stabilisation occurs upon binding by TFIIIC (Bieker et al., 1985; Oettel et al., 1997). Once both TFIIIC and TFIIIA have been recruited, TFIIIB can be brought into the initiation complex (Bieker et al., 1985). Upon TFIIIB recruitment, pol III can then be assembled onto the preformed TFIIIC/TFIIIA/5S gene complex (Bieker et al., 1985) (fig. 1.7.B).

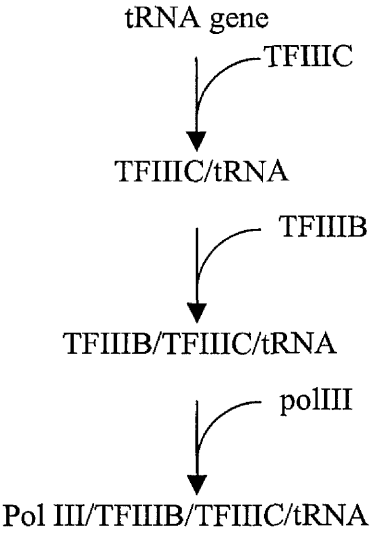
Type III promoters lack ICRs, and so there is no need for TFIIIA or TFIIIC2. The expression of genes with type III promoters requires TFIIIC1 (Yoon et al., 1995),

Fig. 1.7 The order of complex assembly on class III genes.

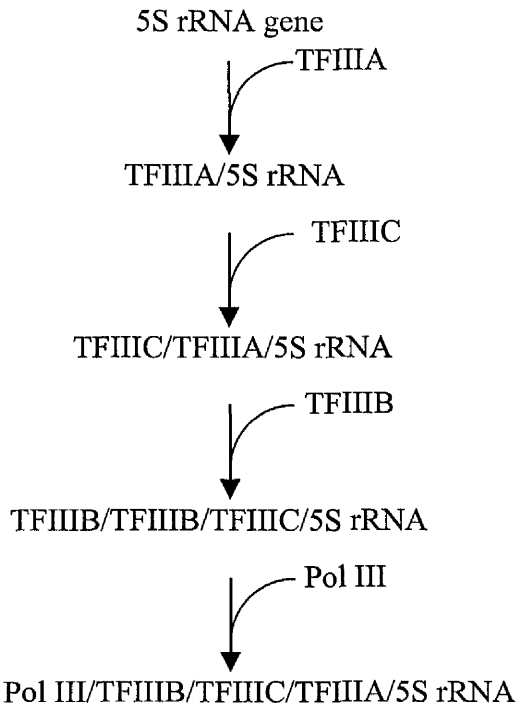
A. On tRNA genes the first transcription factor to bind to the promoter is TFIIIC, which then recruits TFIIIB and lastly pol III.

B. To initiate transcription from 5S rRNA genes, TFIIIA is first recruited to the promoter, then TFIIIC, TFIIIB and finally pol III.

A


tRNA gene
TFIIIC
TFIIIC/tRNA
TFIIIB
TFIIIB/TFIIIC/tRNA
polIII
Pol III/TFIIIB/TFIIIC/tRNA

B


5S rRNA gene
TFIIIA
TFIIIA/5S rRNA
TFIIIC
TFIIIC/TFIIIA/5S rRNA
TFIIIB
TFIIIB/TFIIIB/TFIIIC/5S rRNA
Pol III
Pol III/TFIIIB/TFIIIC/TFIIIA/5S rRNA

an alternative form of TFIIIB (Mital et al., 1996), and the PSE-binding factor (Reddy, 1988; Waldschmidt et al., 1991).

1.11.4 Regulation of RNA polymerase III transcription.

Pol III transcription is regulated by a variety of means, such as virally encoded proteins and various cellular proteins.

During mitosis pol III transcription is repressed (Hartl et al., 1993). TBP is subject to hyperphosphorylation, but this does not appear to be sufficient for the reduction in TFIIIB activity observed (White et al., 1995a). It is proposed that another subunit of TFIIIB is also modified (Leresche et al., 1996; White et al., 1995a). Indeed, pol III transcription appears to be regulated throughout the cell cycle, which is reflected by the activity of TFIIIB, which is low in early G1 phase, but increases as cells reach S phase. The change in activity is apparently due to an alteration of the activity of a TFIIIB-TAF (White et al., 1995b).

The level of pol III transcription varies during the cell cycle and also when cells take an alternative route such as differentiation. For example, when F9 embryonal carcinoma cells differentiate into parietal endoderm there is a down-regulation of pol III transcription (White et al., 1989). This is reflected by a decrease in TFIIIB activity, which is due to a reduction in the abundance of TBP and to a greater extent BRF (Alzuherri and White, 1998).

The level of transcription can be modulated by a variety of virally encoded proteins and cellular proteins. For example, the Tax protein of Human T-cell leukemia virus type I induces a higher level of pol III transcription, and TFIIIB is believed to be the principal target of this effect (Gottesfeld et al., 1996). The ability of a virally encoded protein to enhance pol III transcription is not unique to Tax; for example, adenovirus E1A protein can also up-regulate pol III transcription (Hoeffler et al., 1985).

Cellular proteins can also regulate pol III transcription. For example, Dr1 is a highly conserved 19kD nuclear phosphoprotein (Inostroza et al., 1992). It inhibits transcription upon binding its co-repressor DRAP1 (Mermelstein et al., 1996). Dr1 represses transcription via TBP, and deletion of the TBP binding domain within Dr1 forfeits its ability to repress transcription (Yeung et al., 1994). The binding of Dr1 to TBP does not prevent TBP from binding DNA; instead it prevents TBP from interacting with other transcription factors, such as BRF (White et al., 1994).

The retinoblastoma suspectibility gene and the p53 gene both encode important tumour suppressors. The repression mediated by retinoblastoma gene product shall be discussed later; at present the properties of p53 shall be summarised briefly.

The tumour suppressor protein p53 is lost or mutated in more than half of all human tumours, and its inactivation is considered an important step in carcinogenesis (Hollstein et al., 1991). p53 can arrest cell growth, but it is not required for passage

through the cell cycle. Mice which are homozygously null for the p53 gene develop normally, but display a greater predisposition to cancer (Donehower et al., 1992). It has been shown to be a general repressor of pol III transcription, as it inhibits transcription from all class III promoter types (Cairns and White, 1998; Chesnokov et al., 1996).

Kinases and phosphatases can also regulate pol III transcription, such as the kinase cdc2 which is complexed to cyclin B. This kinase is active as cells enter mitosis, and inactivates TFIIIB specifically when incubated with fractionated factors (Gottesfeld et al., 1994). The highly conserved serine/threonine protein kinase CKII may be involved in growth and cell cycle control (Pepperkok et al., 1994). In yeast CKII has been shown to stimulate tRNA and 5S RNA transcription, but the mechanism for this activation has yet to be elucidated (Hockman and Schultz, 1996).

1.11.5 Chromatin structure of class III genes

The chromatin structure of a gene can often determine its transcriptional activity (Wolffe, 1994). The structure of chromatin is as follows: 146bp of DNA is wrapped approximately twice around a nucleosome core comprising two molecules each of H2A, H2B, H3 and H4 histones. These are arranged as two H2A/H2B dimers associated with a central H3/H4 tetramer (Richmond et al., 1984). The flanking DNA is bound by a single molecule of histone H1 (Pruss et al., 1995). The presence of

histones can often inhibit transcription. For example, a nucleosome on or near a *Xenopus* 5S ICR inhibits transcription initiation in *Xenopus* oocyte nuclear extract (Morse, 1989). The ability of histones to inhibit both initiation and elongation is directly proportional to the density of the nucleosomes (Hansen and Wolffe, 1992).

1.12 RNA polymerase I transcription

1.12.1 Promoter structure of class I genes

Pol I transcription, unlike that displayed by pols II and III, exhibits species specificity. This results from the interaction between transcription factors and the promoter, and between the transcription factors themselves. Very little sequence similarity is observed in pol I promoters from different species, but, the general lay-out is conserved from yeast to human (Paule and White, 2000).

Ribosomal genes are found as multiple copies in head-to-tail clusters. The DNA intervening between these repeats is known as the intergenic spacer (IGS) and contains most of the promoter (Reeder, 1984). 50bp upstream from the initiation site is the core promoter, which is necessary and sufficient for initiation of basal transcription in most species (Paule and White, 2000). The core promoter contains the only conserved rRNA promoter element, the ribosomal initiator or rInr (Perna et al., 1992). This is an AT-rich sequence which surrounds +1 (Radebaugh et al., 1997). Although it resembles a TATA box, it is not an interaction site for TBP.

In most species there are additional elements and factors to help stabilise or assemble the complex formed on the core promoter. One such example is the upstream promoter element (UPE), which extends 150-200 bp upstream of +1 (Paule and White 2000; Reeder, 1984). UPE stimulates transcription driven by the core, but it is not absolutely required for transcription initiation. The UPE binds a factor that mediates the recruitment of the core factor to the promoter. Non-essential DNA separates the core and the UPE; the spacing and helical orientation, however, are important; half helical insertions or deletions are severely compromised in comparison to unaltered promoters (Pape et al., 1990).

In addition to the core and UCE, the IGS contains other sequence elements such as the proximal terminator (Bateman and Paule, 1988), enhancers (Paule and White, 2000), and spacer promoters (Morgan et al., 1983).

1.12.2 Transcription factors utilised by pol I

Two transcription factors are utilised by RNA polymerase I: SL1, which binds the core promoter, and UBF, upstream binding factor, which recognises the UCE. Together they recruit pol I to the initiation site of ribosomal promoters.

1.12.2.1 SL1

In humans the factor which recognises the core promoter is called SL1. It is referred to by other names in different species, for example TIF-IB in mouse (Eberhard et al., 1993), and Rib1 in *Xenopus* (McStay et al., 1991b). It is also the TBP-containing pol I transcription factor. SL1 and TBP were found to co-fractionate. Furthermore, immunodepletion with antibodies against TBP inactivated pol I activity, which could not be restored by recombinant TBP, suggesting the presence of TAFs (Comai et al., 1992). It was subsequently shown that SL1 was composed of TBP and three TAFs of 110, 63 and 48kD (Comai et al., 1992). Each TAF is able to bind TBP, and the two other TAFs (Comai et al., 1994; Paule and White, 2000).

The transcriptional activity of SL1 in an in vitro transcription assay can be reconstituted from purified TBP, TAF_I48, TAF_I63 and TAF_I110 in the presence of UBF and pol I, but not in the absence of TAF_I63 and TAF_I110 (Zomerdijk et al., 1994). SL1 has been demonstrated by coimmunoprecipitation analysis to interact with UBF (Hempel et al., 1996); this interaction is directed by TAF_I48 (Beckmann et al., 1995). The subunits of SL1 TAF_I63 and TAF_I110 have been shown by cross-linking experiments to contact DNA (Beckmann et al., 1995). The inability of SL1 to recognise and bind to TATA boxes in promoters is believed to be due to the ability of TAF_I48 to alter the DNA binding properties of TBP, either by blocking the DNA binding surface or changing its conformation (Beckmann et al., 1995).

1.12.2.2 UBF

UBF binds the UPE and aids core factor assembly of SL1 onto the promoter. The actual requirement for UBF is species dependent; in some species the core factor alone can mediate basal transcription. In human and *Xenopus* are reliant upon UBF (Bell et al., 1988; McStay et al., 1991a).

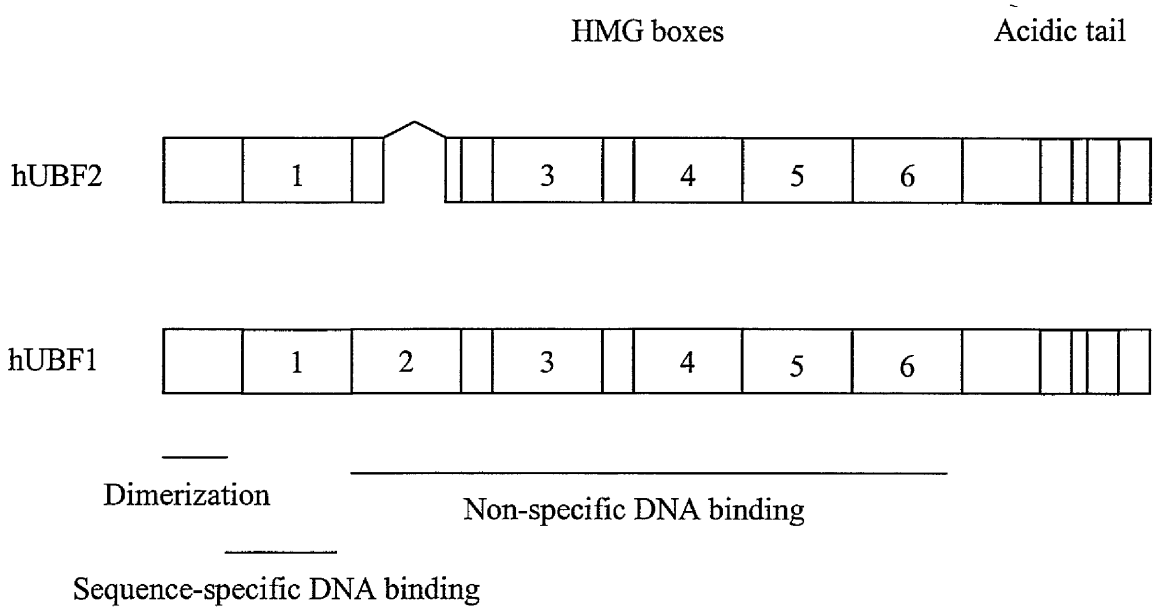
Vertebrate UBF is a dimer of 90-100 kD (O'Mahony and Rothblum, 1991). It has an amino-terminal dimerisation domain (McStay et al., 1991a), followed by four to six HMG box DNA-binding domains and a carboxy-terminal acidic serine-rich tail (fig. 1.8) (Bachvarov and Moss, 1991; Jantzen et al., 1990). The UBF variants result from differential splicing of the UBF message (O'Mahony and Rothblum, 1991). The region spliced is not conserved between species; in *Xenopus* a 22 amino acid region between HMG domains 3 and 4 is spliced from xUBF2, but not xUBF1 (Guimond and Moss, 1992). In mammals, 37 amino acids of HMG domain 2 is spliced from UBF2 but not UBF1. These splice vaiants have specific functions; UBF1 functions in transcription, whilst UBF2 has a role in enhancer function (McStay et al., 1997).

1.12.3 Transcription complex formation on class I genes

The first step in the transcription of ribosomal genes is the binding of UBF to the promoter, thereby establishing an interaction between dimerised UBF and both the core and UCE promoter elements. UBF is able to bind both the core and UCE

Fig. 1.8 Diagrammatic representation of the structure of UBF.

A diagram depicting the two forms of human UBF: UBF1 and UBF2, which has 22 amino acids deleted from its HMG2 box. The N-terminus of UBF is the dimerization domain, whilst the HMG1 box promotes sequence specific DNA binding, and HMG boxes 2-6 are responsible for non-specific DNA binding.


HMG boxes
Acidic tail
hUBF2
1
3
4
5
6
hUBF1
1
2
3
4
5
6
Dimerization
Non-specific DNA binding
Sequence-specific DNA binding

elements because the HMG boxes within UBF can bend the DNA sharply. Alone UBF is sufficient to direct initiation, but instead functions to recruit SL1 to the promoter, via protein-protein interactions involving the TBP and the TAF_I48 subunits of SL1. Once SL1 is recruited to the vicinity of the promoter it is able to bind to the DNA. The final step is the positioning of RNA pol I over the transcription start site (fig. 1.9) (Paule and White, 2000).

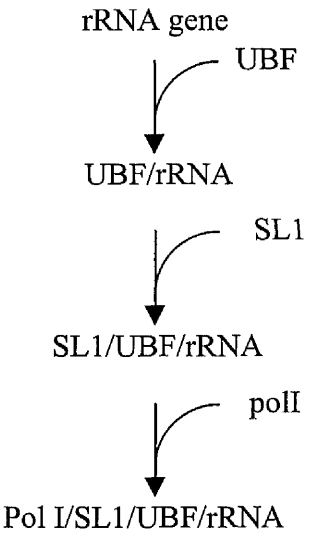
1.12.4 Regulation of RNA polymerase I transcription by UBF

The regulation of rRNA transcription reflects the growth and protein synthetic requirements of a cell. One way to modulate the expression of rRNA genes would be to regulate the activity of UBF and this can be achieved by at least three mechanisms; i) regulation of the amount of UBF, ii) regulation of the phosphorylation status of UBF and iii) sequestration of UBF into inactive complexes.

Sells et al. demonstrated that upon differentiation of L6 myoblasts the rate of rDNA transcription decreased, which was accompanied by a reduction in the amount of UBF in the cells, suggesting a possible casual relationship between the two events (Larson et al., 1993). Upon serum starvation of chinese hamster ovary cells, a reduction in the level of phosphorlyated UBF was observed in comparsion to growing cells. These findings suggest that the phosphorylation of UBF is a prerequisite for the activation of pol I transcription (O' Mahony et al., 1992). An example of a kinase capable of recognising UBF is CKII (Voit et al., 1992). A third means by which the

Fig. 1.9 Complex assembly on class I promoters.

The first step in complex assembly is the recruitment of UBF to the promter, which in turn recruits SL1and then lastly pol I.


rRNA gene
UBF
UBF/rRNA
SL1
SL1/UBF/rRNA
polI
Pol I/SL1/UBF/rRNA

activity of UBF can be regulated is by being sequestered by RB, which will be discussed in more detail below.

1.13 Regulation of pol III transcription by RB

The ability of RB to regulate pol III transcription was discovered by White et al (White et al., 1996). They showed that synthesis of pol III products, VA, 5S rRNA and tRNA was greater in fibroblasts derived from *Rb*-/- mice than fibroblasts from *Rb*+/+ mice (White et al., 1996). In addition, RB was shown to inhibit pol III transcription in a reconstituted system (White et al., 1996). This repression required an intact large pocket domain; RB with deletions within the large pocket was unable to repress transcription (White et al., 1996). RB was shown to inhibit transcription from all pol III promoter types, suggesting that RB targets a general factor of pol III transcription (Larminie et al., 1997). Indeed, RB binds and inactivates the pol III transcription factor TFIIIB (Larminie et al., 1997). RB has also been shown to bind TFIIIC2, however, this association is less than that observed with TFIIIB (Chu et al., 1997).

The physical association between TFIIIB and RB was demonstrated by pull-down assays, coimmunopreipcipitations and cofractionation analyses. (Larminie et al., 1997). As TFIIIB is a multisubunit complex, the next question to ask is which subunit RB targets in order to exert its effect. TBP has been shown not to bind RB (Weintraub et al., 1995) and is unable to relieve RB-mediated pol III repression

(Larminie et al., 1997), and so it is not a likely candidate. However, an affinity purified TFIIIB-TAF fraction is able to relieve such repression (Larminie et al., 1997), suggesting that a TAF subunit of TFIIIB is the target of RB-mediated repression.

1.14 Regulation of pol I transcription by RB

In addition to being able to repress pol II and pol III transcription, RB has also been demonstrated to repress pol I transcription (Cavanaugh et al., 1995). Upon differentiation of a monocyte-like cell line U937, a decline in pol I transcription is observed, as is the accumulation of RB in the nucleoli, the site of pol I transcription (Cavanaugh et al., 1995). Hence, experiments were undertaken to determine if RB was able to regulate pol I transcription. RB was shown to inhibit pol I transcription in an in vitro transcription system (Cavanaugh et al., 1995). The target of this repression was UBF, and the effect was mediated by the large pocket of RB (Cavanaugh et al., 1995).

The ability of RB to repress pol I transcription was supported by observations of Voit et al (Voit et al., 1997). Like Cavanaugh and co-workers, they showed that UBF was targeted in order to exert this effect. However, they proposed that the repression was mediated by the C-terminus of RB (Voit et al., 1997). They showed that the RB-binding site in UBF is HMG boxes 1 and 2 (Voit et al., 1997). In addition, they presented a model of how RB is able to inhibit pol I transcription. RB binds UBF, thereby preventing UBF from binding the pol I promoter. However, it

does not prevent UBF from interacting with either pol I or the TAF subunits of SL1 (Voit et al., 1997).

Hannan et al, have also undertaken investigations to further understand RB-mediated pol I repression. They showed that the A/B pocket of RB is required for its association with UBF. In contrast to Voit et al., they found thet RB binding did not abolish the ability of UBF to bind DNA, but rather its interaction with SL1 (Hannan et al., 2000a). In addition, p130 but not p107 was found to bind UBF. Furthermore, the overexpression of p130 represses ribosomal transcription (Hannan et al., 2000a).

Other circumstances under which RB downregulates pol I transcription have also been investigated. Studies show that upon confluence-induced cell cycle arrest the amount of UBF complexed to RB rises (Hannan et al., 2000b).

1.15 Aims and objectives of these studies

The aim of these studies is to identify the subunit of TFIIIB that RB targets to exert its inhibitory effect on pol III transcription, and to determine the regions of each protein required for this interaction. Also, to determine the effect RB binding to TFIIIB has upon its interaction with other components of the pol III machinery, establishing a mechanism of RB-mediated repression.

In the majority of human cancers RB is inactivated. The effects this has upon the RB/TFIIIB interaction are to be determined.

The physical association between RB and UBF is to be investigated. Also the binding sites responsible in each protein are to be identified.

Lastly, the ability of the other pocket proteins, p107 and p130, to interact with the targets of RB-mediated repression of pol I and pol III transcription, namely UBF and TFIIIB respectively, is to be determined.

Chapter 2

Experimental Protocols

2 Experimental protocols

All chemicals, unless otherwise stated, were supplied by Sigma (Sigma-Aldrich Company Ltd, Poole, Dorset, UK), BDH Laboratory Supplies (Merck House, Poole, Dorset, UK) or GibcoBRL (Life Technologies Ltd, Paisley, Renfrewshire, UK). Protein A sepharose was obtained from Amserham Pharmacia Biotech AB. Radiochemicals were provided by Amserham International, UK.

Plasmids used

yBRF plasmid constructs

The in vitro translatable yBRF and mutant derivatives were kindly provided by George Kassavetis (Kassavetis, 1998).

hUBF and xUBF plasmid constructs

In vitro translatable hUBF, xUBF and xUBF mutant forms were a generous gift from Brain McStay (McStay et al., 1991a).

hBRF plasmid construct

The in vitro translatable hBRF plasmid construct contains the full length sequence and was generously donated by Nouria Hernandez (Mital et al., 1996).

RB plasmid contructs

The RB constructs were kindly provided by Dennis McCance (Kaelin et al., 1991)

2.1 Molecular biology procedures

2.1.1 Determination of nucleic acid concentration

The concentrations of nucleic acid solutions were determined spectrophotometrically using a quartz cuvette (Sambrook, 1989).

1 A_{260} = 50μg/ml dsDNA

1 A_{260} = 40μg/ml ssRNA

2.1.2 Transformations

20μl of competent cells (XL1 Blue for DNA preparations and BL21 (DE3) pLysS for GST fusion and His-tagged protein preparations) were thawed on ice. After thawing, 0.2μg of the appropriate DNA was added and the mixture was placed on ice for 30 minutes. The mixtures were heat-shocked at 42°C for 45 seconds and then cooled on ice for two minutes. 900μl of LB (containing no amp) was added to the cells, which were then incubated at 37°C for 30 minutes. After incubation, the

cells were pelleted and the majority of supernatant aspirated off. The cells were then resuspended in the remaining supernatant, spread on a LB plate (amp. 100μg/ml) and incubated at 37°C overnight.

***E. coli* strains:**

XL1 Blue,

racA1 endA1 gyrA96 thi-1 hsdR17 supe44 relA1 lac[F' proAB lacI^{q}ZΔM15 Tn10 (Tetr^2)]

BL21(DE3)pLysS,

BF$^-$ dem opmT hscB($r_B^-m_B^-$) galλ(DE3) [pLysS Cam^r]

LB: stored at RT

20g of LB mix was dissolved and autoclaved in 1L H_2O, to give

1 % Bacto-tryptone (w/v),

0.5 % Bacto-yeast extract (w/v),

1 % NaCl (w/v).

Amp. was added as required after autoclaving to give 100μg/ml.

Agar Plates: stored at 4°C

1 x LB,

1.2 % (w/v) agar.

The solution was autoclaved. When it was cool enough to be held, amp. was added to give a final concentration of 100μg/ml. The solution was mixed and poured into 100mm diameter plates. Immediately after the surface was flamed with a bunsen burner, and the lids set ajar to allow the plates to dry at RT.

2.1.3 Large scale DNA preparations

Large scale preparations of plasmid DNA was carried out as described in the manufacturers' protocol (Qiagen).

2.1.4 Small scale DNA preparations

Small scale preparations of plasmid DNA were carried out as described in the manufacturers' protocol (Promega).

2.1.5 In vitro translation

In vitro transcription/translation reactions were carried out as specified in the manufacturers' protocol (Novagen). 1μg of plasmid DNA was added to 8μl of the transcription mixture and incubated at 30°C for 30 minutes. To the transcription mixture, 30μl of translation mixture, 40μCi ^{35}S Met and Cys and 6μl dH_2O, was added. The mixture was then incubated at 30°C for one hour.

The in vitro translation of the truncated BRF proteins was undertaken by linearising the construct containing BRF cDNA. These forms were made by incubating 10μg of the BRF construct with 1U of the appropriate restriction endonuclease, either Stu I, Bgl II, Xcm I, Hinc II or EcoR I, for one hour at 37°C. Completion of endonuclease digestion was determined by analysing 1μl of the reaction mixture on a 1% agarose gel at 80V for one hour. The agarose gel was stained with ethidium bromide and viewed with U.V. light. Upon completed endonuclease digestion, the DNA was phenol/chloroform extracted, ethanol precipitated and resuspended in 1 x TE to give a concentration of 0.2μg/μl. The truncated template was in vitro transcribed by adding 1μg of endonuclease digested template DNA with 1mM NTPs, 1 x TSC buffer (Promega), 5mM DTT, 40U RNasin (Promega), 80U T7 RNA polymerase (Promega) and H_2O to 50 μl, then incubating at 37°C for 90 minutes. After incubation, the reaction mixture was phenol/chloroform extracted, and the RNA ethanol precipitated with 1/10 volume 7.5M NH_40Ac. The RNA was resuspended in 50μl DEPC-H_2O; this was aided by

incubating at 65°C. The RNA samples (2µl) were added to an in vitro translation system composed of 35µl reticulocyte lysate (Promega), 7µl DEPC-H_2O, 1µl amino acid mixture minus Met and Cys (Promega), 40U RNasin (Promega) and 40µCi ^{35}S Met and Cys. The mixture was then incubated at 30°C for 90 minutes. Expression of the truncated protein was analysed by resolving 2µl aliquots by SDS-polyacrylamide gel electrophoresis.

Reticulocyte lysate containing in vitro expressed radiolabelled RB was phosphorylated by incubating the RB expressing reticulocyte lysate with 100ng of cdk 4/cyclin D1, cdk 2/cyclin E and cdk 2/cyclin A (a gift from Prof. R. White), for 30 minutes at 30°C. Successful phosphorylation was determined by resolving 2µl aliquots by SDS-polyacrylamide gel electrophoresis and looking for a shift in electrophoretic mobility.

1 % Agarose Gel:

1 % (w/v) agarose,

1 x TAE.

Agarose Gel Running Buffer:

1 x TAE.

1 x TAE: stored at RT

0.04M Tris-acetate,

0.001M EDTA.

2.1.6 Expression of GST fusion proteins

GST fusion proteins were prepared as previously described (Smith and Johnson, 1988). 40ml of LB (amp. 100μg/ml) was inoculated with a single colony from a streaked plate, or from a loopful of glycerol stock, and incubated at 37°C overnight. The following day the overnight culture was diluted 1:10, by adding it to 400ml LB (amp. 100μg/ml), and this culture was then incubated at 30°C. Samples of the culture were taken and the optical density at 600nm was read. Upon the culture reaching an optical density >0.4, the cultures were induced with IPTG to a final concentration of 0.1mM. The cultures were then grown at 30°C for 6 hours. Following incubation, the cells were pelleted by centrifugation at 1367g for 15 minutes, in a Sorvall centrifuge. The supernatant was aspirated off and the bacterial pellet resuspended in 9ml ice-cold PBS, 1% triton, 0.001M PMSF. The resuspended cells were sonicated on ice (2 x 60W), after which the bacterial debris was pelleted by centrifugation at 17,522g, for 5 minutes in a Sorvall centrifuge. The resulting supernatant was removed and incubated with 400μl of packed glutathione-agarose beads for 30 minutes at 4°C. Following binding, the glutathione-agarose beads were pelleted by centrifugation at 828g for 3 minutes at 4°C in a bench top Sorvall. The supernatant was removed and the glutathione-agarose beads washed twice with 50ml of ice-cold PBS, 1% triton, then twice with 50ml of ice-cold PBS. The remaining

glutathione-agarose beads were then transferred to an Eppendorf tube. Successful expression was confirmed by analysing 20µl of bound GST-fusion protein by SDS-PAGE and staining with coomassie blue. The remaining GST-fusion protein was snap frozen on dry-ice and stored at –80°C.

In some instances the GST-fusion protein was eluted from the glutathione-agarose beads. This was achieved by adding one bead volume of 25mM glutathione, 0.05mM Tris-HCl pH 8.0 to the glutathione-agarose beads and vigorously vortexing the mixture at RT for 10 minutes; this procedure was repeated twice. Again, successful expression and elution was confirmed by resolving 10µl aliquots on a SDS-polyacrylamide gel and then staining with coomassie blue. The remaining protein was snap frozen on dry-ice and stored at –80°C.

PBS: stored at 4°C

1 PBS tablet (Sigma), was dissolved in 200ml of water to give 0.01M phosphate buffer,

0.0027M KCl,

0.137M NaCl, pH 7.4,

4mM Na_2HPO_4,

1.8mM KH_2PO_4.

2.1.7 Expression of His-tagged RB (379-928)

40ml of LB (amp. 100μg/μl) was inoculated with a single colony from a streaked plate, and incubated at 37°C overnight. The following day the overnight culture was diluted 1:10 by adding it to 400ml of LB (amp. 100μg/μl). It was then incubated at 30°C. Samples of the culture were taken periodically and the optical density at 600nm was determined. Upon reaching an optical density >0.4, the cultures were induced with IPTG to a final concentration of 0.15mM. The cultures were then grown at 30°C for six hours. Following incubation, the cells were pelleted by centrifugation at 1367g for 15 minutes at RT in a Sorvall centrifuge. The supernatant was removed and the pelleted cells resuspended in 9ml 1 x TBS, 1 % Triton, 0.001M PMSF. The resuspended cells were sonicated on ice (2 x 60W), then the bacterial debris was pelleted by centrifugation at 17,522g for 5 minutes at RT, again in a Sorvall centrifuge. The supernatant was then incubated with 300μl of packed Ni-NTA beads (Qiagen) with imidazole to a final concentration of 10mM, for 30 minutes at 4°C. Following binding, the Ni-NTA beads were pelleted in a bench top Sorvall at 828g for 3 minutes at 4°C and the supernatant removed. The Ni-NTA beads were then washed five times with 20 bead volumes of 5 x TBS, 10mM imidazole, then five times with 20 bead volumes of 5 x TBS, 40mM imidazole. The bound protein was eluted with 1 bead volume of 1 x TBS, 150mM imidazole with vigorous vortexing at RT for 10 minutes. Successful expression was determined by analysing a 10μl aliquot of the purified protein by 7.8 % SDS-PAGE and then staining with coomassie blue.

2.2 Electrophoresis analysis

2.2.1 SDS-PAGE

SDS-PAGE analysis was carried out based on a protocol described previously (Laemmli, 1970), using Biorad Mini-Protean II systems. 7.8%/10%/12% denaturing polyacrylamide gels were poured with low acrylamide/low pH stacking gels. Prior to loading, the samples were prepared in 4 x protein sample buffer, and boiled for 4 minutes. Apart from samples that were immunoprecipitations of solely cell extract, these were vigorously vortexed instead of being boiled. The loaded samples were resolved at 70V, until the dye front had gathered, and then at 140V until the dye front had run off the bottom of the gel. Full range molecular weight rainbow markers (Amersham) were run alongside the samples. Those gels containing radioactive samples were vacuum dried at 80°C. Those containing large amounts of specifically purified proteins were stained in coomassie blue for 1 hour, then destained. Other gels were immunoblotted.

4 x Protein Sample Buffer: stored at RT

250mM Tris-HCl pH6.8,

2% SDS,

20% β-mercaptoethanol,

40% glycerol,

0.5% bromophenol blue.

10 x Protein Running Buffer: stored at RT

0.1M Tris-HCl,

0.768M glycine,

20% SDS, pH 8.8.

4 x Upper Buffer: stored at RT

1.5M Tris-HCl, pH 8.8,

0.4% SDS.

4 x Lower Buffer: stored at RT

0.5M Tris-HCl pH 6.8,

0.4% SDS.

SDS-PAGE composition table:

	Stacking buffer	7.8%	10%	12%
4 x lower buffer	-	1.25ml	1.25ml	1.25ml
4 x upper buffer	1.25ml	-	-	-
30% acrylamide	0.7ml	1.3ml	1.7ml	2.0ml
dH_20	3ml	2.4ml	2.0ml	1.7ml

+ 11µl TEMED

30µl 10% (w/v) APS

Staining and destaining of polyacrylamide gels

Polyacrylamide gels were stained by placing them in a plastic box containing coomassie blue, then placing that on a tilting platform for one hour. In order to visualise the protein bands, the coomaasie blue was drained off, and destain was added to the box, which was returned to the tilting platform for another hour. After destaining, the polyacrylamide gel was removed from the box and vacuum dried.

Coomassie Blue: stored at RT

1% (w/v) Coomassie Blue,

50% (v/v) Methanol,

10% (v/v) Glacial acetic acid.

Destain: stored at RT

30% (v/v) Methanol,

10% (v/v) Acetic acid.

2.2.2 Immunoblotting

Immunoblotting was carried out as described previously (Burnette, 1981; Towbin, 1979). Samples were transferred onto 0.2μM pore nitrocellullose (Biorad) in 1 x Western Blotting Buffer, using a Biorad Mini-Protean II system. The samples

were blotted at 37V, with a set limit of 1 mA for 30 minutes. The transblotter was kept cool by an ice block. Upon completion of transfer, the nitrocellolose filter was washed in 1 x TBS, then transferred into Milk Buffer (MB) for three washes of two minutes, after which the primary antibody diluted 1:1000 in MB was added and the filter probed overnight at 4°C. The following day the filter was washed three times for two minutes each in MB, after which the secondary antibody, either rabbit polyclonal or mouse monoclonal depending on the primary antibody, again diluted 1:1000 in MB, was added to the filter, and further probed at RT for 1 hour. After this, the filter was washed three times for two minutes each with MB, then twice for 15 minutes with MB. It was then transferred into 1 x TBS for 5 minutes before being developed by ECL (Amersham), apart from fig. 6.3.B which was developed by alkaline phosphatase. Both methods were undertaken as instructed in the manufacturers' protocols.

50 X Western Blotting Buffer: stored at RT

1M Na_2HPO_4 was brought to pH 6.7 by the addition of 1M NaH_2PO_4.

10 x TBS: stored at RT

25mM Tris-HCl pH 7.6,

150mM NaCl.

Milk Buffer: made as required

1 x TBS,

0.5% (v/v) Tween-20,

4% (w/v) powdered milk.

Antibodies

M19	αTAF$_I$48 (Santa Cruz)
M18	αTAF$_I$95/110 (Santa Cruz)
C15	αRB (Santa Cruz)
C18	αp107 (Santa Cruz)
C19	αTAF$_I$48 (Santa Cruz)
C20	αp130 (Santa Cruz)
C21	αOCT-1 (Santa Cruz)
SD9	αp107 (Santa Cruz)
XZ91	αRB (Santa Cruz)
IF8	αRB (Santa Cruz)
G99-549	αRB (Pharminogen)
G3-245	αRB (Pharminogen)
MTBP-6	αTBP (Pruzan et al., 1992)
SL30	αhTBP (Ruppert et al., 1996)
αhUBFAb	αhUBF (Santa Cruz)
αRb2mAb	αp130 (Affiniti)
4286-4	αTFIIICβ (Generated against a peptide encompassing residues 897-911 of TFIIICβ)

BN51 113	αpol III subunit (Ittmann et al., 1993)
128-4	αBRF (Generated against a peptide encompassing amino acids 533-547 of BRF)
330-4	αBRF (Generated against a peptide encompassing the residues 664-677 of BRF)

2.3 Immunoprecipitations

2.3.1 Purification of antibodies

100µl of packed protein A sepharose was washed twice with 1ml of ice cold PBS, and poured into a column (Biorad). The pH of the antibody preparation was adjusted to pH 8.0, then poured into a Bio-rad column. Once the antibody solution had passed through the column it was recycled five times. After recycling the sample, the column was washed with 10 column volumes of 100mM Tris-HCl pH 8.0, then with 10 column volumes of 10mM Tris-HCl, pH 8.0. The antibody was eluted 5 times with 1 column volume of 100mM glycine pH 3.0, into eppendorf tubes containing 1/10 column volume 1M Tris pH 8.0. The fractions containing the antibody were identified by mini bradford assays.

2.3.2 Mini bradford assays

Bradford reagent was diluted 10 fold with dH_2O, 100µl aliquots were added to the wells of a 96 well ELISA assay tray to which 10µl of eluted antibody was added. The intensity of colour change related to the amount of protein in that fraction.

2.3.3 Coupling of antibodies to protein A beads

20µl of packed protein A sepharose beads (Pharmacia) was washed twice with 100µl ice-cold LDB (LDB is 20mM Hepes pH 7.0, 100mM KCl, 12mM $MgCl_2$, 0.1mM EDTA, 17% glycerol, 2mM DTT). The beads were pelleted in a microfuge at 16,060g for 30 seconds, and the supernatant removed. The beads were then coupled to the appropriate antibody, by adding 2µl of the antibody of choice and 18µl of LDB; coupling was commenced by placing the mixture on a vibrating platform at 4°C for one hour. After coupling, the beads were washed twice with 100µl of ice-cold LDB, then pelleted in a microfuge at 16,060g for 30 seconds, and the supernatant decanted.

2.3.4 Immunoprecipitations using reticulocyte lysate expressed proteins

20µl of protein A sepharose beads (Pharmacia) conjugated to an anti-TBP antibody were washed twice with 200µl LDB, the beads were pelleted in a microfuge at 16,060g for 30 seconds, and the supernatant decanted. 20µl of reticulocyte lysate (Novagen) containing in vitro expressed ^{35}S radiolabelled hTBP or hBRF from the T7 expression vectors pARFLIID and pCITED1, respectively, were added as indicated to the beads. Alternatively, as specified, reticulocyte lysate containing truncated forms of hBRF were analysed. In one experiment a third protein was included in the immunoprecipitation namely RB purified from baculovirus Sf9 cells, a generous gift from S. Mittnacht. The immunoprecipitation reactions were placed on a vibrating platform for 3 hours at 4°C. After which they were washed five times with 150µl LDB. The beads were pelleted in a microfuge at 16,060g for 30 seconds and the supernatant was aspirated off. 16µl of 1 x protein sample buffer was added and the samples were boiled for 4 minutes, prior to being loaded onto a 7.8% SDS-polyacrylamide gel and visualised by autoradiography.

2.3.5 Immunoprecipitations involving reticulocyte lysate expressed proteins and cellular extract

150µg of the desired cellular extract was mixed with 15µl of the reticulocyte lysate expressing the desired ^{35}S labelled protein and, as indicated, 200ng of eluted

GST fusion protein or His-tagged RB. These mixtures were added to the beads coupled to the antibody of choice, and were placed on the vibrating platform for 3 hours. After binding, the beads were washed five times with 150µl LDB. 16µl of 1 x protein sample buffer was added and the samples boiled prior to being resolved by SDS-polyacrylamide gel electrophoresis and visualised by autoradiography.

2.3.6 Immunoprecipitation from cellular extract

150µg of the cellular extract of choice, and as specified 200ng of the appropriate eluted GST-fusion protein or His-tagged RB, were added to the antibody coupled beads, and the mixture placed on a vibrating platform for 3 hours. Following incubation, the beads were washed five times with 150µl LDB. 16µl of 1 x protein sample buffer was added, and the samples vigorously vortexed, before being resolved on a SDS-polyacrylamide gel and visualised by western analysis.

2.4 GST-fusion protein pull down assays

Equal volumes of purified GST proteins were resolved by SDS-polyacrylamide gel electrophoresis and stained with coomassie blue. The relative amounts of GST proteins present were estimated, and equal amounts of GST-fusion proteins were compared in the pull-down assays. The reticulocyte lysate expressing

the required ^{35}S-labelled protein was added to the beads and the mixture incubated on a vibrating platform at 4°C for 3 hours. After vibrating, the samples were washed five times with 700µl LDB. 16µl of 1 x protein sample buffer was added, the sampled boiled and the reactions resolved by SDS-polyacrylamide gel electrophoresis and visualised by autoradiography.

2.5 Random polymerisation assays

The desired immunoprecipitation reactions were set up as described above. After washing with LDB, the random polymerisation assays were carried out, as described previously (Roeder, 1974). 25µl of incubation buffer was added to each sample, which were then mixed thoroughly and incubated at 30°C for 20 minutes. After incubation, the mixture was bound to DEAE discs and the discs washed for five minutes with 0.5M Na_2HPO_4. This wash step was repeated five times. The discs were then washed twice for 5 minutes each with dH_20, then 2 x 5 minutes with 96% ethanol, and lastly with diethyl ether. After the last wash, the discs were blotted with whatman paper and left to air-dry. Once the discs were dry, they were placed in scintillation vials containing 5ml of scintillation fluid, and the incorporated ^{32}P counted in a scintillation counter.

TC(NE): stored at –80°C

6.25mM rATP,

6.25mM rCTP,

6.25mMrGTP,

1.75mM EDTA.

Polymerase Buffer: stored at 4°C

0.7M Tris-HCl pH 7.9,

0.9M NH_4SO_4 ,

28mM MnCl.

Incubation mixture: made fresh as required

240µl polymerase buffer,

78µl TC(NE),

40nM UTP,

80µg polydAdT,

160µCi α^{32}P UTP,

0.08% (w/v) BSA.

2.6 Oligonucleotide binding assay

2.6.1 Oligonucleotide binding assay

The immunoprecipitation reactions of interest were set up as described above. After the immunoprecipitation reactions had been washed five times with LDB, 1ng of ^{32}P-radiolabelled B-box probe, 1μg of polydIdC, and as indicated 100ng of specific or non-specific competitor oligonucleotide were added to the reaction samples. The samples were mixed well and incubated at 30°C for 30 minutes. After incubation, the samples were washed five times with 150μl LDB. The probe retained on the beads was eluted by the addition of 20μl of 1M KCl, and then bound to DEAE discs. The discs were blotted dry with whatman paper. Once dry, they were placed in a scintillation vial containing 5ml scintillation fluid and bound ^{32}P was measured in a scintillation counter.

2.6.2 Manufacture of B-box probe

The oligonucleotide RJW1 contains the upper strand of the B-block TFIIIC recognition sequence from a B2 gene (White et al., 1989). It was labelled by incubating 40ng of the oligonucleotide with 20μCi of γ-^{32}P ATP, 10U of T4 polynucleotide kinase (Promega) and 1 x T4 buffer (Promega), at 37°C for 1 hour. The reaction was heat inactivated at 65°C for 10 minutes. After heat inactivation, the

probe was phenol/chloroform extracted and ethanol precipitated, washed five times with 70% ethanol, and resuspended in 20µl of 1 x TE. 100ng of the complementary oligonucleotide RJW2 was added and the nucleic acids denatured at 90°C for 2 minutes; the probe was then cooled overnight to allow annealing to occur. The following day, the sample was diluted with 1 x TE to give a final concentration of 0.5ng/µl.

2.6.3 Oligonucleotides used in the oligonucleotide binding assay

RJW1

5'-AGAGGTCCTGAGTTCAAATCCCAGCAACC-3'

RJW2

5'-GGTTGCTGGGATTTGAACTCAGGACCTCT-3'

RJW19

5'-CCGTGCCTGATCATGATATTCTTTGGG-3'

RJW20

5'-CCCAAAGAATATCATGATCAGGCACGG-3'

1 x TE: stored at RT

10mM Tris-HCl pH 7.5,

1mM EDTA pH 8.0.

2.7 Tissue culture

2.7.1 Passaging of cell lines

SAOS2 and C33A cells were cultured as adherent monolayers in Dulbeccos' modification of Eagles Medium (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS-GiboBRL), 4mM L-glutamine (GibcoBRL), antibiotics; 10mg/ml streptomycin and 100U/ml penicillin (GibcoBRL). The cells were grown at 37°C, in 5 % CO_2/H_2O saturated atmosphere. The cells were cultured in 75 cm^2 tissue culture flasks (NUNC) and seeded at one in four. Cells were passaged by treatment with trypsin (Gibco BRL, dissolved in FCS-free DMEM, 0.05% (w/v)) at 37°C for 1 minute to dislodge the cells from the polystyrene. Trypsinisation was arrested by the addition of complete media, in which the cells were resuspended.

2.7.2 Cyro-storage of cells

Live cells were subjected to long term storage by resuspending near confluent 75cm^2 flasks in 2ml media containing 10% DMSO. The suspension was

aliquoted into cryotubes (NUNC) and placed at -80°C overnight to ensure slow freezing, before being transferred to liquid nitrogen.

Cells were thawed at RT and poured into a 50ml tube, 30ml of complete media was added to the cells, the mixture was then centrifuged at 298g for 5 minutes at RT. After centrifugation, the supernatant was aspirated off and the cells resuspended in 12ml of complete media before being transferred to a 75 cm^2 flask. Recovery was almost 100% and cells were always passaged once before being used after thawing.

2.7.3 Preparation of protein microextracts

Preparation of protein microextracts was based on a protocol described previously (Schöler, 1989). C33A cells were harvested when cells were near confluence. The plates were washed twice with 6ml of ice-cold PBS; then harvested in 6ml of ice-cold PBS, with plate scraping. The PBS/cell mixture was placed in a 50ml tube and centrifuged at 298g for 8 minutes at 4°C in a bench top Sorvall. The supernatant was aspirated off, and the pellet transferred to an eppendorf tube. The pellet was resuspended in 1 volume of microextraction buffer, and immediately snap frozen on dry ice. After freezing, the cells were thawed at 30°C and the freeze/thaw process was repeated twice. After the last thawing process, the mixture was centrifuged in a microfuge at 16,060g for 10 minutes at 4°C. After centrifugation,

the supernatant was collected and the protein concentration measured by a bradford assay.

Microextraction stock: stored at RT

0.02M Hepes-HCl pH 7.8,

0.45M NaCl,

25% (v/v) Glycerol,

0.05M NaF,

0.2mM EDTA.

Microextraction buffer: made fresh as required

974µl microextraction stock,

0.5mM PMSF,

1mM DTT,

20µl PIM.

PIM: stored at –20°C

1.6ml Bestatin (2.5mg/ml),

50µl Leupeptin (1mg/ml),

100µl Trypsin inhibitor (1mg/ml),

24µl Aprotinin (2.2mg/ml),

70µl Pepstatin (1mg/ml),

150µl E-64 (2mg/ml).

2.7.4 Bradford assay

A standard Bradford assay curve was constructed by determining the optical densities at 595nm of 0μg, 2μg, 4μg, 6μg, 8μg, 10μg, 12μg, 14μg, 16μg, and 18μg of BSA in 1ml of Bradfords reagent (Bio-Rad) diluted 1:5 with dH_2O. The readings were plotted on a graph, (x-axis protein concentrations, y-axis absorbency readings) and a standard curve fitted. 4μl aliquots of the microextraction samples were taken and diluted 1:4 with dH_2O. 2μl, 4μl and 8μl of the diluted microextraction samples were added to 1ml of Bradfords reagent diluted 1:5 with dH_2O, and the optical density at 595nm determined. These readings were then converted into protein concentrations by reading from the standard curve. For those readings obtained from 2μl of diluted microextraction buffer the reading was doubled, that obtained for the 4μl sample was left unaltered, whilst those for the 8μl sample was halved. These figures were then averaged to find the protein concentration of the microextraction samples.

2.7.5 Transient transfections

SAOS2 cells were transiently transfected using the calcium-phosphate-precipitate method. Cells were plated at a density of 1 x 10^6 cells in a 100mm diameter dish (NUNC) and incubated at 37°C for 24 hours. 4 hours prior to the

addition of the precipitated DNA, the medium was aspirated off and 6ml of fresh medium was added.

The donor DNA was placed in a plastic bijou flask and the volume made up to 450µl with 0.1mM EDTA pH 8.0, 1mM Tris-HCl pH 8.0. 1/10 volume of $CaCl_2$ was added, and the solution mixed. The DNA mixture was then added a drop at a time, with continuous vortexing to 500µl 2 x Hepes buffer saline in a second plastic bijou. When the addition was completed, the mixture was further vortexed for 15 seconds, then left to stand for 30 minutes at RT. After precipitation, the DNA was added a drop at a time to a plate of SAOS2 cells. The cells were incubated at 37°C overnight to allow absorption of the DNA-calcium phosphate precipitate into the cells. The following morning, the cells were washed twice with 6ml PBS, fresh medium was added, and the transfected cells incubated at 37°C for 72 hours. Total RNA was harvested from the cells using TRI-reagent (Sigma), according to the manufacturer's protocol. To assess for the expression of the transfected genes, parallel plates were harvested for protein by the addition of 100µl 1 x protein sample buffer, in which the cells were resuspended. Harvesting was quantitated by resolving 10µl of the suspension by 7.8% SDS-polyacrylamide gel electrophoresis, followed by staining with coomassie blue. Relative amounts of protein were estimated by visual comparsions. Equal amounts of protein were subsequently resolved by 7.8% SDS-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membrane and probed with the appropriate antibody.

2 x Hepes Buffer Saline: stored at 4°C

0.28M NaCl,

50mM Hepes,

1.5mM $Na_2HPO_4.2H_20$.

2.8 Primer extensions assays

2.8.1 Manufacture of probes

The VA_1 and CAT probes were both radiolabelled by incubating 50ng of the appropriate oligonucleotide with 40U T4 polynucleotide kinase (Promega), 1 x T4 kinase buffer (Promega), and 80μCi γ-^{32}P ATP, in DEPC-H_2O, at 37°C for 1 hour. Upon completion of the reaction, the samples were heat inactivated at 65°C for 10 minutes.

2.8.2 Primer extension reactions

To each primer extension reaction, containing up to 10μg of RNA (but not exceeding a final volume of 10μl), 1μl of probe and 9μl of 1st strand buffer (Promega) was added. The nucleic acids were denatured at 80°C for 10 minutes, then annealed at 50°C for 2 hours. Following annealing, 1.7mM DTT, 0.83mM

dNTPs, 2nM actinomcyin D, 20U RNasin (Promega), 100U superscript (Promega) and dH_2O to a final volume of 30µl were added to each reaction, and the mixtures incubated at 42°C for 1 hour. After extension, the samples were ethanol precipitated at –20°C, then centrifuged in a microfuge at 16,060g for 15 minutes at RT. The pellets were washed with 75% ethanol and centrifuged at 16,060g for 5 minutes at RT. The pellets were dried at 50°C for 5 minutes and resuspended in 4µl of formamide running buffer by vortexing vigorously for 30 minutes. Prior to resolution on a 7% sequencing gel for 1 hour at 40V, the samples were boiled for 2 minutes and then immediately placed on ice.

Formamide running buffer: stored at RT

98% formamide,

0.01% Bromophenol blue,

0.01% Xylene cyanol pp,

5mM EDTA.

7 % sequencing gel mix: stored at RT (in foil)

7 % (v/v) acrylamide,

46 % (w/v) urea,

0.5 X TBE.

The mixture for one gel consisted of 50mls of 7 % sequencing gel mix, to which 300µl of 10% (w/v) APS and 30µl TEMED were added. Prior to loading of the samples, the gel was pre-run for 30 minutes at 40V.

7 % sequencing gel running buffer:

0.5 X TBE.

0.5 X TBE: stored at RT

0.045M Tris-borate,

0.001M EDTA pH 8.0.

2.8.3 Oligonucleotides used in primer extension analysis

VA_1 specific

5'-CACGCGGGCGGTAACCGCATG-3'

CAT specific

5'-CGATGCCATTGGGATATATCA-3'

Chapter 3

Characterisation of the interaction between the pol III transcription factor TFIIIB and the tumour suppressor protein RB

3 Characterisation of the interaction between the pol III transcription factor TFIIIB and the tumour suppressor protein RB

3.1 Introduction

The retinoblastoma protein (RB) is a 105kDa nuclear phosphoprotein that is encoded by the important tumour suppressor gene *Rb* (Weinberg, 1995; Herwig and Strauss, 1997; Grana et al., 1998; Mulligan and Jacks, 1998). In normal cells RB provides a mechanism for constraining cell growth and proliferation. In the absence of RB, the ability of a cell to regulate these functions is compromised (Weinberg, 1995; Whyte, 1995). RB is found mutated in many human tumours, such as retinoblastomas, small cell lung carcinomas, sarcomas and bladder carcinomas (Weinberg, 1995; Herwig and Strauss, 1997; Grana et al., 1998; Mulligan and Jacks, 1998). In many other tumours, the pathway by which the activity of RB is regulated is aberrant (Pines, 1995; Weinberg, 1995). It is therefore of great importance to understand how RB influences cellular activity.

In 1996, White et al. (White et al., 1996) demonstrated that RB is capable of suppressing pol III transcription both *in vitro* and *in vivo*. Initial investigations demonstrated that bacterially purified RB could inhibit pol III transcription from a system reconstituted from fractionated factors. In addition, overexpression of RB repressed transcription from pol III templates in transfected cells (White et al., 1996). Primary fibroblasts from *Rb*-/- mice and *Rb*+/+ mice were analysed for pol III activity. Such studies revealed that the fibroblasts derived from *Rb*-/- mice had

greater pol III activity than fibroblasts derived from *Rb*+/+ mice (White et al., 1996). Comparison of the osteosarcoma cell lines U2OS, which is RB positive, and SAOS2, which lacks a functional RB protein, demonstrated that the RB-deficient cells displayed increased pol III activity relative to the RB-positive cells (White et al., 1996). The ability of RB to repress pol III activity was alleviated by mutations in the RB pocket domain, such as RB null mutants isolated from small cell lung carcinomas (Horowitz et al., 1990). RB repression was also abolished by the presence of the adenoviral transforming protein E1A, which binds and inactivates RB (DeCaprio et al., 1988; Dyson et al., 1989b; Munger et al., 1989).

Transcription by pol III involves at least two general factors, named TFIIIB and TFIIIC (Willis, 1993; Geiduschek and Kassavetis, 1995; White, 1998). TFIIIB has been shown to be a multisubunit complex, which consists of the TATA-binding protein TBP (Hernandez, 1993; Rigby, 1993), and at least two TBP-associated factors or TAFs. One of these TAFs has been shown to be homologous to the pol II transcription factor TFIIB; because of this similarity, this subunit has been named TFIIB-Related Factor, or BRF (Buratowski and Zhou, 1992; Colbert and Hahn, 1992; Lopez-de-Leon et al., 1992; Khoo et al., 1994; Wang and Roeder, 1995; Mital et al., 1996), the other TFIIIB-TAF is called B" (Kassavetis et al., 1995). Yeast TFIIIB has been reconstituted from recombinant polypeptides (Kassavetis et al., 1995; Roberts et al., 1996; Ruth et al., 1996), in contrast to mammalian TFIIIB, which is far less well characterised.

The majority of pol III templates lack a TATA box and so in these cases TFIIIB is not brought to the promoter by DNA-protein interactions instigated by TBP. Instead, TFIIIB is recruited by protein-protein interactions with the DNA-binding protein TFIIIC (Willis, 1993; Geiduschek and Kassavetis, 1995; White, 1998).

Investigations by Larminie et. al. shed some light upon the mechanism whereby RB regulates pol III transcription (Larminie et al., 1997). Work undertaken showed that the activity of a pol III preinitiation complex, reconstituted from fractionated factors, was inactivated by the addition of bacterially purified RB, irrespective of being added before, during or after preinitiation complex assembly (Larminie et al., 1997).

In vitro transcription assays illustrated the ability of RB to repress pol III transcription from all three pol III promoter types, suggesting that RB is a general pol III repressor (Larminie et al., 1997). The ability of RB to repress pol III transcription was further localised to the large pocket domain. The GST fusion protein GST-RB (379-928) encompasses the large pocket domain and is able to repress pol III transcription. In contrast, the GST fusion protein GST-RB (612-711), which encompasses much of the B domain of the RB large pocket, was not able to repress pol III transcription in this assay (Larminie et al., 1997).

As RB can inactivate pol III transcription from all pol III promoter types, the ability of RB to target a general component of pol III transcription, such as the pol III

enzyme or the general pol III transcription factors TFIIIB and TFIIIC, was examined by add-back experiments. VA_1 transcription was reconstituted using partially purified factors; upon the addition of recombinant RB to this reconstituted system, transcription was repressed. The repression mediated by RB could be relieved by the addition of TFIIIB, but not by the addition of TFIIIC or pol III. Therefore, the add-back experiments demonstrated that TFIIIB is the target of RB repression, as only the addition of TFIIIB could relieve RB-mediated inhibition of pol III (Larminie et al., 1997).

The ability of RB to interact with endogenous TFIIIB was suggested by the fact that a population of RB molecules cofractionates with TFIIIB through a variety of purification steps, including phosphocellulose and DEAE-Sephadex columns and glycerol gradients. In contrast, RB did not cofractionate with TFIIIC or pol III. When phosphocellulose fractions containing TFIIIB were applied to a heparin-sepharose column and the bound protein eluted in a linear salt gradient, close cofractionation was observed between TFIIIB and RB. These observations suggest that there is a stable and specific interaction between endogenous TFIIIB and RB (Larminie et al., 1997).

The ability of TFIIIB and RB to interact was also analysed by immunoprecipitations. Antiserum against RB was found to be able to co-precipitate TBP and BRF. The presence of a specific competitor abolished the co-precipitation of TBP and BRF by the anti-RB antibody. These immunoprecipitation data provide

further evidence that endogenous TFIIIB interacts with endogenous RB at physiological ratios (Larminie et al., 1997).

The ability of RB to target TFIIIB was further established by studying extracts from primary cells isolated from wild-type or RB-knockout mice. The RB-negative extracts displayed elevated levels of pol III transcription when compared to RB-positive extracts. The level of TFIIIC and pol III activity between these two extract types varied only slightly. However, the activity of TFIIIB was found to be much greater in the RB-deficient extracts when compared to the RB-positive extracts, again suggesting that RB targets TFIIIB specifically (Larminie et al., 1997).

Additional add-back experiments were undertaken in order to determine which subunit of TFIIIB is targeted by RB to repress pol III transcription. VA_1 transcription was reconstituted using partially purified factors, and again repressed by the addition of recombinant RB. The ability of recombinant TBP and affinity-purified TFIIIB-TAFs to reverse this inhibition was examined. It was shown that TBP was unable to reverse repression, whereas the affinity-purified TFIIIB-TAFs were able to reverse inhibition in a dose-dependent manner. These add-back experiments suggest that RB targets one or more of the TFIIIB-TAFs to repress pol III transcription (Larminie et al., 1997).

The next step was to determine more precisely how RB interacts with TFIIIB. As mentioned above, TBP does not relieve RB-mediated pol III repression whereas a TFIIIB-TAF does (Larminie et al., 1997). Also, TBP has previously been reported to

be unable to bind RB (Weintraub et al., 1995). Hence it was decided to determine if RB targeted the BRF subunit of TFIIIB. Work presented here shows that recombinant and endogenous RB interact with BRF. The binding interfaces of these interactions were mapped. In addition, the interactions between TBP and BRF were characterised and found not to be disrupted by the addition of bacterially purified RB. In contrast, the interactions between TFIIIB and TFIIIC and between TFIIIB and pol III were both found to be disrupted by the presence of RB. These observations culminated in the proposal of a model for how RB represses pol III transcription.

3.2 Results

3.2.1 Endogenous and recombinant RB associate with BRF - a subunit of TFIIIB

Immunoprecipitations were undertaken in order to determine if endogenous RB could interact with BRF. HeLa cell extract containing wild type RB was mixed with reticulocyte lysate containing radiolabelled BRF. The mixtures were immunoprecipiated using a variety of anti-RB antibodies immobilised on protein A beads. The immunoprecipitated complexes were washed extensively, and the precipitated material examined by SDS-PAGE and autoradiography (fig. 3.1. and 3.2). Both antisera against RB were found to coprecipitate detectable amounts of BRF (lane 2 in both figures). The interaction was dependent on the presence of cell extract

FIG. 3.1 Endogenous RB coimmunoprecipitates with the BRF subunit of TFIIIB.

Reticulocyte lysate (15μl) containing in vitro-translated BRF was immunoprecipitated (IP) in the presence of 20μl LDB (lane 1), or 150μg HeLa whole-cell extract (lane 2) using anti-RB antibody G3-245. Precipitated material was resolved on a 7.8% SDS-polyacrylamide gel and visualised by autoradiography.

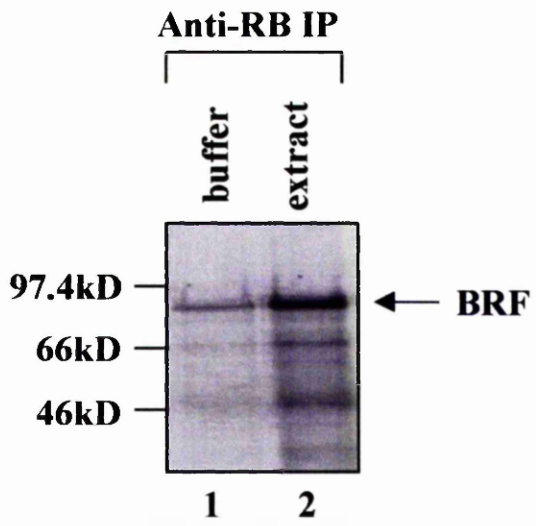
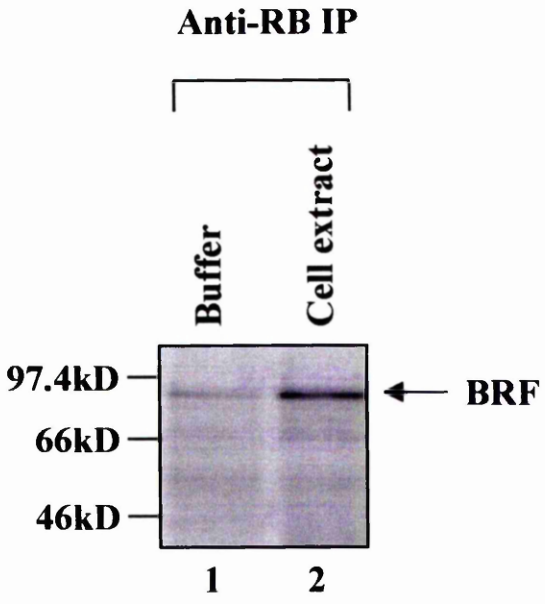

Anti-RB IP
buffer
extract
97.4kD
66kD
46kD
BRF
1
2

FIG. 3.2 Endogenous RB coimmunoprecipitates with the BRF subunit of TFIIIB.

Reticulocyte lysate (15µl) containing in vitro-translated BRF was mixed with 20µl LDB (lane 1) or 150µg of HeLa whole cell extract (lane 2) and immunoprecipitated (IP) in the presence of anti-RB antibody G99-549. Proteins retained after extensive washing were resolved on a SDS-7.8% polyacrylamide gel and visualised by autoradiography.


Anti-RB IP
Buffer
Cell extract
97.4kD
66kD
46kD
BRF
1
2

and presumably the RB within, since on replacing the extract with buffer the amount of BRF precipitated was greatly reduced (lane 1 in both figures).

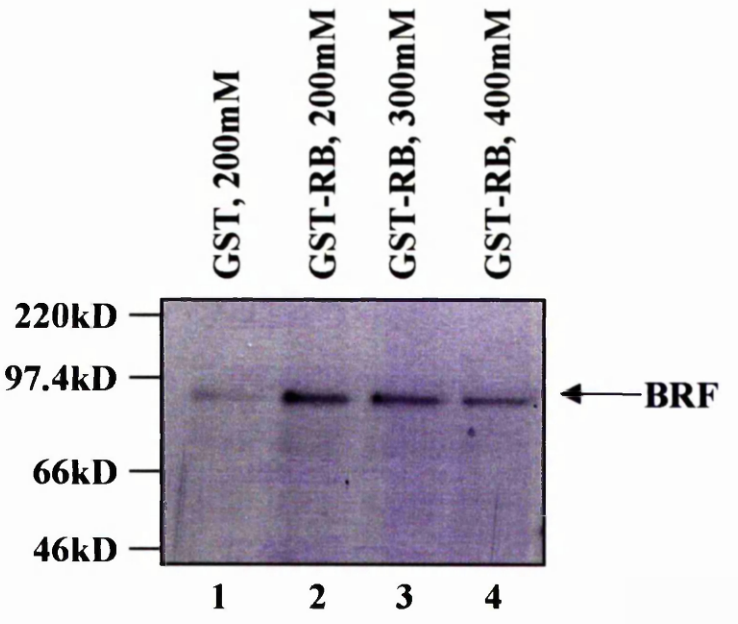
To determine if recombinant RB could interact with BRF and if this was a stable interaction, a variety of GST fusion proteins were purified and immobilised on glutathione-agarose beads. GST alone and GST-RB (379-928) were incubated with radiolabelled BRF that had been translated *in vitro* using reticulocyte lysate. The beads were washed with buffer containing 200mM, 300mM and 400mM KCl, the protein retained on the beads was resolved by SDS-PAGE and visualised by autoradiography (fig. 3.3). GST alone brought down a low, background amount of BRF (lane 1) in contrast to GST-RB (379-928), which brought down considerably more BRF (lanes 2-4). The ability of GST-RB (379-928) to pull down BRF is specific to the RB portion of the GST fusion protein, as GST alone pulled down only a small amount of BRF. The interaction between RB and BRF is demonstrated to be stable, as it can withstand washes of increased stringency (up to 400mM KCl) (lanes 2-4). These immunoprecipitation data and pull down assay suggest that RB and BRF interact with one another, and that this interaction is stable.

3.2.2 The identification of the BRF-binding domain in RB

The BRF-binding domain in RB was identified by the use of a variety of GST-RB fusion proteins, namely GST-RB (379-928), which encompasses the large pocket domain of RB, GST-RB (379-792) which contains only the small pocket,

FIG. 3.3 Recombinant RB interacts with the BRF subunit of TFIIIB.

Reticulocyte lysate containing in vitro-translated BRF (15μl) was incubated in the presence of glutathione-agarose beads carrying equal amounts of GST (lane 1) or GST-RB (379-928) (lanes 2-4). Proteins retained after washing with 200mM KCl (lanes 1 and 2), 300mM KCl (lane 3) and 400mM KCl (lane 4) were resolved on a SDS-7.8% polyacrylamide gel and visualised by autoradiography.

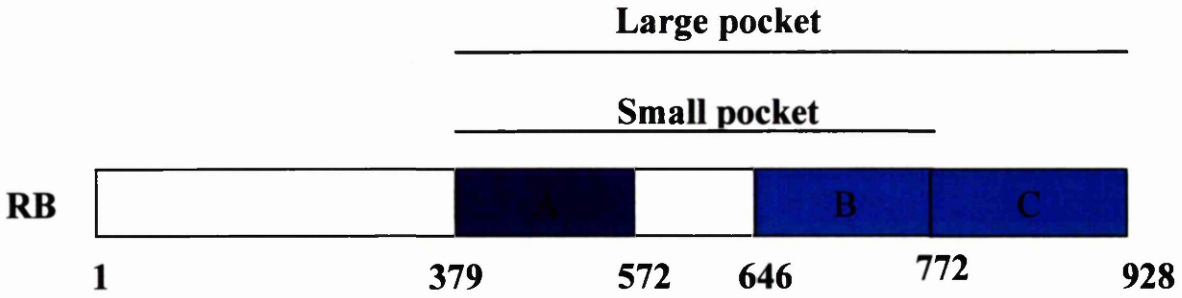

GST, 200mM
GST-RB, 200mM
GST-RB, 300mM
GST-RB, 400mM
220kD
97.4kD
66kD
46kD
BRF
1
2
3
4

and lastly GST-RB (763-928), which contains the C-domain. These proteins are represented in a schematic diagram (fig 3.4). Each of these GST fusion proteins were purified and immobilised on glutathione-agarose beads, and incubated with reticulocyte lysate containing radiolabelled BRF. The proteins retained on the beads were resolved by SDS-PAGE and visualised by autoradiography (fig. 3.5). As can be seen, the GST alone pulled down a minimal background amount of BRF (lane 2), whereas GST-RB (379-928) brought down substantially more BRF (lane 3). The ability of GST-RB (379-792) (lane 4) and GST-RB (763-928) (lane 5) to retain BRF is reduced compared to that observed for GST-RB (379-928) (lane 3). Indeed, the amount of BRF that is bound by GST-RB (379-792) is only slightly greater than the background observed with GST alone (lane 4 versus lane 2). These pull down data therefore suggest that the large pocket of RB is the binding domain for BRF.

Transient transfection assays were carried out to test if the region of RB that binds to BRF is the same as the region required to repress pol III transcription *in vivo*. The human osteosarcoma cell line SAOS2, which lacks wild-type RB (Shew et al., 1990), was transfected with expression vectors encoding the large or small pocket of RB. Primer extension analysis was used to determine the levels of transcription of a co-transfected VA_1 gene, which is used as a pol III reporter. A control plasmid was included to normalise for transfection efficiency; this contained the CAT gene, driven by the simian virus 40 early promoter. VA_1 and CAT RNA levels were assayed by primer extension (fig 3.6.A). From the VA_1 transcripts resolved on a sequencing gel, it can be seen that the transfected RB large pocket had a far greater ability to repress pol III transcription than the RB small pocket (lane 2 compared to lane 3). The primer

FIG. 3.4 A schematic representation of the GST-RB fusion proteins.

The diagram illustrates the regions of RB encompassed by the various GST-fusion proteins employed.



GST-RB(379-928)

GST-RB(379-792)

GST-RB(763-928)

BRF binding domain in RB

FIG. 3.5 Identification of the BRF-binding domain in RB.

Glutathione-agarose beads carrying equal amounts of GST alone (lane 2), GST-RB (379-928) (lane 3), GST-RB (379-792) (lane 4), and GST-RB (763-928) (lane 5) were incubated with 15μl of reticulocyte lysate containing expressed BRF. Proteins remaining after extensive washing were resolved on a SDS-7.8% polyacrylamide gel and visualised by autoradigraphy. Lane 1 shows 10% of the input reticulocyte lysate containing in vitro-translated BRF.

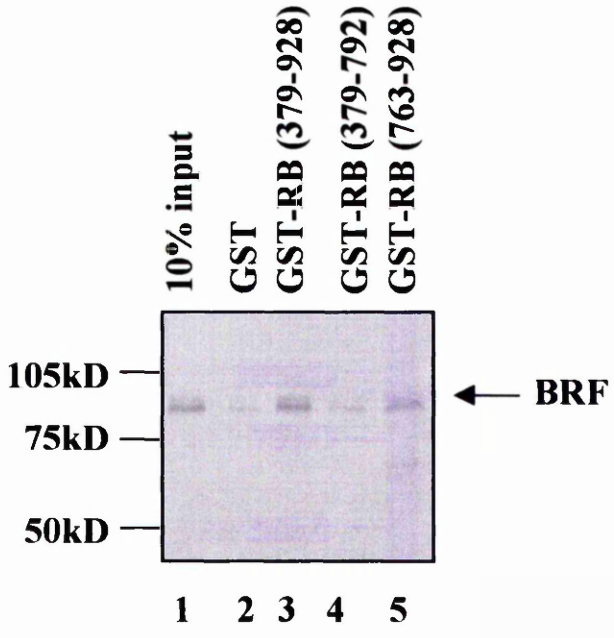
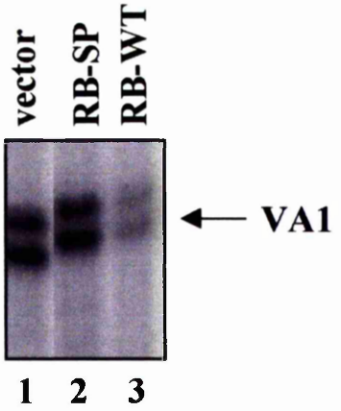

10% input
GST
GST-RB (379-928)
GST-RB (379-792)
GST-RB (763-928)
105kD
75kD
50kD
BRF
1
2
3
4
5

FIG. 3.6 Overexpression of full length RB inhibits pol III transcription in vivo.

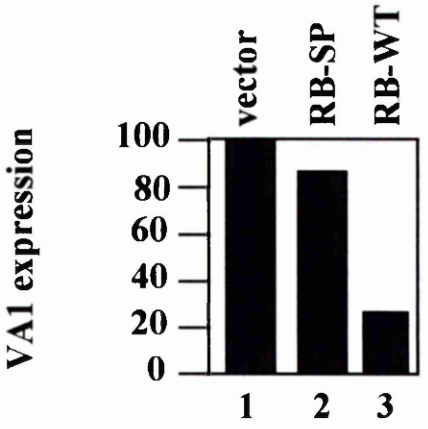
(A) SAOS2 cells were transfected with pVA_1 (4 μg), pCAT (4 μg), pSG5 (10μg lane 1, 2μg lanes 2 and 3), pRBSP (8 μg lane 2) and pRBWT (8 μg lane 3). VA_1 and CAT levels were assayed by primer extension analysis.

(B) The primer extension was quantitated by phosphorimager. The values shown for VA_1 have been normalised to the levels of CAT to correct for transfection efficiency, and are expressed relative to the value obtained for pSG5 control.

A


vector
RB-SP
RB-WT
VA1
1 2 3

B


VA1 expression
vector
RB-SP
RB-WT
100
80
60
40
20
0
1 2 3

extension was quantified by using a phosphorimager and then normalising to the levels of CAT to correct for transfection efficiency. It can be observed that whilst small pocket of RB is able to repress pol III transcription very slightly, the greatest effect is observed with the large pocket of RB (fig. 3.6.B lane 2 compared to lane 3). The primer extension analysis in conjunction with the pull down assays therefore indicate that the large pocket domain of RB is not only the domain responsible for binding BRF, but also for repressing pol III transcription. The data described above provide a correlation between the ability of RB to bind BRF and the ability of RB to repress pol III transcription.

To ensure that the differences in pol III transcript abundance were not due to variation in the expression of the RB proteins after transfection, protein was harvested from parallel transfections, resolved by SDS-PAGE, and probed for the presence of RB by western analysis. The untransfected cells have little RB, whereas those which have been transfected with RB-containing vectors have much greater amounts of RB (data not shown). The full-length and small pocket RB were both expressed with equal proficiency, thus indicating that the difference in pol III repression was due to the difference in the encoded proteins and not their levels of expression.

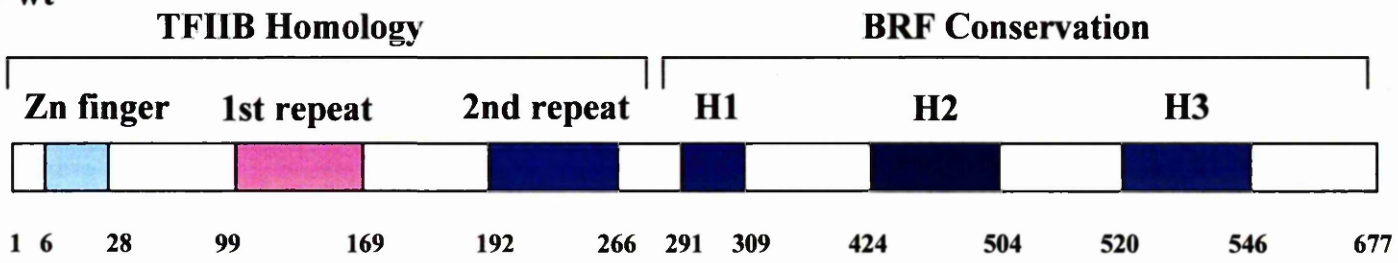
3.2.3 Identification of the RB-binding region in hBRF

Once the BRF-binding domain in RB had been identified, the inverse was to be determined, namely the RB-binding domain in BRF. In order to do this, a variety

FIG 3.7 A schematic representation of the hBRF truncations generated.

A diagrammatic representation of the conserved regions of BRF present in the truncations generated by endonuclease digestion.

hBRF wt



hBRF/Stu I (1-593)

hBRF/Bgl II (1-533)

hBRF/Xcm I (1-341)

hBRF/Hinc II (1-275)

hBRF/EcoR 1 (1-194)

of BRF truncated proteins were expressed (see fig. 3.7 for a schematic representation). The truncated proteins were generated by incubating the vector pCITE D1, which contains the cDNA sequence of hBRF, with various endonuclease restriction enzymes whose cleavage sequences were unique in hBRF. Complete cleavage was confirmed by analysing a sample on a 1% agarose gel. The cleaved DNA was then purified and the encoded transcript generated by transcription with T7 RNA polymerase. The RNA generated was purified and the protein expressed and radiolabelled using rabbit reticulocyte lysate. Successful expression was confirmed by resolving an aliquot by SDS-PAGE and visualising by autoradiography. The ability of the truncated proteins to bind to RB was determined by incubating the proteins with GST alone or GST-RB (379-928) purified and immobilised on glutathione-agarose beads. After incubation, the beads were washed extensively and the protein remaining bound to the beads was resolved by SDS-PAGE and visualised by autoradiography (fig. 3.8). As can be seen, the cleavage of BRF with either Stu I, which removes most of the C-terminal tail, or Bgl II, which removes the H3 region, does not abolish the ability of BRF to interact with RB (lanes 3 and 4 respectively). These GST pull down assays suggest that neither the C-terminal tail or H3 region of BRF contribute to the RB binding domain in BRF. To further define the RB-binding domain of BRF, alternative unique endonuclease restriction sites were identified, from which truncated proteins were expressed and their ability to interact with RB assessed (fig. 3.9). As is observed, cleavage of the BRF gene with Hinc II, which removes the entire BRF conservation domain, and EcoR I, which also removes the majority of the 2nd N-terminal imperfect repeat, generated truncated proteins which were able to interact with RB (lanes 6 and 9

FIG. 3.8 Identification of the RB-binding region in hBRF.

Reticulocyte lysate (15μl) containing in vitro-translated BRF (lanes 1 and 2), BRF/StuI (lane 3) or BRF/BglII (lane 4), were incubated in the presence of glutathione-agarose beads carrying equal amounts of GST (lane 1) or GST-RB (379-928) (lanes 2, 3 and 4). Proteins retained after extensive washing were resolved by SDS-7.8% polyacrylamide gel electrophoresis and then visualised by autoradiography.

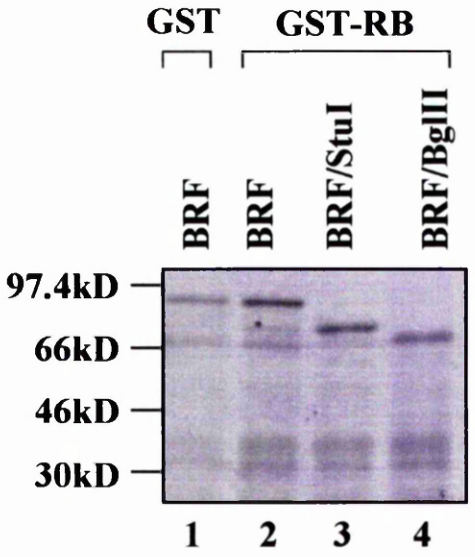
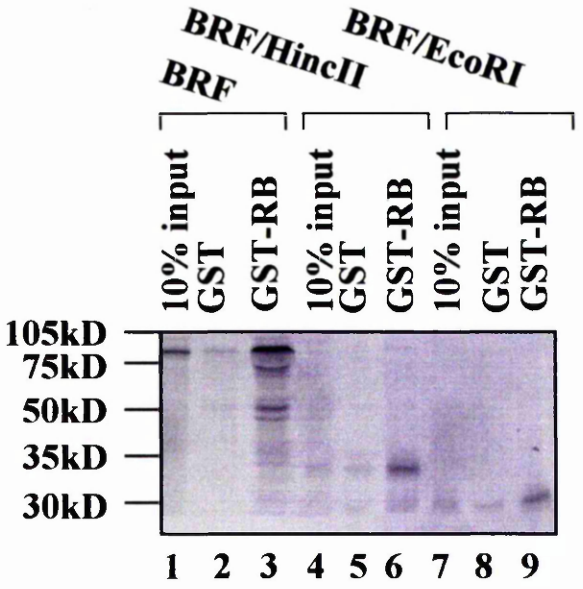

GST
GST-RB
BRF
BRF
BRF/StuI
BRF/BglII
97.4kD
66kD
46kD
30kD
1
2
3
4

FIG. 3.9 Identification of the RB-region domain in hBRF.

Glutathione-agarose beads carrying equal amounts of GST (lanes 2, 5, and 8) or GST-RB (379-928) (lanes 3, 6 and 9) were incubated with reticulocyte lysate (15μl) containing BRF (lanes 2 and 3), BRF/HincII (lanes 5 and 6) or BRF/EcoRI (lanes 8 and 9). Proteins remaining on the beads after extensive washing were resolved by SDS-7.8% polyacrylamide gel electrophoresis and visualised by autoradiography. Lanes 1, 4 and 7 all show 10% of the input reticulocyte lysate containing the relevant in vitro-translated BRF or BRF truncations.


BRF
BRF/HincII
BRF/EcoRI
10% input
GST
GST-RB
10% input
GST
GST-RB
10% input
GST
GST-RB
105kD
75kD
50kD
35kD
30kD
1 2 3 4 5 6 7 8 9

respectively). These GST pull down assays suggest that the RB-binding domain in BRF resides upstream of the 2nd N-terminal imperfect repeat. Within the BRF cDNA sequence, there are no more unique endonuclease restriction sites upstream of the EcoR I site; hence an alternative means of determining the RB binding domain in BRF was sought. Fortunately, an extensive collection of yBRF truncations was available. These yBRF truncations had been previously characterised (Kassavetis et al., 1998), and were so comprehensive in their design, that every conserved domain within BRF could be assayed for its contribution towards the binding of RB.

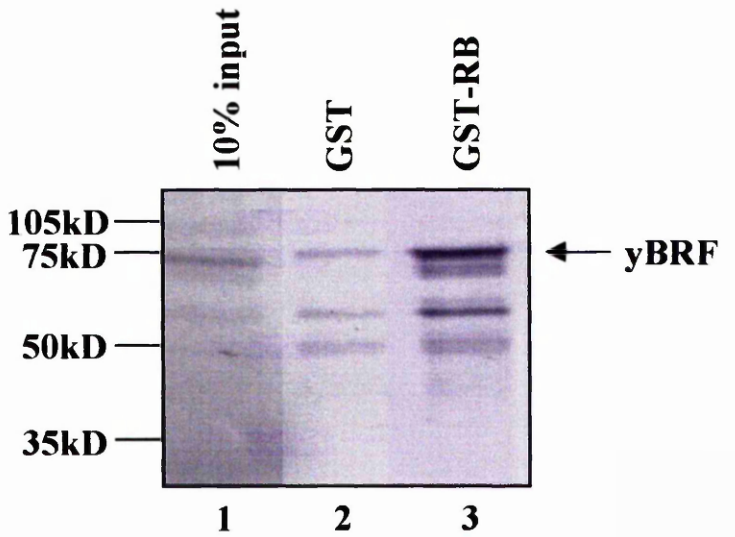
3.2.4 Identification of the RB-binding region in yBRF

The first step in employing the yBRF truncations was to determine if yBRF could bind to human RB. To investigate this, yBRF was radiolabelled *in vitro* using reticulocyte lysate and incubated with GST alone and GST-RB (379-928) immobilised on glutathione-agarose beads. After incubation, the proteins retained on the beads were resolved by SDS-PAGE and visualised by autoradiography. As is shown (fig. 3.10), yBRF is able to bind GST-RB (379-928) to a far greater extent than yBRF binds GST. This is an important observation, as the yBRF truncations can consequently be employed to determine the RB binding domain in yBRF.

To determine where RB binds to in yBRF, the collection of yBRF truncations were expressed and radiolabelled *in vitro* using reticulocyte lysate. The expression of these proteins was confirmed by resolving 2μl aliquots by SDS-PAGE, and visualised

FIG. 3.10 yBRF interacts with recombinant RB.

Reticulocyte lysate containing yBRF (15μl) was incubated with glutathione-agarose beads carrying equal amounts of GST (lane 2) or GST-RB (379-928) (lane 3). After extensive washing, the retained proteins were resolved on a SDS-7.8% polyacrylamide gel and visualised by autoradiography. Lane 1 shows 10% of the input reticulocyte lysate containing in vitro-translated yBRF.


10% input
GST
GST-RB
105kD
75kD
50kD
35kD
yBRF
1
2
3

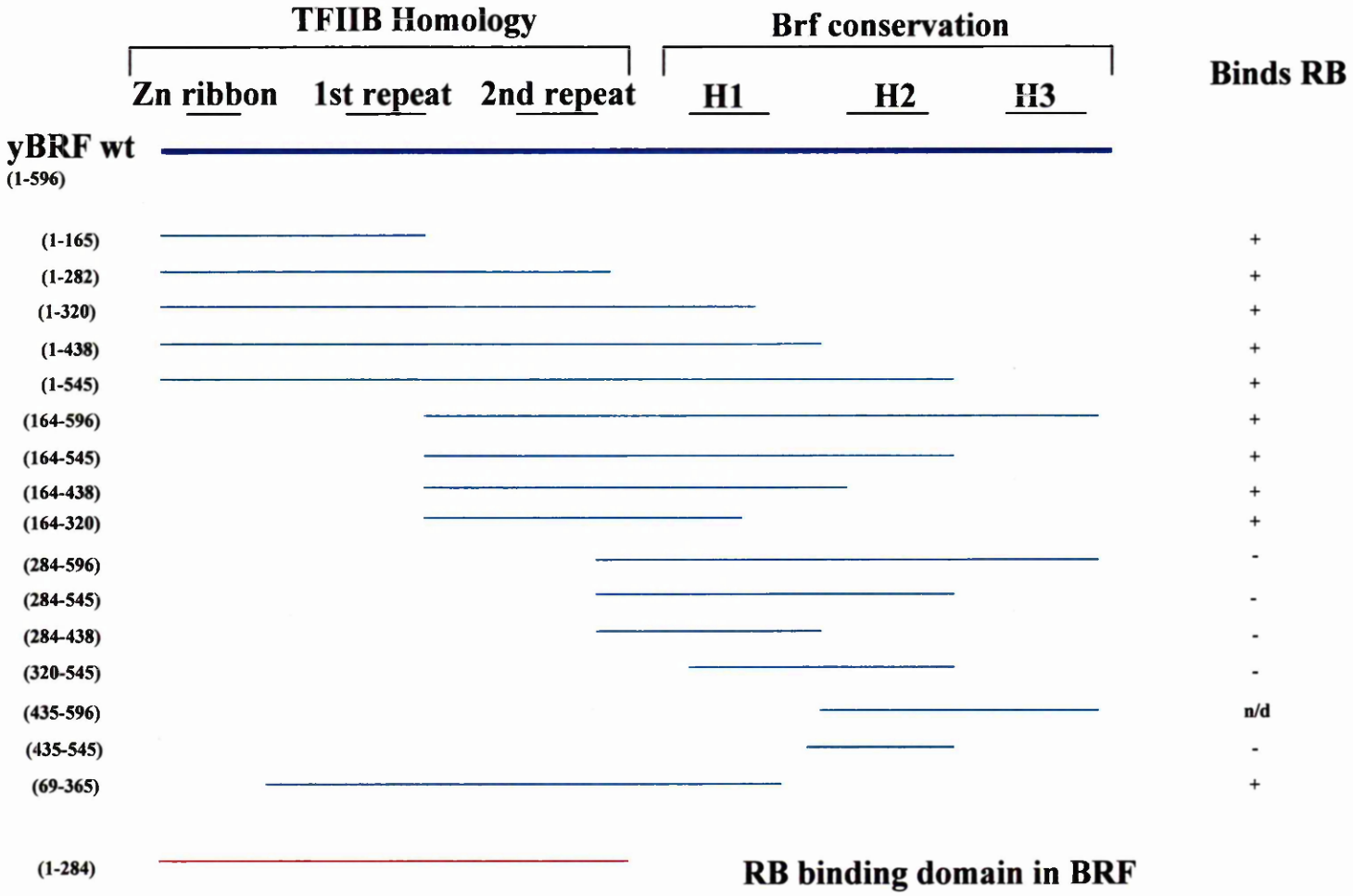
by autoradiography. Once the proteins had been successfully expressed, they were incubated with GST alone or GST-RB (379-928) immobilised on glutathione-agarose beads. After incubation, they were extensively washed and the bound protein was resolved by SDS-PAGE and visualised by autoradiography. The results of these GST pull down assays are summarised in figure 3.11.A, and the pull down assay data from the most informative truncations are shown in fig. 3.11.B. As can be seen from the pull down analysis, the yBRF truncations which were able to bind RB contained either part or all of the N-terminal TFIIB homology domain of yBRF (lanes 3, 6, 12, 15). Those yBRF truncations which are composed solely of the C-terminal BRF homology domain and lack any part of the TFIIB homology domain are unable to bind RB (lane 9). These data demonstrate that the N-terminal TFIIB homology domain of yBRF is responsible for binding to RB. This confirms the result obtained using hBRF. Although many yBRF truncations were available, we were unable to localise more precisely the RB-binding domain within the N-terminal half of BRF since it was not possible to identify any sequence that was essential for interaction. Although yeast residues 1-165 proved sufficient to bind to RB, binding was also obtained with residues 164-438. These observations might be explained if the two direct repeats (aa 119-159 and aa 217-257) each provide a region that can interact with RB. Further experiments will be required to test this possibility.

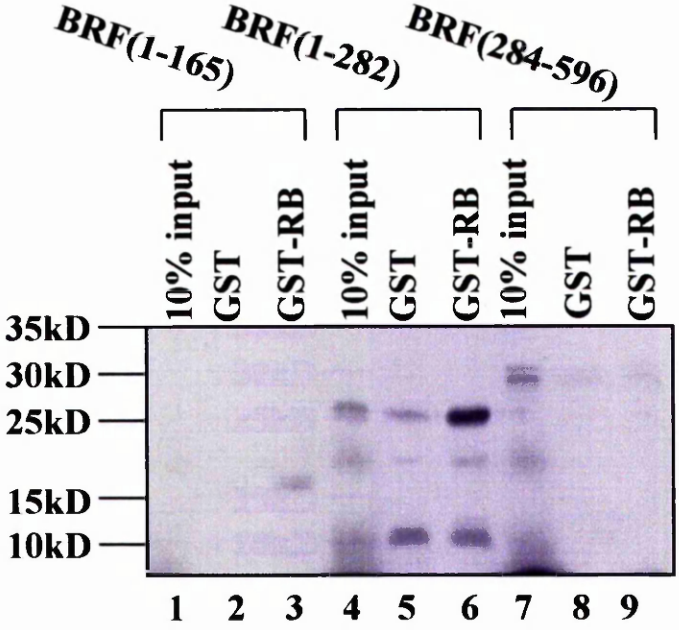
It was important to confirm that the failure of the C-terminal domain to interact with RB was not due to its denaturation. To ensure the viability of the yBRF truncation, BRF (aa 284-596), a control experiment was undertaken. Previous work (Kassavetis et al., 1998) and work that will be discussed in section 3.2.5 show that

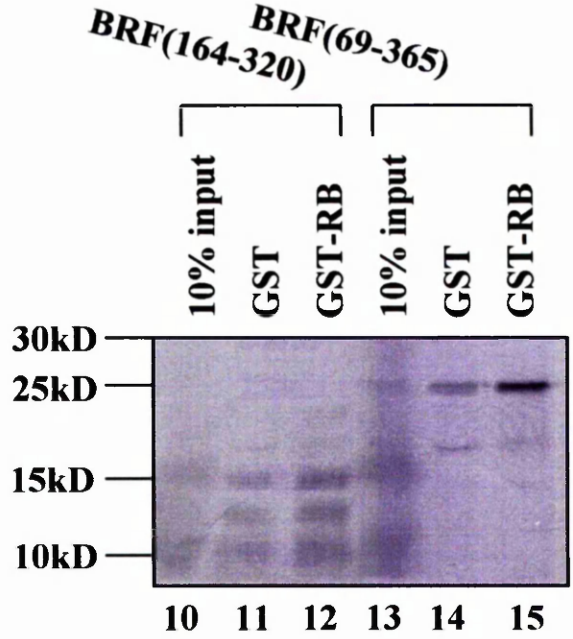
FIG. 3.11 Identification of the RB-binding region in yBRF.

(A) A schematic representation of the collection of yBRF truncations, and their ability to interact with RB.

(B) Glutathione-agarose beads carrying equal amounts of GST (lanes 2, 5, 8, 11 and 14) or GST-RB (379-928) (lanes 3, 6, 9, 12 and 15) were incubated with reticulocyte lysate (15μl) containing BRF (1-165) (lanes 2 and 3) , BRF (1-282) (lanes 5 and 6), BRF (284-596) (lanes 8 and 9), BRF (164-320) (lanes 11 and 12), or BRF (69-365) (lanes 14 and 15). Retained protein was resolved on a SDS-12% polyarcylamide gel and visualised by autoradiography. Lanes 1, 4, 7, 10, and 13 shows 10% of the input reticulocyte lysate containing the appropriate in vitro-translated yBRF truncations.


TFIIB Homology
Brf conservation
Zn ribbon
1st repeat
2nd repeat
H1
H2
H3
Binds RB
yBRF wt
(1-596)
(1-165) +
(1-282) +
(1-320) +
(1-438) +
(1-545) +
(164-596) +
(164-545) +
(164-438) +
(164-320) +
(284-596) -
(284-545) -
(284-438) -
(320-545) -
(435-596) n/d
(435-545) -
(69-365) +
(1-284)
RB binding domain in BRF


BRF(1-165)
BRF(1-282)
BRF(284-596)
10% input
GST
GST-RB
10% input
GST
GST-RB
10% input
GST
GST-RB
35kD
30kD
25kD
15kD
10kD
1 2 3 4 5 6 7 8 9


BRF(164-320)
BRF(69-365)
10% input
GST
GST-RB
10% input
GST
GST-RB
30kD
25kD
15kD
10kD
10 11 12 13 14 15

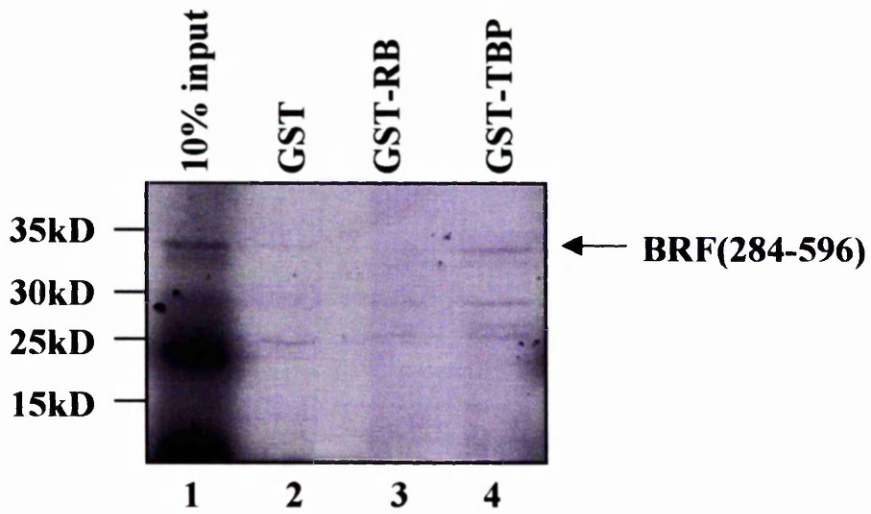
the main TBP-binding domain in BRF is the H2 region, which is located in the C-terminal half of BRF. A variety of GST fusion proteins were expressed, purified and immobilised on glutathione-agarose beads, namely, GST alone, GST-RB (379-928), and GST-TBP. As the main TBP binding region in BRF is the H2 region, present in BRF(284-596), it is expected that BRF(284-596) will be able to bind GST-TBP if it has retained its structure. BRF(284-596) was expressed and radiolabelled *in vitro* with reticulocyte lysate and incubated with each of the aforementioned GST fusion proteins. After incubation, the beads were washed and retained protein resolved by SDS-PAGE and visualised by autoradiography, (fig. 3.12). As can be seen BRF(284-596), did not bind either GST alone, or GST-RB (379-928) (lane 2 and 3 respectively); however it was retained by GST-TBP (lane 4). These GST pull down data demonstrate that this yBRF truncation has retained its structure, as it is able to bind to TBP. Collectively, the pull down assays demonstrate that the RB binding domain lies in the N-terminal half of BRF.

3.2.5 Characterisation of the interactions between the components of B', namely TBP and BRF

As discussed above, the RB-binding domain in BRF resides in the N-terminal half of the protein. To determine if RB binding to BRF could affect the ability of BRF to bind TBP, the TBP/BRF interaction was investigated.

FIG. 3.12 The yeast truncation BRF (284-596) is able to bind TBP.

Reticulocyte lysate containing BRF (284-596) (15μl), was incubated with glutathione-agarose beads carrying equal amounts of GST (lane 2), GST-RB (379-928) (lane 3) and GST-TBP (lane 4). Retained protein was resolved on a SDS-12% polyacrylamide gel and visualised by autoradiography. Lane 1 shows 10% input of the reticulocyte lysate containing BRF(284-596).


10% input
GST
GST-RB
GST-TBP
35kD
30kD
25kD
15kD
BRF(284-596)
1
2
3
4

The initial experiment undertaken was to show that TBP and BRF interact. TBP and BRF were expressed and radiolabelled in vitro by reticulocyte lysate, and immunoprecipitated with TBP antiserum immobilised on protein A beads. After incubation, the coprecipitate was resolved by SDS-PAGE and visualised by autoradiography (fig. 3.13). As is observed, no TBP is retained by protein A beads alone (lane 1), but TBP is coprecipitated with the antiserum raised against TBP (lane 2), thus illustrating that the coprecipitation of TBP is dependent on the TBP antiserum immobilised on the protein A beads. In the absence of TBP, BRF is not immunoprecipitated (lane 3), but in the presence of TBP, BRF is coprecipitated (lane 4), thus demonstrating that TBP and BRF interact.

The ability of TBP and BRF to interact was further confirmed by GST pull down assays. In this instance, GST alone and GST-TBP were expressed, purified and immobilised on glutathione-agarose beads. Each of these fusion proteins were incubated with reticulocyte lysate containing radiolabelled BRF. After extensive washing, the bound protein was analysed by resolving on a SDS-polyacrylamide gel and visualised by autoradiography (fig. 3.14). As can be seen, BRF is not brought down with GST alone (lane 1) but it is pulled down with GST-TBP (lanes 2-4). This experiment also shows that the interaction between BRF and TBP is stable, as increasing the stringency of the washes, by raising the ionic concentration of the washing buffer, did not disrupt the ability of TBP and BRF to interact (lanes 2-4). Thus, from these GST pull down data it can be concluded that TBP and BRF form an interaction stable to high ionic strength.

FIG. 3.13 Recombinant BRF interacts with recombinant TBP.

Reticulocyte lysate (15μl) containing either in vitro-translated TBP (lanes 1 and 2), or in vitro-translated BRF (lane 3) or both (lane 4), were immunoprecipitated with the anti-TBP antibody, MTBP-6 (lanes 2-4) or incubated with only protein A beads (lane 1). The precipitated material was resolved on a SDS-7.8% polyarcylamide gel and visualised by autoradiography.

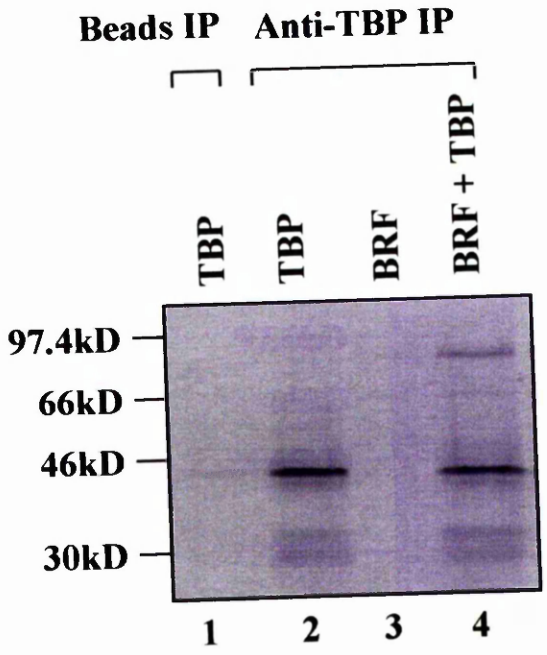
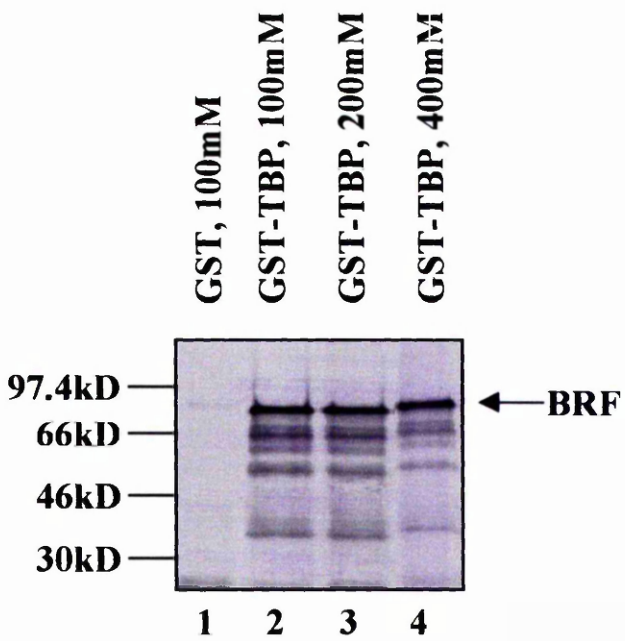

Beads IP
Anti-TBP IP
TBP
TBP
BRF
BRF + TBP
97.4kD
66kD
46kD
30kD
1
2
3
4

FIG. 3.14 Recombinant BRF interacts with recombinant TBP and this interaction is stable to 400mM KCl.

Reticulocyte lysate containing in vitro-translated BRF (15μl), was incubated with glutathione-agarose beads carrying equal amounts of GST alone (lane 1), or GST-TBP (lanes 2-4). The protein retained on the beads were washed with 100mM KCl (lanes 1 and 2), 200mM KCl (lane 3), and 400mM KCl (lane 4), and resolved on a SDS-7.8% polyacrylamide gel and visualised by autoradiography.

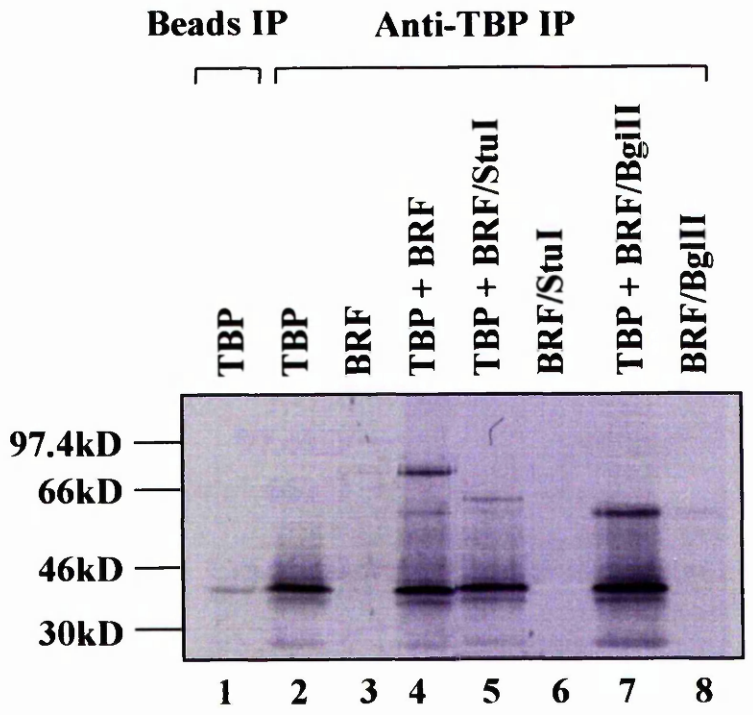

GST, 100mM
GST-TBP, 100mM
GST-TBP, 200mM
GST-TBP, 400mM
97.4kD
66kD
46kD
30kD
BRF
1
2
3
4

Having demonstrated the interaction between TBP and BRF, the next step was to identify the TBP binding domain within BRF. This was undertaken by incubating pCITE D1, a construct containing the cDNA sequence of BRF, with various restriction endonucleases, whose cleavage sites were unique in hBRF. Completion of cleavage was confirmed by analysing an aliquot on a 1% agarose gel. Once the DNA was completely cleaved, it was purified and transcribed using T7 RNA polymerase. The RNA was then purified and the encoded protein expressed and radiolabelled in vitro by reticulocyte lysate. Protein expression was confirmed by resolving a 2μl aliquot by SDS-PAGE, followed by autoradiography. The ability of these BRF truncated proteins to interact with TBP was determined by immunoprecipitations. Reticulocyte lysate containing either radiolabelled BRF truncations or TBP were incubated with TBP antiserum immobilised on protein A beads. After incubation the beads were washed and the coprecipitated material was examined by resolving on a SDS-polyacrylamide gel and autoradiography (fig. 3.15). Again it was found that the coprecipitation of BRF is dependent on the presence of TBP (lanes 1-4). Furthermore, the truncated proteins BRF/Stu I, which lacks the C-terminal tail of BRF (lane 5), and BRF/Bgl II, which lacks the H3 region (lane 7), are both able to bind TBP. These immunoprecipitation data indicate that the C-terminal tail and the H3 region are not required for BRF to bind TBP.

The ability of these BRF truncations to interact with TBP was confirmed by GST pull down assays. GST alone and GST-TBP were expressed, purified and immobilised on glutathione-agarose beads. Each of these fusion proteins was

FIG 3.15 Recombinant TBP interacts with BRF truncations.

Reticulocyte lysate (15μl) containing in vitro-translated BRF (lanes 3 and 4), BRF/Stu I (lanes 5 and 6) or BRF/Bgl II (lanes 7 and 8), with the addition of reticulocyte lysate containing in vitro-translated TBP (lanes 1, 2, 4, 5 and 7), were immunoprecipitated using the anti-TBP antibody MTBP-6. The precipitated protein was washed extensively, resolved on a SDS-7.8% polyacrylamide gel and visualised by autoradiography.


Beads IP
Anti-TBP IP
TBP
TBP
BRF
TBP + BRF
TBP + BRF/StuI
BRF/StuI
TBP + BRF/BglII
BRF/BglII
97.4kD
66kD
46kD
30kD
1
2
3
4
5
6
7
8

incubated with reticulocyte lysate containing radiolabelled full length BRF or a truncated form of BRF. The beads were extensively washed, and the retained protein resolved by SDS-PAGE and visualised by autoradiography (fig 3.16). As is shown, GST alone retains a minimal background amount of BRF (lanes 1-3). However, there is a much stronger interaction between all of the BRF forms and TBP (lanes 4-6). It can be concluded from these immunoprecipitation data and GST pull down analysis that neither the C-terminal tail nor the H3 region contribute to the ability of BRF to interact with TBP.

The TBP-binding domain in BRF was further analysed by generating another BRF truncation using restriction enzyme Xcm I, in which the H2 region had been removed. Again the ability of this truncation to interact with TBP was determined by immunoprecipitation. Various truncations of BRF were expressed and radiolabelled in reticulocyte lysate, and incubated with TBP antiserum immobilised on protein A beads. TBP expressed in reticulocyte lysate, but not radiolabelled was also added; in this instance expression was confirmed by resolving an aliquot by SDS-PAGE, and staining with coomassie blue. After incubation and washing, the coprecipitate was resolved by SDS-PAGE and visualised by autoradiography (fig. 3.17). From the autoradiogram, it can be seen that the truncated forms of BRF previously examined were capable of binding TBP (lanes 4-7). However the truncated form in which the H2 region was removed, namely BRF/Xcm I, is not capable of binding TBP (lanes 8 and 9). Therefore it can be concluded from these immunoprecipitation data that the TBP binding domain in BRF resides in the H2 region. The TBP-binding domain in BRF is therefore separate from the domain responsible for interacting with RB.

FIG. 3.16 Recombinant TBP interacts with BRF truncations.

Glutathione-agarose beads carrying equal amounts of GST alone, (lanes 1, 2, and 3) or GST-TBP (lanes 4, 5 and 6) were incubated with reticulocyte lysate (15μl) containing in vitro-translated BRF (lanes 1 and 4), BRF/Stu I (lanes 3 and 6) or BRF/Bgl II (lanes 2 and 5). The retained protein was washed extensively, and resolved by SDS-7.8% polyacrylamide gel and visualised by autoradiography.

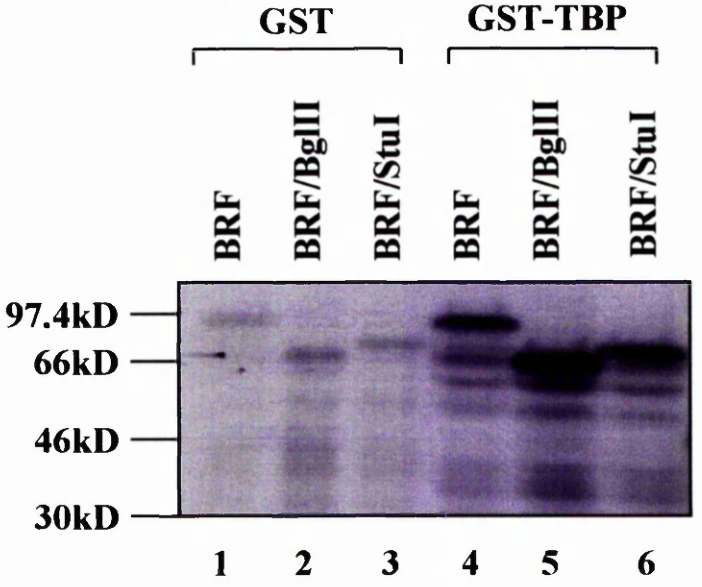
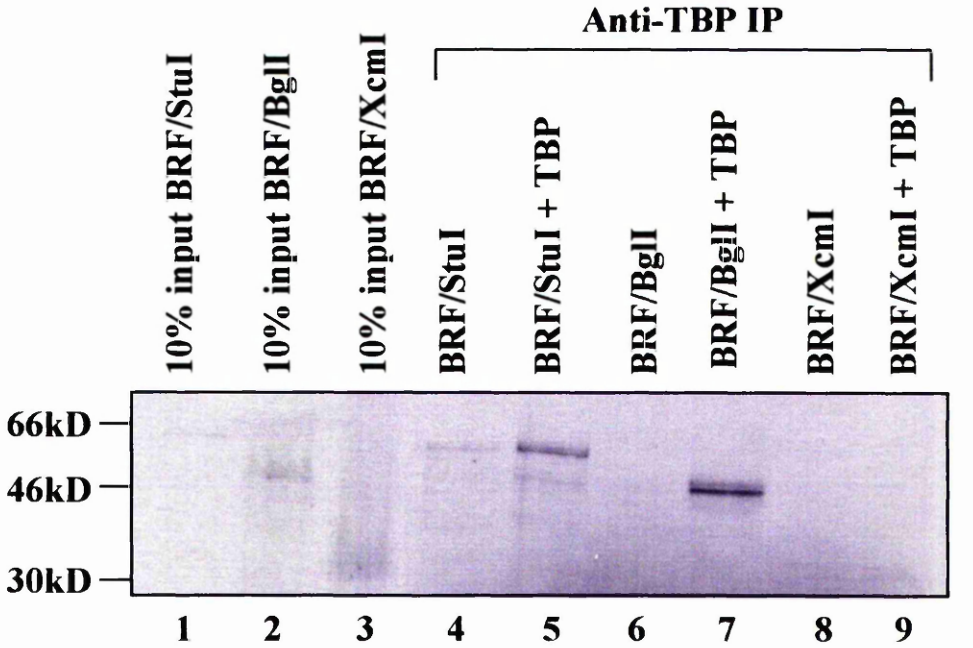

GST
GST-TBP
BRF
BRF/BglII
BRF/StuI
BRF
BRF/BglII
BRF/StuI
97.4kD
66kD
46kD
30kD
1
2
3
4
5
6

FIG. 3.17 A BRF truncation which does not interact with TBP.

Reticulocyte lysate (15μl) containing in vitro-translated BRF/Stu I (lanes 4 and 5), BRF/Bgl II (lanes 6 and 7) or BRF/Xcm I (lanes 8 and 9), with the addition of reticulocyte lysate containing in vitro-translated but not radiolabelled TBP (lanes 5, 7, and 9), were immunoprecipitated with the anti-TBP antibody MTBP-6. The precipitated protein was washed and resolved on a SDS-7.8% polyacrylamide gel and visualised by autoradiography. Lanes 1, 2, and 3 show 10% of the input reticulocyte lysate containing in vitro-translated BRF truncations.


Anti-TBP IP
10% input BRF/StuI
10% input BRF/BglI
10% input BRF/XcmI
BRF/StuI
BRF/StuI + TBP
BRF/BglI
BRF/BglI + TBP
BRF/XcmI
BRF/XcmI + TBP
66kD
46kD
30kD
1
2
3
4
5
6
7
8
9

Hence it was decided to investigate the effect of RB upon the interaction between TBP and BRF.

3.2.6 RB does not disrupt the interaction between BRF and TBP

As it has previously been demonstrated that TFIIIB is the target for RB repression of pol III transcription (Larminie et al., 1997), it was decided to test if RB exerts its effect by disrupting the TFIIIB complex. This was investigated by immunoprecipitations. Reticulocyte lysates containing radiolabelled BRF and TBP were incubated with TBP antiserum immobilised on protein A beads, in the absence or presence of RB purified from baculovirus cells. After incubation, the coprecipitate was resolved by SDS-PAGE and visualised by autoradiography (fig. 3.18). It can be seen that TBP and BRF can coprecipitate (lane 4) and the ability of TBP and BRF to coprecipitate is not affected by the presence of RB (lane 5). These immunoprecipitation data suggest that RB does not disrupt the interaction between TBP and BRF.

The effect of RB upon the interaction between endogenous TBP and BRF was also investigated by immunoprecipitations. HeLa extracts were incubated with protein A beads coupled to BRF antiserum or its corresponding preimmune as the negative control. After incubation, the coprecipitate was extensively washed and resolved by SDS-PAGE. The presence of TBP was determined by western blotting (fig.3.19). As can be observed, the preimmune serum does not coprecipitate TBP

FIG. 3.18 RB does not disrupt the interaction between recombinant TBP and recombinant BRF.

15μl of reticulocyte lysate containing in vitro-translated TBP (lanes 1 and 2), or in vitro-translated BRF (lane 3), or both (lanes 4 and 5), with the addition of baculovrius purified RB (lane 5) were immunoprecipitated by the anti-TBP antibody MTBP-6 (lanes 2-5) or incubated with protein A beads (lane 1). The precipitated protein was washed and resolved by on a SDS-7.8% polyacrylamide gel and visualised by autoradiography.

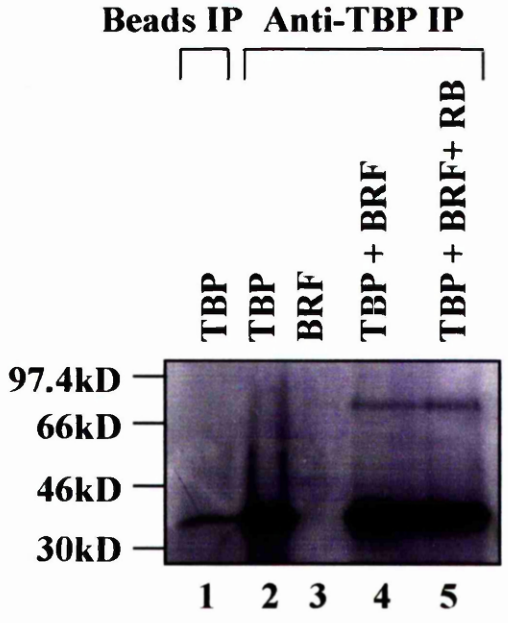
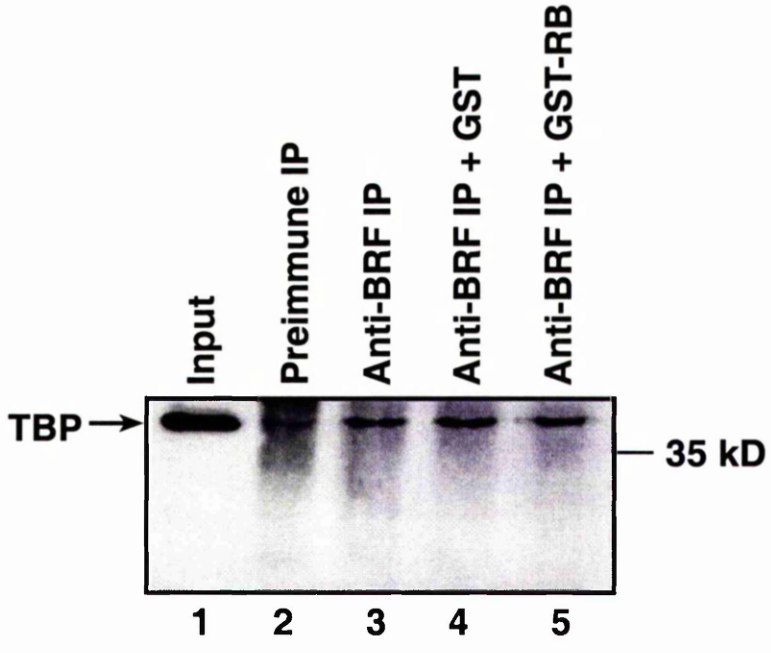

Beads IP
Anti-TBP IP
TBP
TBP
BRF
TBP + BRF
TBP + BRF+ RB
97.4kD
66kD
46kD
30kD
1
2
3
4
5

FIG. 3.19 RB does not abolish the interaction between endogenous TBP and BRF.

HeLa cell extract (150 μg) alone (lanes 2 and 3), or mixed with 200ng of GST (lane 4) or GST-RB (379-928) (lane 5) was immunoprecipitated (IP) using BRF 128 antiserum (lanes 3–5) and the corresponding preimmune (lane 2). The precipitated material was resolved on a SDS-7.8% polyacrylamide gel. The presence of TBP was determined by western analysis with the anti-TBP antibody SL30. Lane 1 shows 25% input of the HeLa extract.


Input
Preimmune IP
Anti-BRF IP
Anti-BRF IP + GST
Anti-BRF IP + GST-RB
TBP
35 kD
1
2
3
4
5

(lane 2), whereas the BRF antiserum does, demonstrating the specificity of this interaction (lanes 3). The presence of GST alone does not affect the interaction between TBP and BRF (lane 4), and neither does the addition of GST-RB (379-928) (lane 5). These observations lead to the conclusion that RB does not affect the interaction between TBP and BRF. It was therefore decided to determine if RB represses pol III transcription by preventing TFIIIB from interacting with TFIIIC, the pol III enzyme or both.

3.2.7 Characterisation of the effects of RB upon the interaction between TFIIIB and TFIIIC

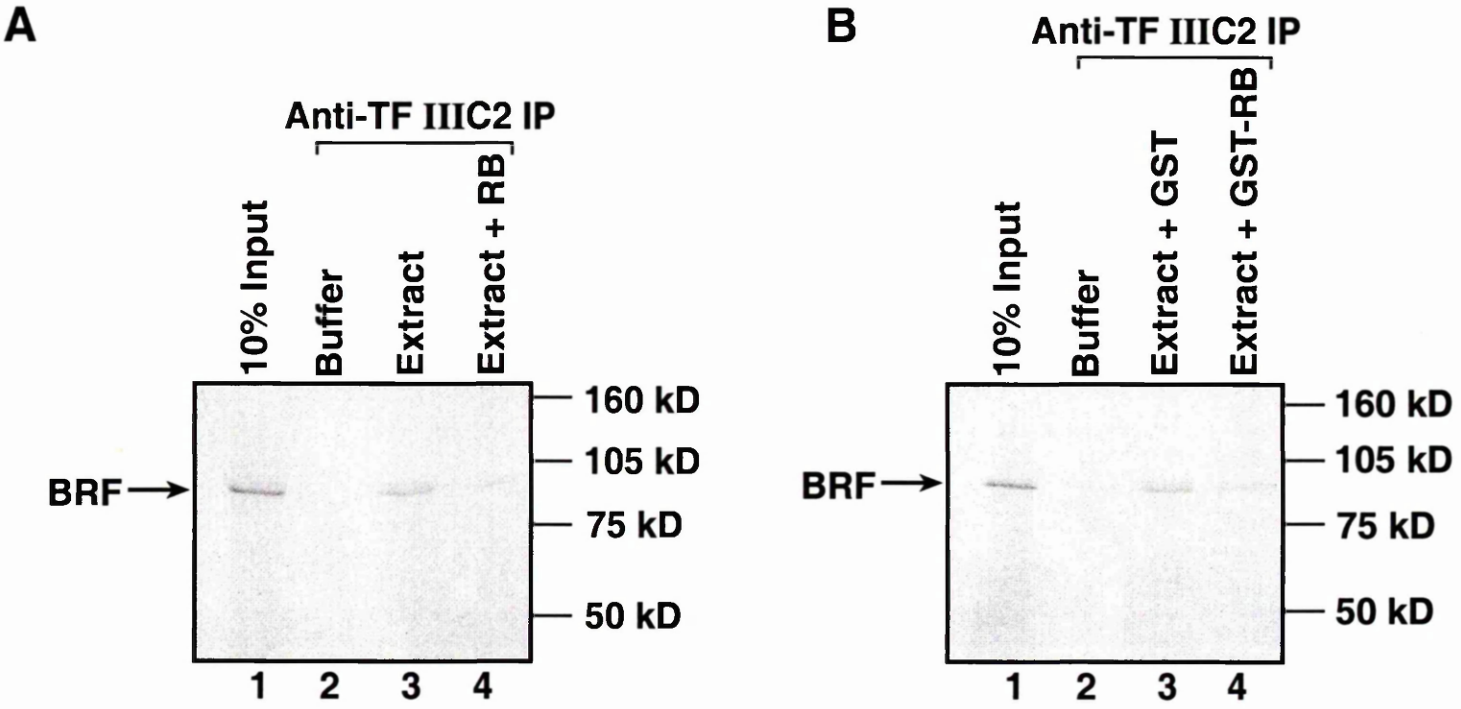
The effect of RB upon the interaction between TFIIIB and TFIIIC2 was investigated by immunoprecipitations. HeLa extract was mixed with in vitro translated BRF in the presence of His-tagged RB (379-928). The mixtures were then incubated with TFIIIC2 antiserum immobilised on protein A beads. After incubation and extensive washing, the coprecipitated material was resolved by SDS-PAGE and visualised by autoradiography (fig. 3.20.A). The antiserum raised against TFIIIC2 coprecipitates BRF (lane 3); however, the addition of His-tagged RB (379-928) greatly reduces the level of BRF coprecipitated by the TFIIIC2 antiserum (lane 4).

To ensure these observations were not a peculiarity of His-tagged fusion proteins, an alternative bacterially expressed RB protein was utilised in additional immunoprecipitation experiments. HeLa extract was mixed with radiolabelled BRF,

FIG. 3.20 RB disrupts the interaction between endogenous TFIIIC2 and recombinant BRF.

(A) HeLa cell extract (150 μg) (lanes 3 and 4) or buffer (lane 2) was mixed with reticulocyte lysate containing in vitro-translated BRF, with the addition of 100ng of His-tagged RB (lane 4), was immunoprecipitated using TFIIIC2 antiserum 4286 (lanes 2–4). The precipitated material was resolved on a SDS-7.8% polyacrylamide gel and visualised by autoradiography. Lane 1 shows 10% input of the reticulocyte lysate containing in vitro-translated BRF.

(B) Reticulocyte lysate containing in vitro-translated BRF (15μl), was mixed with buffer (lane 2) or HeLa cell extract (150 μg) (lanes 3 and 4), and 200ng of GST alone (lane 3) or 200ng of GST-RB (379-928) (lane 4), then immunoprecipitated with TFIIIC2 4286 antiserum (lanes 2-4). The precipitated material was resolved on a SDS-7.8% polyacrylamide gel and visualised by autoradiography. Lane 1 shows 10% input of reticulocyte lysate containing in vitro-translated BRF.


A
Anti-TF IIIC2 IP
10% Input
Buffer
Extract
Extract + RB
BRF
160 kD
105 kD
75 kD
50 kD
1
2
3
4
B
Anti-TF IIIC2 IP
10% Input
Buffer
Extract + GST
Extract + GST-RB
BRF
160 kD
105 kD
75 kD
50 kD
1
2
3
4

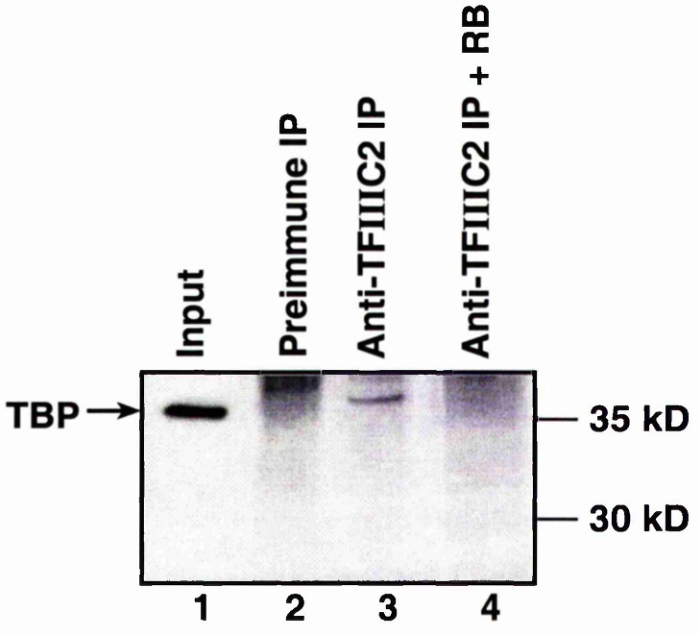
with the addition of either GST, or GST-RB (379-928), then incubated with TFIIIC2 antiserum immobilised on protein A beads. The coprecipitate was resolved by SDS-PAGE and visualised by autoradiography (fig. 3.20.B). The antiserum against TFIIIC2 once again coprecipitated BRF in the presence of GST (lane 3); however, the addition of GST-RB (379-928) greatly reduces the amount of BRF retained (lane 4). These data suggest that the presence of RB compromises the interaction between TFIIIB and TFIIIC.

The effect of RB upon the interaction between endogenous TFIIIB and TFIIIC was also examined by immunoprecipitations. HeLa extract was mixed with GST alone or GST-RB (379-928) and incubated with TFIIIC2 antiserum immobilised on protein A beads. After incubation, the coprecipitate was extensively washed and resolved by SDS-PAGE, and the presence of TFIIIB was determined by probing for TBP by western analysis (fig. 3.21). The preimmune does not coprecipitate TBP (lane 2), in contrast to the TFIIIC2 antiserum which does coprecipitate TBP, and is capable of doing so in the presence of GST alone (lane 3). The addition of GST-RB (379-928) greatly reduces the amount of TBP coprecipitated by the TFIIIC2 antiserum (lane 4). These immunoprecipitations therefore demonstrate that RB is able to disrupt the interaction between TFIIIB and TFIIIC.

An alternative means of assessing the effect of the RB upon the interaction between TFIIIB and TFIIIC was provided by assaying the DNA-binding activity of precipitated material. These oligonucleotide binding assays involve

FIG. 3.21 RB disrupts the interaction between endogenous TBP and TFIIIC2.

HeLa cell extract (150 μg) alone (lanes 2 and 3), or mixed with 200ng of GST-RB (379-928) (lane 4) was immunoprecipitated using a column purified TFIIIC2 4286 antiserum, (lanes 3 and 4), or the corresponding preimmune serum (lane 2). The precipitated material was resolved by SDS-7.8% polyacrylamide gel electrophoresis. The presence of TBP was determined by western analysis using anti-TBP antibody SL30. Lane 1 shows 20 % input of HeLa extract.


Input
Preimmune IP
Anti-TFIIIC2 IP
Anti-TFIIIC2 IP + RB
TBP
35 kD
30 kD
1
2
3
4

immunoprecipitations as before, then incubating the coprecipitated material with a radiolabelled DNA probe, and then removing unbound probe by washing. The amount of probe bound was subsequently determined by scintillation counting. The initial oligonucleotide binding assay was to determine if such an experimental approach was viable. TFIIIC2 4286 antiserum and its corresponding preimmune were immobilised on protein A beads and incubated with HeLa extract. After incubation, the beads were extensively washed and incubated with a radiolabelled B box probe, that contains a recognition sequence of the internal pol III promoter bound by TFIIIC2. After the second incubation, the beads were again washed, the retained DNA eluted, bound to DEAE discs, and scintillation counted (fig 3.22.A). As can be seen, significantly more B box binding activity is immunoprecipitated by the TFIIIC2 antiserum than the corresponding preimmune (compare lane 2 to lane 1). The ability of the immunoprecipitated material to bind the B box probe is partially specific (fig. 3.22.B), as the introduction of unlabelled B box competitor oligonucleotide greatly reduces the amount of probe immunoprecipitated (lane 2). In contrast, incubation with a non-specific competitor oligonulceotide does not have such a great effect (lane 3). The effect of RB upon the interaction between TFIIIC and TFIIIB was determined by immunoprecipitating HeLa extract with the BRF antiserum 330 immobilised on protein A beads (fig. 3.22.C). As can be seen, the presence of GST-RB (379-928) greatly reduces the ability of the complex coprecipitated by the BRF antiserum to retain the radiolabelled B box probe (lane 3), in contrast to GST alone which had much less effect (lane 2). In order to confirm that the inclusion of RB specifically affected the TFIIIC/TFIIIB interaction and not the ability of precipitated TFIIIC2 to bind the B-box probe, TFIIIC2 antiserum was used to

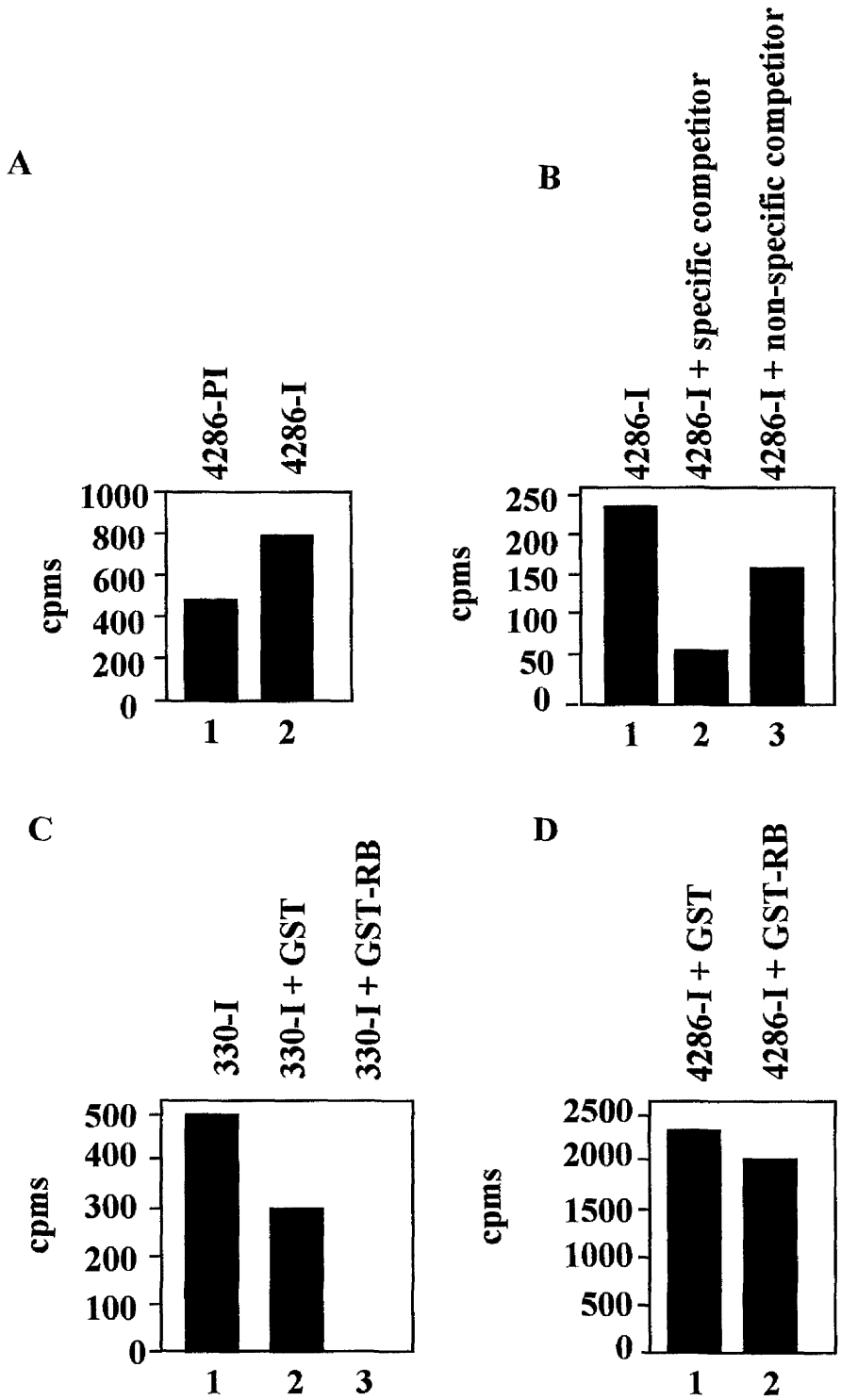
FIG. 3.22 RB disrupts the interaction between endogenous TFIIIC2 and endogenous TFIIIB.

(A) HeLa cell extract (150 μg) was immunoprecipitated with TFIIIC2 antiserum 4286 (lane 2) and the corresponding preimmme (lane 1). The precipitated material was incubated with radiolabelled B box probe. After extensive washing, the amount of retained probe was measured by scintillation counting.

(B) HeLa cell extract (150 μg) was immunoprecipitated with TFIIIC2 antiserum 4286 (lanes 1-3). The precipitate was incubated with radiolabelled B box probe, with the addition of a specific competitor oligonucleotide (lane 2), and a non-specific competitor oligonucleotide (lane 3). After incubation, the precipitate was washed and the amount of probe retained determined.

(C) HeLa cell extract (150 μg) alone (lane 1), or mixed with 200ng of GST (lane 2) or GST-RB (379-928) (lane 3) was immunoprecipitated with BRF antiserum 330 (lanes 1-3). The precipitate was incubated with radiolabelled B box probe; retained probe was measured.

(D) HeLa cell extract (150 μg) with addition of 200ng of GST alone (lane 1) or GST-RB (379-928) (lane 2) was immunoprecipitated with TFIIIC2 antiserum 4286 (lanes 1 and 2). The precipitate was incubated with radiolabelled probe and the retained probe was measured


A
4286-PI
4286-I
cpms
1000
800
600
400
200
0
1
2
B
4286-I
4286-I + specific competitor
4286-I + non-specific competitor
cpms
250
200
150
100
50
0
1
2
3
C
330-I
330-I + GST
330-I + GST-RB
cpms
500
400
300
200
100
0
1
2
3
D
4286-I + GST
4286-I + GST-RB
cpms
2500
2000
1500
1000
500
0
1
2

immunoprecipitate HeLa extract in the presence of recombinant RB (fig. 3.22.D). The addition of GST-RB (379-928) only slightly reduces the ability of the TFIIIC2 antiserum to immunoprecipitate a complex which has the capacity to bind the B box probe. The immunoprecipitation data and oligonucleotide binding assays suggest that RB compromises the ability of TFIIIC2 to coimmunoprecipitate with BRF, indicating that RB disrupts the interaction between TFIIIB and TFIIIC.

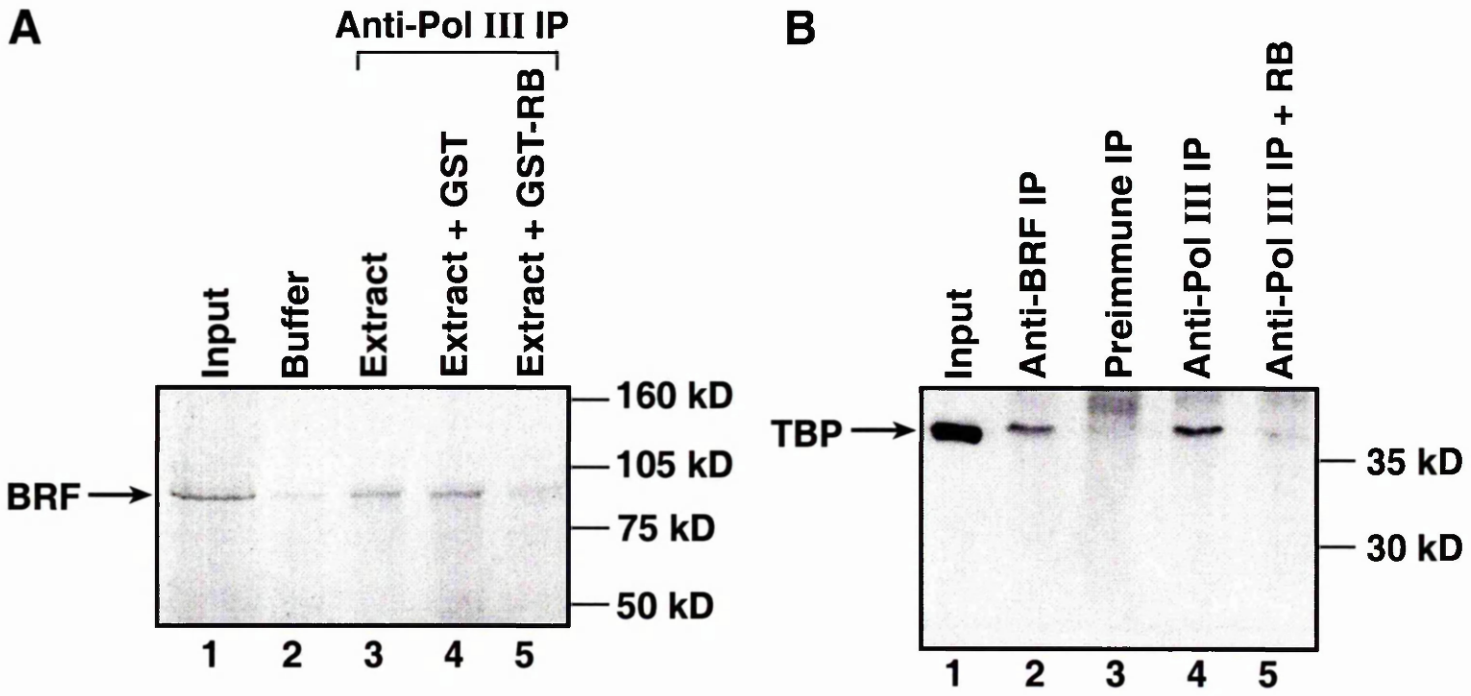
3.2.8 Characterisation of the effect of RB upon the interaction between TFIIIB and pol III

The ability of RB to disrupt the pol III/TFIIIB interaction was investigated by immunoprecipitations. Pol III antiserum was immobilised on protein A beads and incubated with HeLa extract mixed with reticulocyte lysate containing radiolabelled BRF, with the addition of GST alone or GST-RB (379-928). After incubation, the coprecipitate was washed and resolved by SDS-PAGE and visualised by autoradiography (fig. 3.23.A). As is shown, the pol III antiserum is able to coprecipitate BRF (lane 3) and this ability is not negated by the presence of GST alone (lane 4). However, when GST-RB (379-928) was added to the immunoprecipitation reaction the ability of the pol III antiserum to coprecipitate BRF was reduced (lane 5). These immunoprecipitation data suggest that RB disrupts the interaction between TFIIIB and pol III.

FIG. 3.23 RB disrupts the interaction between pol III and BRF.

(A) 15μl of reticulocyte lysate containing in vitro-translated BRF, was mixed with buffer (lane 2) or HeLa extract (lanes 3-5), with the addition of 200ng of GST alone (lane 4) or GST-RB (379-928) (lane 5), then immunoprecipitated with column purified pol III antiserum BN51 (lanes 2–5). The precipitate was resolved on a SDS-7.8% polyacrylamide gel and visualised by autoradiography. Lane 1 shows 10% input of reticulocyte lysate containing in vitro-translated BRF.

(B) HeLa cell extract (150 μg) (lanes 2-5) mixed with 100ng of His-tagged RB (379-928) (lane 5), was immunoprecipitated with the BRF antiserum 330 (lane 2), the corresponding preimmune (lane 3), and the pol III antiserum BN51 (lanes 4 and 5). The precipitate was resolved on a SDS-7.8% polyacrylamide gel. The presence of TBP was determined by western analysis with the anti-TBP antibody SL30. Lane 1 shows 25% input of HeLa.


A
Anti-Pol III IP
Input
Buffer
Extract
Extract + GST
Extract + GST-RB
BRF
160 kD
105 kD
75 kD
50 kD
1
2
3
4
5
B
Input
Anti-BRF IP
Preimmune IP
Anti-Pol III IP
Anti-Pol III IP + RB
TBP
35 kD
30 kD
1
2
3
4
5

The effect of RB upon the interaction between endogenous TFIIIB and endogenous pol III was also investigated by immunoprecipitations. Pol III and BRF antisera immobilised on protein A beads were incubated with HeLa extract, in the absence or presence of His-tagged RB (379-928). After incubation, the coprecipitate was washed and resolved by SDS-PAGE. The presence of TFIIIB was determined by probing for TBP by western analysis (fig. 3.23.B). As is shown, the preimmune serum (lane 2) does not precipitate TBP, but the BRF antiserum does (lane 3). The pol III antiserum coprecipitates TBP (lane 4), but upon the addition of His-tagged RB (379-928) this effect is abolished (lane 5). These observations suggest that the presence of RB disrupts the interaction between TFIIIB and pol III.

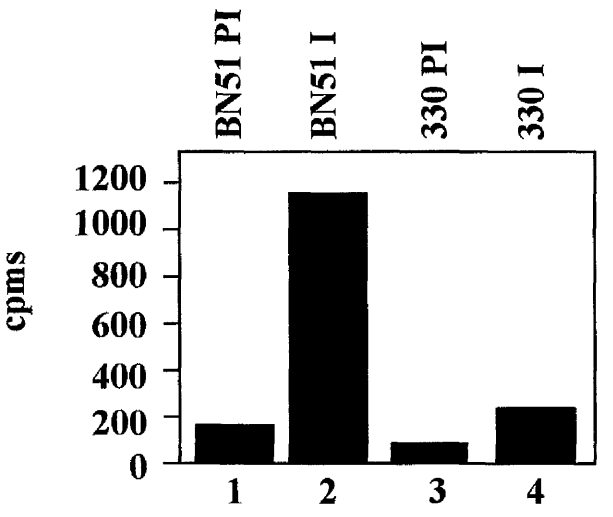
An alternative means of detecting RNA polymerase was employed, namely polymerisation assays using poly(dA-dT) as template. This experimental approach allowed for further assessment of the TFIIIB-pol III interaction. Anterisera against BRF and pol III, along with the corresponding preimmunes as negative controls, were immobilised on protein A beads, incubated with HeLa extract, then assayed for polymerase activity (fig. 3.24A). As is shown, both antisera immunoprecipitated more pol III activity than their corresponding preimmunes. / Sera To determine the effect of RB upon the interaction between TFIIIB and pol III, BRF antiserum was immobilised on protein A beads and incubated with HeLa extract with the addition of GST alone or GST-RB (379-928). The coprecipitate was washed and assayed for polymerase activity. It can be seen that (fig. 3.24.B), the presence of GST-RB (379-928) greatly reduced the ability of pol III and TFIIIB to

FIG. 3.24 RB disrupts the interaction between endogenous TFIIIB and endogenous pol III.

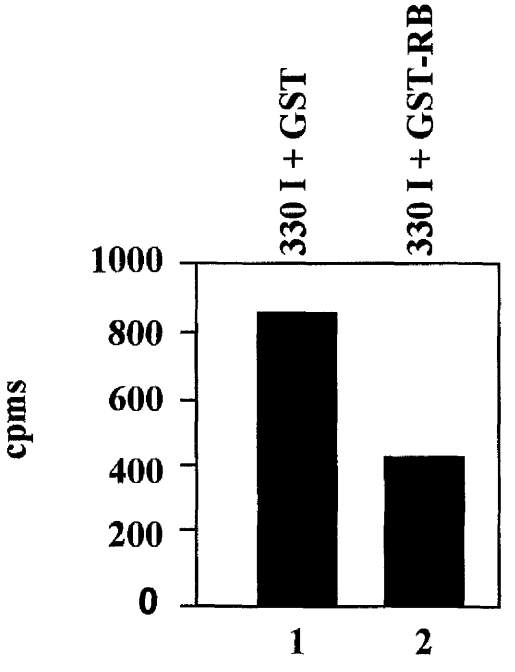
(A) HeLa cell extract (150 μg) was immunoprecipitated by the pol III antiserum BN51 (lane 2) and the corresponding preimmune (lane 1), and the BRF antiserum 330 (lane 4) and its corresponding preimmune (lane 3). The presence of pol III was detected by a polymerisation assay.

(B) HeLa cell extract (150 μg) mixed with 200ng of GST alone (lane 1) or GST-RB (379-928) (lane 2) was immunoprecipitated with BRF antiserum. Again, pol III was detected by a polymerisation assay.

A


BN51 PI
BN51 I
330 PI
330 I
cpms
1200
1000
800
600
400
200
0
1
2
3
4

B


330 I + GST
330 I + GST-RB
cpms
1000
800
600
400
200
0
1
2

interact. Thus, these assays again demonstrate that RB is capable of disrupting the interaction between TFIIIB and pol III.

3.2.9 Model whereby RB represses pol III transcription

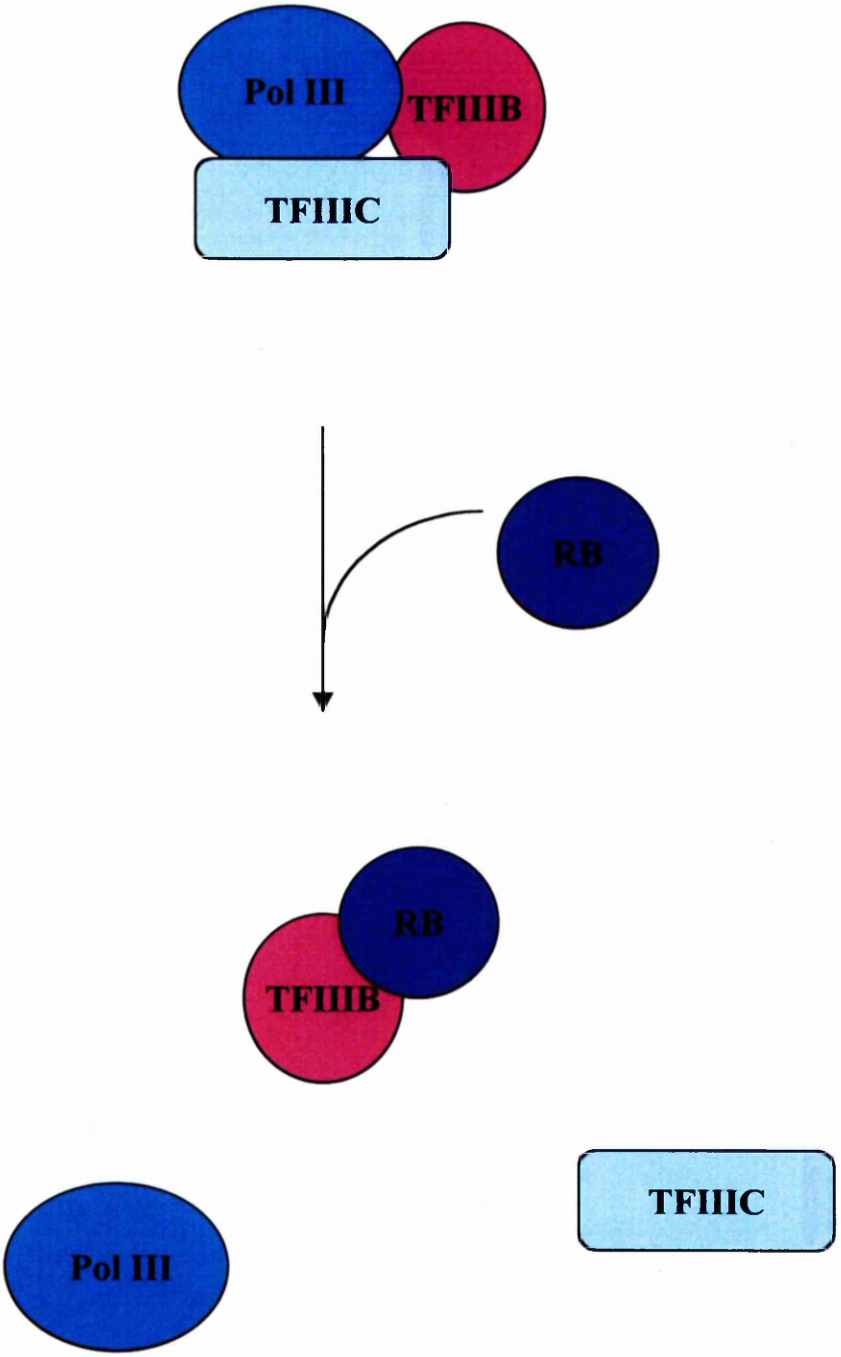
The observations discussed above culminate in the following model: RB represses pol III transcription by targeting the BRF subunit of the pol III general transcription factor TFIIIB; RB exerts this effect not by disrupting the TFIIIB multisubunit complex, but instead RB prevents TFIIIB from interacting with both TFIIIC and pol III (fig. 3.25).

3.3 Discussion

Relatively recently it was discovered that RB is able to inhibit pol III transcription (White et al., 1996; Larminie et al., 1997). The work presented above has elucidated a mechanism for this repression; namely, that RB interacts with the BRF subunit of TFIIIB and prevents it from physically associating with the other components of the transcription machinery. The domain of RB responsible for binding BRF and repressing pol III transcription is the large pocket of RB (White et al., 1996). Since this is also the domain necessary for growth inhibition (Qin et al., 1992), perhaps a correlation exists between these events.

FIG. 3.25 Model whereby RB represses pol III transcription.

RB targets the BRF subunit of TFIIIB, thereby making it unavailable to interact with both TFIIIC and pol III.


Pol III
TFIIIB
TFIIIC
RB
RB
TFIIIB
TFIIIC
Pol III

The RB-binding domain in BRF was elucidated. Truncations of yeast and human BRF identified the region as being in the TFIIB homology half. Since deletion of either imperfect repeat did not abolish RB binding, it could be that both of these repeats is a RB-binding region. The stiochiometry of the RB/BRF interaction is not known and it could be that RB binding to BRF is cooperative, resulting in two RB molecules being bound to a BRF protein by virtue of the imperfect repeats. Whether this is spatially feasible will only be apparent upon determination of the tertiary structure of BRF.

RB represses pol III transcription via two effects. Firstly, it prevents TFIIIB from interacting with TFIIIC2, the DNA binding protein which acts to recruit TFIIIB to most class III promoters. However, there are some genes that are transcribed by pol III in a TFIIIC2-independent fashion, such as human U6 snRNA genes (White, 1998). These genes, unlike other pol III templates, have an upstream TATA sequence which can be recognised directly by the TBP subunit of TFIIIB (White, 1998). If RB only affected the interaction between TFIIIB and TFIIIC2, then this could not account for the ability of RB to repress class III promoter types. However, there is another means by which RB represses pol III transcription, namely by disrupting the interaction between TFIIIB and pol III. The ability of RB to disrupt this interaction provides a mechanism for RB to be a general repressor of pol III transcription, as the recruitment of pol III by TFIIIB is an essential step in the transcription of all pol III genes (Willis, 1993; Geiduschek and Kassavetis, 1995; White, 1998).

The ability of RB to disrupt the interaction between TFIIIB and pol III is unlike the mechanism of RB-mediated repression reported for the other transcription systems, pol I (Cavanaugh et al., 1995; Voit et al., 1997) and pol II (Ross et al, 1999). For example, RB does not disrupt the interaction between the pol I transcription factor UBF and the pol I enzyme (Voit et al., 1997). However, there would appear to be an analogy between the pol II and pol III systems, regarding the ability of RB to prevent a physical association between a TBP-containing complex and a promoter-recognising protein. Thus, RB binds TFIIIB and prevents it from interacting with the promoter-recognition factor TFIIIC2, whilst in the pol II system RB binds E2F, a promoter-recognising factor, and prevents it from associating with TFIID, a TBP-containing complex (Ross et al., 1999).

Although it appears that RB represses pol III transcription by two distinct mechanisms, why this is so is not clear. The ability of RB to disrupt the interaction between TFIIIB and pol III would suffice to repress transcription from all pol III promoter types. As mentioned above, the RB binding site has been located to the TFIIB-homology half of BRF, more precisely the imperfect repeats. It could be that RB binding to BRF covers the regions needed for BRF to interact with both TFIIIC2 and pol III. In yeast, the domains in BRF that interact with TFIIIC and pol III have been identified as being situated around the 2 N-terminal repeats (Khoo et al., 1994; Chaussivert et al., 1995; Kassavetis et al., 1997), the same region which has been shown to be responsible for binding RB. It is likely that the same regions are responsible for such interactions in human BRF. Hence, upon binding to BRF, RB

disrupts the interaction between BRF and TFIIIC and pol III by masking the binding sites of these two proteins.

The model proposed above does not fully comply with previous observations regarding RB-mediated pol III repression. Previous work has suggested that RB targets two general pol III transcription factors, TFIIIC and TFIIIB (Chu et al., 1997). Other studies (Larminie et al., 1997) and the work presented above imply that TFIIIB is the specific target of repression, and that upon addition of RB the interaction between TFIIIB and TFIIIC is abolished.

Larminie et al. suggested that RB represses pol III transcription by disrupting the TFIIIB complex (Larminie et al., 1997). Work presented here demonstrates that RB does not disrupt the physical association between TBP and BRF, unlike Dr1, which represses pol III transcription by competing with BRF for binding sites on TBP (White et al., 1994). This is consistent with the fact that TBP and BRF can be coimmunoprecipitated with antibodies against RB (Larminie et al., 1997). However, mammalian TFIIIB is believed to be composed of at least one more subunit besides TBP and BRF, probably one which shows homology to the B" subunit of yeast TFIIIB (Lobo et al., 1992; Teichmann and Seifart, 1995; Mital et al., 1996; Teichmann et al., 1997). It cannot be ruled out that RB disrupts the interactions between the components of B' and an as of yet unidentified subunit(s) of TFIIIB. At present the molecular reagents are not available for such investigations to be undertaken. They are also not available to examine the possible interaction between RB and the ill-defined TFIIIC1 component of TFIIIC.

In conclusion, it appears that RB represses pol III transcription by disrupting the initiation complex, preventing TFIIIB from interacting with TFIIIC and pol III.

Chapter 4

Disruption of the interaction between BRF and RB in cancer cells

4 Disruption of the interaction between BRF and RB in cancer cells

4.1 Introduction

A hallmark of cancer is uncontrolled proliferation and deregulation of the cell cycle. The fundamental task of the cell cycle is to faithfully replicate DNA, and ensure equal distribution of the chromosomes between two daughter cells (Heichman and Roberts, 1994). During G1, cells are receptive to extracellular stimuli, which signal to the cells to either proceed towards division by transit through S, G2, and M-phases or to withdraw from the cell cycle into a resting state called G0 (Pardee, 1989). Cancer cells differ from normal cells by being unreceptive towards many extracellular stimuli; these signals are ignored and the cells tend to remain cycling, rather than going into a resting state that may facilitate differentiation. There are various control or check-points throughout the cell cycle, an example of which occurs during late G1 and is known as the restriction or R point. Once a cell passes the restriction point it is committed to cycling (Pardee, 1989). As the restriction point constitutes a major regulatory feature of the cell cycle, it is of considerable importance to understand the mechanisms which act at the restriction point, and if deregulation of this machinery allows cancerous cells to continuously cycle.

A means of regulating the passage of cells through the restriction point is by the cyclin-dependent protein kinases (cdks). The activity of cdks requires cyclin binding; which occurs in a progressive fashion through the cell cycle by cyclins D, E, A and B. (Morgan, 1995; Resnitzky et al., 1994). cdk activity also depends on both

positive and negative regulatory phosphorylation and can be compromised by cdk inhibitory proteins (Sherr and Roberts, 1995). A target of cyclin-dependent kinases is RB; this gives rise to the RB pathway which converges on the restriction point (Weinberg, 1995).

As the activity of the cdks is controlled by various cyclins throughout the cell cycle, so is the phosphorylation status of RB. Upon the appropriate extracellular signals, cyclin D is expressed giving rise to the formation of a cdk4/6-cyclin D complex by mid-G1. The cdk4/6-cyclin D1 complex phosphorylates RB, preventing it from acting as a cell cycle brake; hence cells can pass through the restriction point and exit G1 (Sherr, 1993). At the G1-S transition, cyclin E is the most predominant cyclin and maintains the hyperphosphorylation status of RB in a mitogen-independent fashion (Sherr, 1993). The other cyclins, A and B, maintain the hyperphosphorylated status of RB as cells proceed through the cell cycle. RB-dephosphorylation does not occur until cells complete mitosis and re-enter G1 phase (or G0) (Sherr, 1996). A pattern for RB phosphorylation throughout the cell cycle can be proposed: RB is hypophosphorylated in G1 phase; as cells enter S phase, RB is hyperphosphorylated; it remains in this state until end of M phase, whereupon it is dephosphorylated (Weinberg, 1995). Like RB, pol III transcription levels fluctuate as a cell passes through the cell cycle; pol III transcription increases gradually through G1, reaching a maximal level during S and G2, then decreasing as cells enter M (White et al., 1995b). During interphase, the pattern of RB phosphorylation in the cell cycle coincides with the model for elevated pol III transcription, this suggests a correlation

between the phosphorylation of RB and the upregulation of pol III transcription, namely phosphorylated RB is unable to repress pol III transcription.

In cancerous cells, as one may have expected, the situation is altered. For example in a variety of transformed cells, the RB kinases becomes deregulated, ultimately resulting in the inactivation of RB by hyperphosphorylation. In this state RB can not act as a cell cycle brake at the restriction point, or inhibit transcription, and so cells continuously cycle and display deregulated transcriptional activity. RB may become hyperphosphorylated in a variety of ways, such as the overexpression of cyclin D1, which is prevalent in many human cancers as a result of gene amplification or translocations targeting the cyclin D1 locus. Such overexpression is observed in more than 50% of breast cancers, and in esophageal and squamous cell carcinomas (Hunter and Pines, 1994; Pines, 1995; Sherr, 1994; Weinberg, 1995). A similar means of inactivating RB would be the amplification of cdk4, as observed in glioblastomas and some gliomas (He et al., 1994; Schmidt et al., 1994). Alternatively, RB hyperphosphorylation can be achieved by the deletion of genes encoding the inhibitors of cdk4, such as p15 and p16; such mutations are observed in esophageal squamous cell carcinomas (Mori et al., 1994; Nobori, 1992). The mutational inactivation of p15 and p16 presumably mimics the overexpression of cyclin D1 or cdk4; the consequence of each is the hyperphosphorylation of RB and subsequent physiological inactivation.

In addition to phosphorylation, there are two other means by which RB can become physiologically inactivated, namely by mutation and through sequestration

by a transforming protein. Mutational inactivation of RB has been observed in retinoblastomas and osteosarcomas (Weinberg, 1995). Several mutant forms of RB have also been isolated from various cell lines and demonstrated to be inactive, giving rise to null mutants. The small-cell lung cancer cell line NCI-H209 has been shown to express an aberrant, underphosphorylated form of RB (Kaye et al., 1990). This mutated RB contains a single point mutation within exon 21, which results in the substitution of phenylalanine for cysteine at residue 706 (Kaye et al., 1990). This substitution mutation is sufficient to alleviate phosphorylation, prevent binding to the viral transforming proteins SV40 large T antigen and adenovirus E1A (Kaye et al., 1990), and abolish RB-mediated repression of pol III transcription (White et al., 1996).

Examination of two other small-cell lung carcinoma cell lines revealed additional mutations in RB. The small-cell lung carcinoma cell line NCI-H69C contains an abnormal RB species in which the 38 amino acids of exon 22 have been deleted; this form is unable to interact with adenovirus E1A or SV40 large T antigen (Horowitz et al., 1990). Another small-cell lung carcinoma cell line, NCI-H1436 contains a RB form in which the 35 amino acids of exon 21 have been eliminated; this RB mutant is also unable to bind to adenovirus E1A or SV40 large T antigen (Horowitz et al., 1990).

The osteosarcoma cell line SAOS2 contains an abnormal RB protein of 95kD (Shew et al., 1990). This protein has a C-terminal truncation resulting from the deletion of exons 21-27 (Shew et al., 1990). This RB truncation is not phosphorylated

and neither does it bind to SV40 large T antigen (Shew et al., 1990). In addition, the introduction of wild type RB into SAOS2 cells suppresses their neoplastic phenotype, suggesting that the endogenous RB was inactivated (Shew et al., 1990).

The final means by which RB becomes physiologically inactive is through sequestration by a transforming protein such as adenovirus E1A, SV40 large T antigen or human papillomavirus E7 protein (DeCaprio et al., 1988; Dyson et al., 1989; Vousden, 1995; Whyte et al., 1988).

There are forms of cancer associated with particular transforming viruses. For example, HPVs are associated with 80% of cervical cancer, the second leading cause of cancer death in women in the world (Schoell et al., 1999). They are classified as high-risk or low-risk types, depending on whether or not they are found in cervical cancers (Swan et al., 1994). High-risk HPV types HPV-16, and -18 express oncoproteins E6 and E7, which interact strongly with p53 and RB, respectively. The binding of E7 to RB has been shown to abolish the interaction between RB and E2F (Chellappan et al, 1992). Upon HPV transformation, E7 is expressed and binds RB, which disrupts the RB-E2F complex. Hence, E2F is released and can subsequently promote cellular proliferation.

Various transformed cell types display abnormally elevated levels of pol III activity (White, 1998). This was first observed upon transformation of murine fibroblast lines with SV40 (Scott et al., 1983; Singh et al., 1985; Carey et al., 1986b; White et al., 1990). This effect is due to the elevated expression of TFIIIC2 and the

ability of SV40 large T antigen to bind and neutralise RB (White et al., 1990; Larminie et al., 1999). It is of interest to note that the level of pol III transcription correlates directly with the degree of transformation. Thus, cells which were most highly transformed have the greatest level of pol III transcription, whereas those less tumorigenic cells show lower levels of pol III activity (Scott et al., 1983; White et al., 1990).

The work presented here demonstrates that the various ways by which RB becomes physiologically inactivated reduce its ability to interact with the BRF subunit of TFIIIB. For example, the RB/BRF interaction is severely compromised following SV40 transformation. This interaction is also perturbed when RB is mutated; the deletion of the C-terminus of RB, or the deletion of 4 amino acids in the RB pocket also compromises the ability of RB to interact with BRF. Null mutant forms of RB are also shown to be unable to alleviate the association between TFIIIB and TFIIIC, or between TFIIIB and pol III, unlike their wild-type counterpart. Lastly, the phosphorylation of RB has also been shown to prevent a physical association between BRF and RB.

4.2 Results

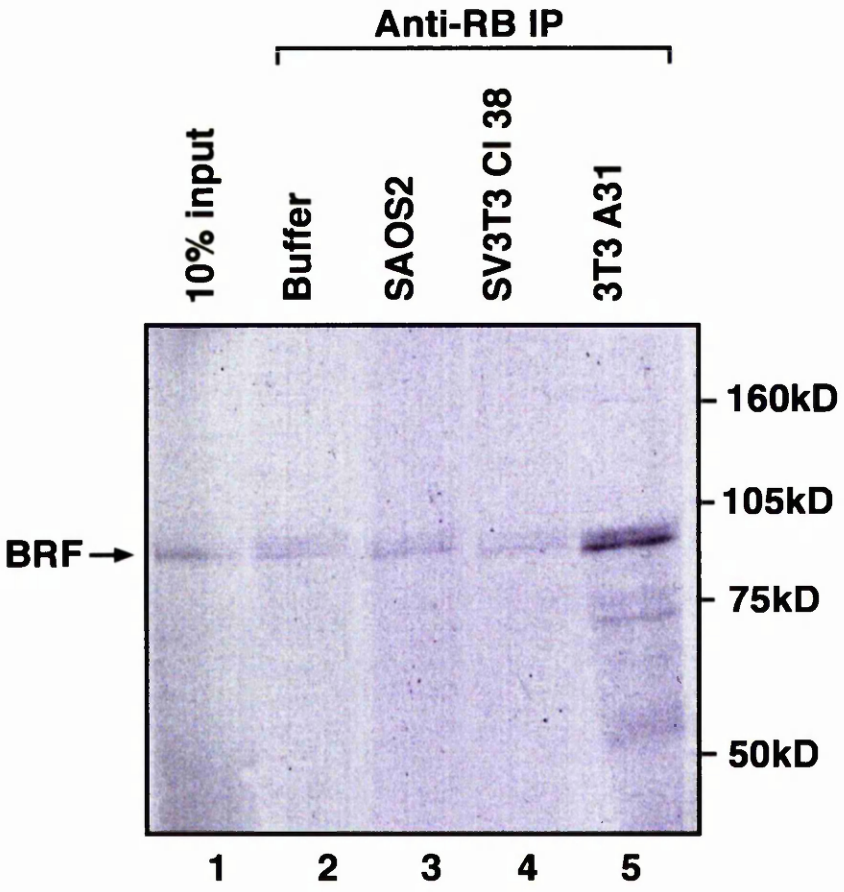
4.2.1 The interaction between TFIIIB and RB is deregulated following SV40 transformation

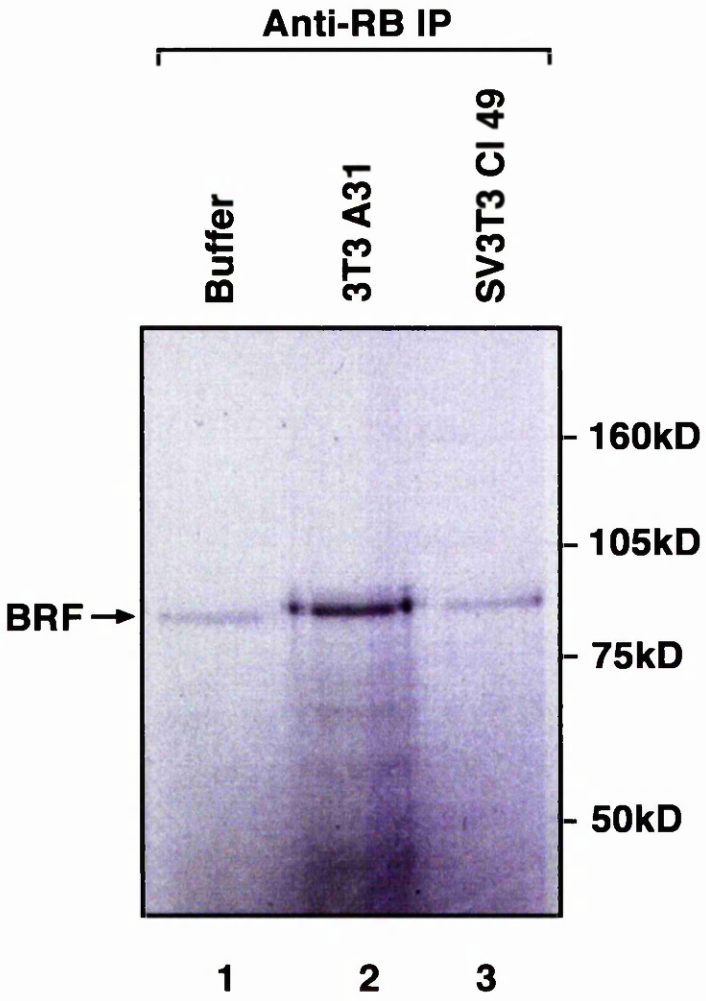
Previous investigations have shown that in murine fibroblasts RB regulates the activity of TFIIIB (Larminie et al., 1997). Immunoprecipitations were undertaken in order to determine the effect of SV40 transformation upon the physical association between TFIIIB and RB. The BRF subunit of TFIIIB was expressed and radiolabelled in vitro using reticulocyte lysate and incubated with whole cell extract. The mixtures were then immunoprecipitated using the RB antibody G3-245. The precipitate was washed and resolved by SDS-PAGE and visualised by autoradiography (fig 4.1.A). As can be seen, significant amounts of BRF were coprecipitated from A31 cell extract, an untransformed murine cell line derived from random-bred Swiss mouse embryo cultures (Aaronson and Todaro, 1968) (lane 5). This interaction is specific and is not due to cross-reaction, since only background levels of BRF were observed when the cell extract was replaced by buffer (lane 2). These background levels were also observed when RB was coprecipitated from SAOS2 cells (lane 3) which express an inactive truncated form of RB (Shew et al., 1990), and SV3T3 Cl38 cells, a SV40-transformed derivative of the A31 cell line (lane 4), even though Cl38 cells contain wild-type full length RB. A similar experiment was carried out with a different antibody, wherein reticulocyte lysate containing in vitro translated and radiolabelled BRF was incubated with cell extract and immunoprecipitated with the anti-RB antibody C-15; the coprecipitated BRF was analysed by SDS-PAGE and

FIG. 4.1 The BRF subunit of TFIIIB interacts with endogenous RB in A31 cell extract, but this interaction is compromised in extracts from SV3T3 Cl38 and Cl49.

(A) Reticulocyte lysate (15μl) containing radiolabelled BRF was immunoprecipitated in the presence of buffer (20μl) (lane 2), 150μg of SAOS2 cell extract (lane 3), 150μg of Cl38 cell extract (lane 4), and 150μg of A31 cell extract (lane 5), using the anti-RB antibody G3-245. The precipitated material was resolved on a SDS-7.8% polyacrylamide gel and visualised by autoradiography. Lane 1 contains 10% of the input of the reticulocyte lysate containing in vitro translated BRF.

(B) 20μl of buffer (lane 1), 150μg of A31 cell extract (lane 2) or 150μg of Cl49 cell extract (lane 3), was mixed with 15μl of reticulocyte lysate containing in vitro translated BRF and immunoprecipitated using the anti-RB antibody C-15. The precipitated material was resolved on a SDS-7.8% polyacrylamide gel and visualised by autoradiography.


Anti-RB IP
10% input
Buffer
SAOS2
SV3T3 Cl 38
3T3 A31
BRF
160kD
105kD
75kD
50kD
1
2
3
4
5


Anti-RB IP
Buffer
3T3 A31
SV3T3 Cl 49
160kD
105kD
75kD
50kD
BRF
1
2
3

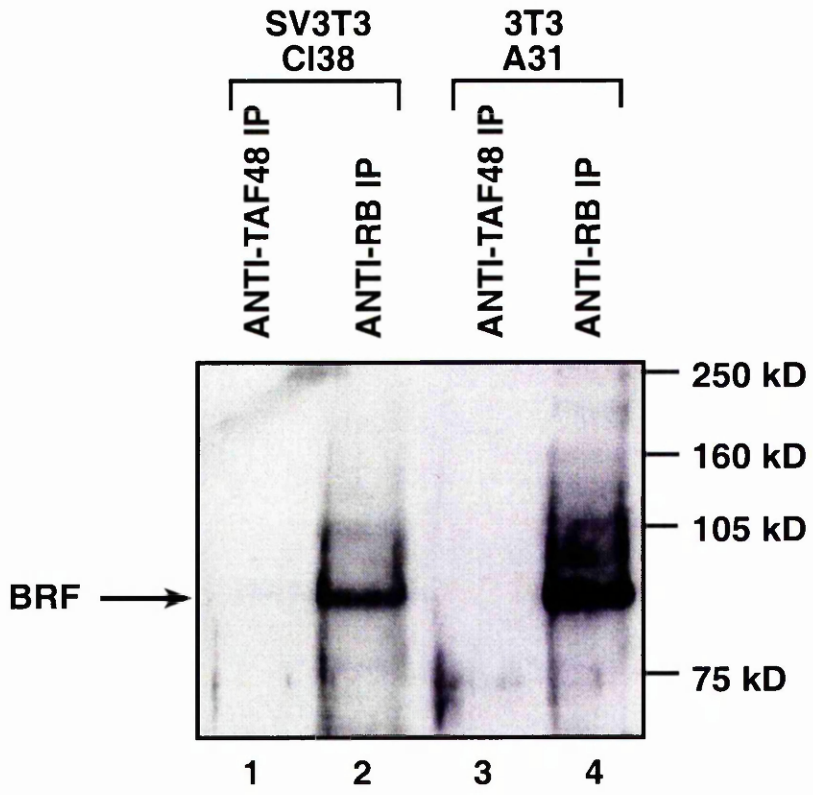
visualised by autoradiography (fig. 4.1.B). Once again, a considerable amount of BRF was coprecipitated from the A31 whole cell extract (lane 2). Upon replacement of the cell extract with buffer, only a background amount of BRF was coprecipitated (lane 1). This background level was comparable to that precipitated from SV3T3 Cl49 cell extract, another SV40-transformed A31 cell line (lane 3). The difference between the parental A31 cell extract and the SV3T3 Cl38 and Cl49 cell extracts is that the latter have been transformed by SV40. These immunoprecipitation data suggest that transformation of a cell line by SV40 compromises the interaction between TFIIIB and RB.

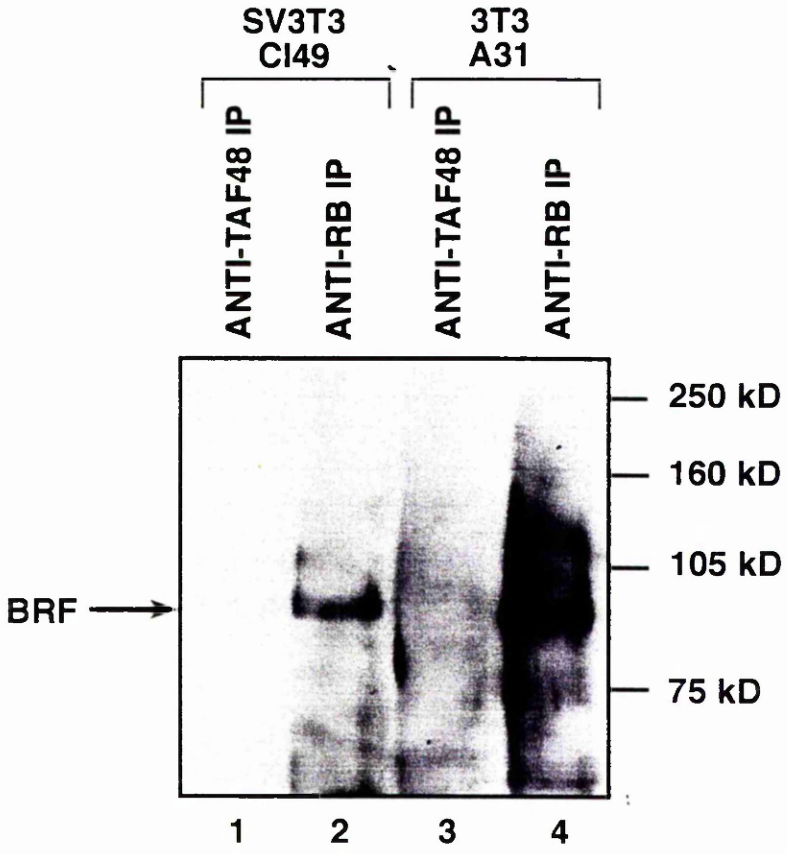
Immunoprecipitations were also carried out to assess the effect of SV40 transformation on the interaction between endogenous TFIIIB and endogenous RB. Whole cell extracts were immunoprecipitated with an anti-RB antibody, or an irrelevant, negative control antibody against TAF48, a subunit of the pol I transcription factor SL1. The precipitated material was resolved by SDS-PAGE and the presence of BRF was probed for by western blotting (fig 4.2.A). It can be seen that the amount of BRF coprecipitated from the A31 cell extract (lane 4) is greater than that coprecipitated from the SV3T3 Cl38 cell extract (lane 2). Both of these interactions are specific, as immunoprecipitation with the anti-TAF48 antibody did not coprecipitate BRF (lanes 1 and 3). A similar effect is observed when SV3T3 Cl49 cell extracts were immunoprecipitated with anti-RB and anti-TAF48 antibodies (fig. 4.2.B). Here it shows that RB coprecipitates more BRF from the A31 cell extract (lane 4) than from the SV3T3 Cl49 cell extract (lane 2). Again this interaction is specific, as the anti-TAF48 antibody did not coprecipitate BRF (lanes 1 and 3). These

FIG. 4.2 The association between endogenous TFIIIB and endogenous RB is greater in A31 extracts compared to Cl38 and Cl49 extracts.

(A) 150μg of A31 cell extract (lanes 3 and 4) and 150μg of Cl38 cell extract (lanes 1 and 2) were immunoprecipitated with the anti-RB antibody C-15 (lanes 2 and 4) or the anti-TAF48 antibody M-19 (lanes 1 and 3). The coprecipitated material was analysed by SDS-7.8% polyacrylamide gel electrophoresis and BRF was visualised by western analysis.

(B) 150μg of A31 cell extract (lanes 3 and 4) and 150μg of Cl49 cell extract (lanes 1 and 2) were immunoprecipitated with the anti-RB antibody C-15 (lanes 2 and 4), and anti-TAF48 antibody M-19 (lanes 1 and 3). The coprecipitated material was resolved by SDS-7.8% polyacrylamide gel electrophoresis and the BRF was detected by western analysis.


SV3T3
Cl38
3T3
A31
ANTI-TAF48 IP
ANTI-RB IP
ANTI-TAF48 IP
ANTI-RB IP
250 kD
160 kD
105 kD
75 kD
BRF
1
2
3
4


SV3T3
CI49
3T3
A31
ANTI-TAF48 IP
ANTI-RB IP
ANTI-TAF48 IP
ANTI-RB IP
250 kD
160 kD
105 kD
75 kD
BRF
1
2
3
4

data confirm those previously observed with the immunoprecipitations using radiolabelled BRF, that the interaction between endogenous TFIIIB and endogenous RB is severely compromised following transformation by SV40.

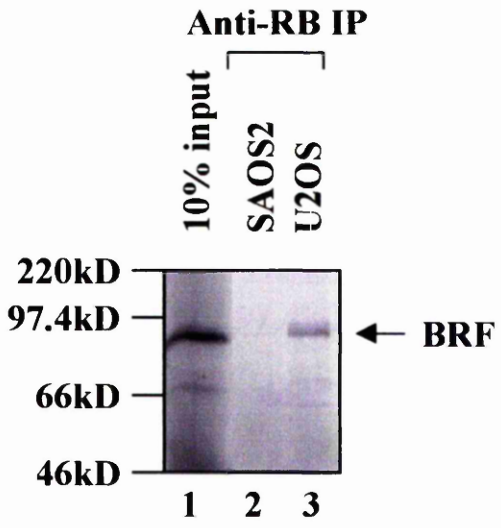
4.2.2 The interaction between TFIIIB and RB is diminished in SAOS2 cells but not U2OS cells

The work described above suggests that transforming proteins bind and inactivate RB, resulting in the diminished ability of RB to bind to the BRF subunit of TFIIIB. Other RB-inactivating mechanisms were examined for their effect upon the RB/BRF interaction, such as mutation or phosphorylation of RB.

The ability of mutant forms of RB to interact with the BRF subunit of TFIIIB was investigated by immunoprecipitations. Reticulocyte lysate containing radiolabelled BRF was incubated with cell extract and immunoprecipitated with an anti-RB antibody. The coprecipitate was analysed by SDS-PAGE and visualised by autoradiography (fig. 4.3). As can be seen, a considerable amount of BRF was coprecipitated from the U2OS cell extract, an osteosarcoma cell line containing wild type RB (lane 2). In comparison, BRF coprecipitated from cell extract prepared from SAOS2 cells, an osteosarcoma cell line containing an inactive truncated RB (Shew et al., 1990), (lane 1) is greatly reduced. These immunoprecipitation data indicate that mutational inactivation of RB can prevent it from interacting with the BRF subunit of TFIIIB.

FIG. 4.3 The BRF subunit of TFIIIB interacts with endogenous RB in U2OS cell extract, but this interaction is compromised in SAOS2 cell extract.

Reticulocyte lysate (15µl) containing in-vitro translated BRF was incubated with 150µg of U2OS cell extract (lane 3) or 150µg of SAOS2 cell extract (lane 2) and immunoprecipitated with the anti-RB antibody G3-245. The coprecipitated material was resolved on a SDS-7.8% polyacrylamide gel and visualised by autoradiography. Lane 1 contains 10% input of the reticulocyte lysate containing in vitro-translated BRF.

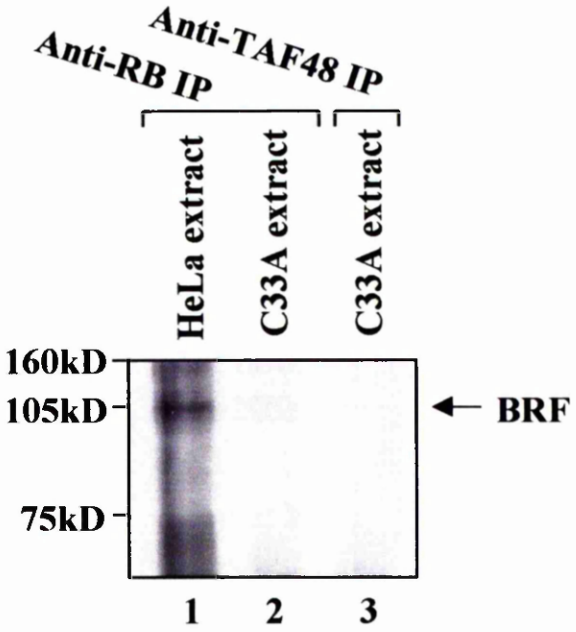

Anti-RB IP
10% input
SAOS2
U2OS
220kD
97.4kD
66kD
46kD
BRF
1
2
3

4.2.3 A mutant form of RB from a cervical carcinoma does not interact with the BRF subunit of TFIIIB

To further investigate the diminished interaction between mutated forms of RB and BRF, cell extracts were prepared from C33A cells. These cells are derived from a cervical carcinoma, in which the *Rb* gene contains a deletion mutation of the first four amino acids encoded by exon 20 (Scheffner et al., 1991). The ability of the deletion mutant RB to physically associate with the BRF subunit of TFIIIB was determined by immunoprecipitations. Whole cell extract prepared from C33A cells and HeLa cells were immunoprecipitated with an anti-RB antibody. The coprecipitated protein was resolved by SDS-PAGE, and the presence of BRF was determined by western analysis (fig. 4.4). As is shown, a greater amount of BRF is coprecipitated from extract prepared from HeLa cells, which contain wild type RB (lane 1), than extracts prepared from C33A cells, which contain a deletion mutant form of RB (lane 2). This interaction is specific, as an irrelevant, negative control antibody against TAF48 does not coprecipitate BRF from C33A cell extract (lane 3). These immunoprecipitation data therefore suggest that the deletion of four amino acids in the pocket domain of RB is sufficient to compromise the ability of RB to interact with the BRF subunit of TFIIIB.

FIG. 4.4 Endogenous BRF and RB interact in HeLa cell extract, but not in C33A cell extract.

150μg HeLa cell extract (lane 1) and 150μg C33A cell extract (lanes 2 and 3) were immunoprecipitated with anti-RB antibody C-15 (lanes 1 and 2), or anti-TAF48 antibody M19 (lane 3). The coprecipitated material was resolved by SDS-7.8% polyacrylamide gel electrophoresis and probed for BRF by western analysis.


Anti-TAF48 IP
Anti-RB IP
HeLa extract
C33A extract
C33A extract
160kD
105kD
75kD
BRF
1
2
3

4.2.4 The BRF subunit of TFIIIB does not interact with the RB null mutant Δ21

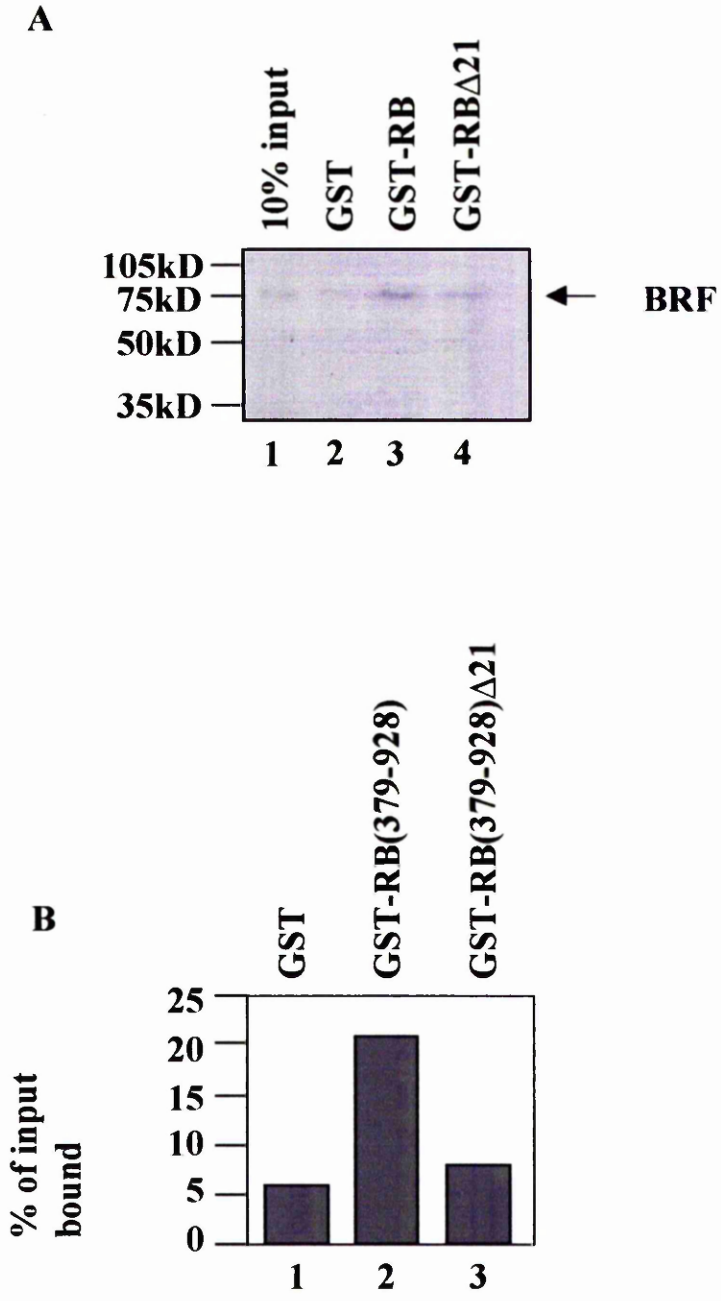
A pull down assay was carried out to test if BRF is able to bind to the Δ21 null mutant form of RB, from small cell lung carcinoma cell line NCI-H1436 in which residues 703-737 are deleted (Horowitz et al., 1990). A variety of GST fusion proteins were expressed, purified and immobilised on glutathione-agarose beads. Each of these fusion proteins was incubated with radiolabelled BRF that had been expressed in vitro using reticulocyte lysate. The beads were extensively washed and the proteins remaining bound were resolved by SDS-PAGE and visualised by autoradiography (fig. 4.5.A). As can be seen, GST alone brought down relatively little BRF (lane 2), whereas GST-RB (379-928) pulled down a readily detectable amount (lane 3). The ability of GST-RB (379-928)Δ21, to pull down BRF was greatly reduced (lane 4). Indeed, when the results were quantitated by using a phosphorimager (fig. 4.5.B), GST alone was shown to pull down 6% of the input, in comparison to GST-RB (379-928) which pulled down 21% of the input (lanes 1 and 2 respectively). GST-RB (379-928)Δ21 pulled down 8% of input, which is comparable to the background level observed with GST alone (lane 3 versus lane 1).

The GST pull down data described in chapter 3 suggest that the large pocket domain of RB is required for binding to BRF. The presence of the Δ21 mutation within the large pocket domain dramatically reduces the ability of RB to interact with

FIG. 4.5 The ability of the BRF subunit of TFIIIB to interact with a null mutant form of RB is severely compromised.

(A) Reticulocyte lysate containing in vitro-translated BRF (15μl) was incubated in the presence of glutathione-agarose beads carrying equal amounts of GST (lane 2), GST-RB (379-928) (lane 3), or GST-RB (379-928)Δ21 (lane 4). After extensive washing, the retained protein was resolved by SDS-7.8% polyacrylamide gel electrophoresis and visualised by autoradiography. Lane 1 shows 10% of the input reticulocyte lysate containing in vitro-translated BRF.

(B) The amount of BRF retained on the glutathione-agarose beads was quantitated by using a phosphorimager. The values are shown as a percentage of the total input bound. GST alone (lane 1), GST-RB (379-928) (lane 2) and GST-RB (379-928)Δ21 (lane 3).


A
10% input
GST
GST-RB
GST-RBΔ21
105kD
75kD
50kD
35kD
BRF
1
2
3
4
B
GST
GST-RB(379-928)
GST-RB(379-928)Δ21
% of input bound
25
20
15
10
5
0
1
2
3

BRF, hence it would appear that an intact large domain is required for this interaction.

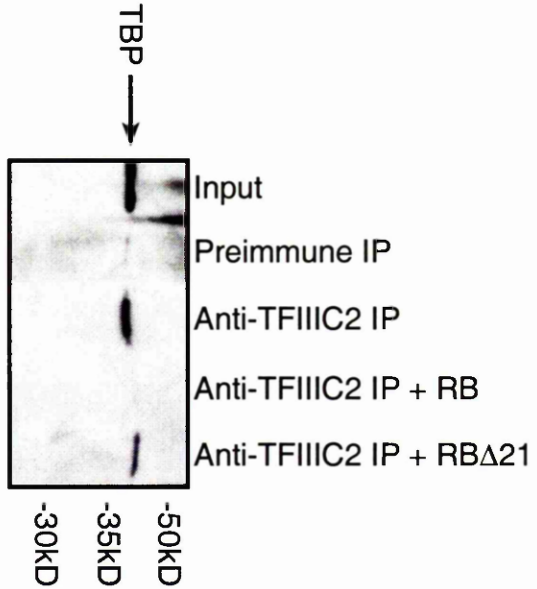
4.2.5 The RB null mutant Δ21 does not disrupt the interaction between TFIIIB and TFIIIC

The effect of the RB null mutant Δ21 upon the interaction between endogenous TFIIIB and TFIIIC was examined by immunoprecipitations. HeLa extract was mixed with GST-RB (379-928) or GST-RB (379-928)Δ21 and incubated with TFIIIC2 antiserum immobilised on protein A beads. After incubation, the coprecipitate was extensively washed and resolved by SDS-PAGE and the presence of TFIIIB was determined by probing for TBP by western analysis (fig. 4.6). Due to the interaction between TFIIIB and TFIIIC, the TFIIIC2 antiserum is able to coprecipitate TBP. However, upon the addition of GST-RB (379-928) the ability of the TFIIIC2 antiserum to coprecipitate TBP is greatly reduced (lane 4). Strikingly, the presence of GST-RB (379-928)Δ21 in the immunoprecipitation reaction did not abolish the ability of the TFIIIC2 antiserum to coprecipitate TBP (lane 5).

Additional immunoprecipitation reactions were undertaken to further substantiate these observations. HeLa cell extract was mixed with reticulocyte lysate expressing in vitro-translated BRF and purified GST alone, GST-RB (379-928) or GST-RB (379-928)Δ21. These reaction mixtures were immunoprecipitated using

FIG. 4.6 A null mutant form of RB is not able to disrupt the interaction between TFIIIB and TFIIIC2.

HeLa cell extract (150 μg) alone (lanes 2 and 3) or mixed with 200ng of GST-RB (379-928) (lane 4) or GST-RB (379-928)Δ21 (lane 5) was immunoprecipitated using affinity-purified TFIIIC2 antiserum 4286 (lanes 3-5), and the corresponding preimmune serum (lane 2). The precipitated material was resolved on a SDS-7.8% polyacryamide gel. The presence of TBP was determined by western analysis using anti-TBP antibody SL30. Lane 1 shows 20% input of HeLa.


TBP
Input
Preimmune IP
Anti-TFIIIC2 IP
Anti-TFIIIC2 IP + RB
Anti-TFIIIC2 IP + RBΔ21
-30kD
-35kD
-50kD

TFIIIC2 antiserum 4286. The precipitate was washed, resolved by SDS-PAGE and visualised by autoradiography (fig. 4.7). As is illustrated, BRF is precipitated in the presence of GST (lane 2). Upon inclusion of GST-RB (379-928) in the immunoprecipitation reaction, the amount of BRF immunoprecipitated is greatly reduced (lane 3). However, the presence of GST-RB (379-928)Δ21 (lane 4) did not compromise the immunoprecipitation of BRF. These immunoprecipitation analyses demonstrate that a RB null mutant is not able to disrupt the interaction between TFIIIB and TFIIIC, unlike wild-type RB.

4.2.6 RB null mutants do not disrupt the interaction between TFIIIB and pol III

Immunoprecipitations were also used to determine the effect of a RB null mutant upon the interaction between TFIIIB and pol III. BRF antiserum immobilised on protein A beads was incubated with HeLa extract in the presence of GST alone, GST-RB (379-928) or GST-RB (379-928)Δ21. The coprecipitate was washed and a random polymerase assay undertaken to detect pol III associated with the precipitated material. The addition of GST alone does not affect the interaction between pol III and TFIIIB (fig. 4.8.A lane 1). However, the addition of GST-RB (379-928) greatly reduced the ability of pol III and TFIIIB to interact (lane 2). The presence of GST-RB (379-928)Δ21 in the immunoprecipitation reaction did not compromise the interaction between TFIIIB and pol III (lane 3). The immunoprecipitations suggest

FIG. 4.7 The RB null mutant Δ21 cannot compromise the association between TFIIIC2 and TFIIIB.

Reticulocyte lysate containing in vitro-translated BRF (15μl) was mixed with 150μg HeLa extract (lanes 3 to 5), with the addition of 200ng of GST alone (lane 2), 200ng of purified GST-RB (379-928) (lane 3), or 200ng of purified GST-RB (379-928)Δ21 (lane 4), then immunoprecipitated with the TFIIIC antiserum 4286. The immunoprecipitated material was resolved by SDS-7.8% polyacrylamide gel electrophoresis and visualised by autoradiography. Lane 1 shows 10% of the input reticulocyte lysate containing in vitro-translated BRF.

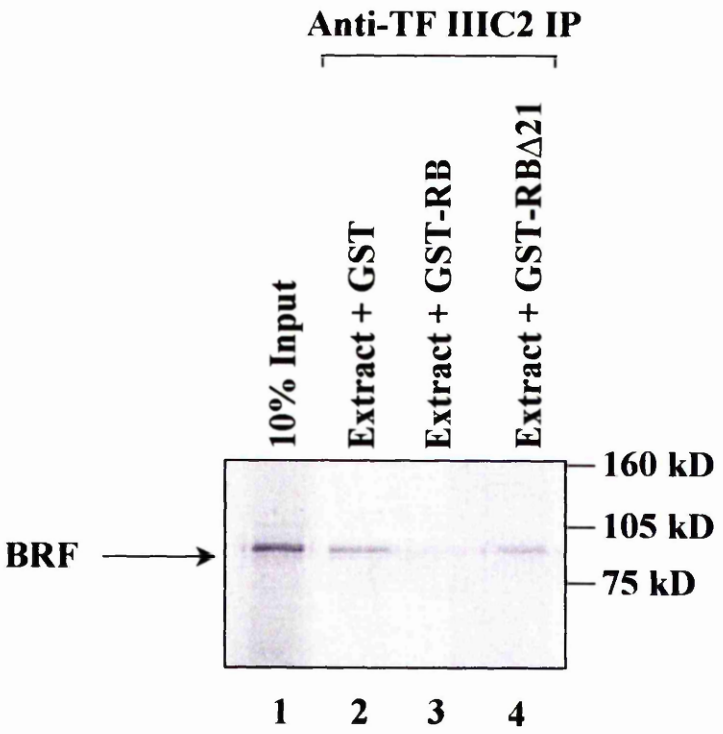
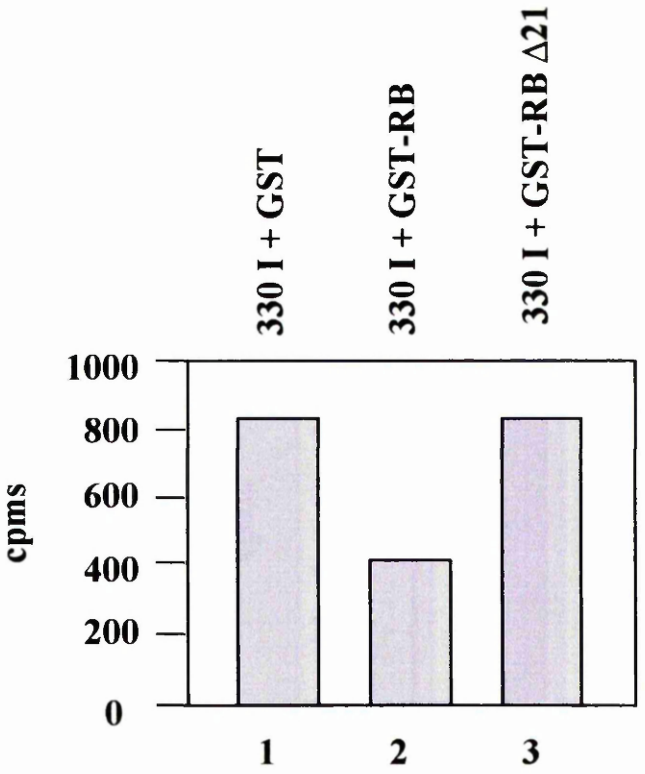

Anti-TF IIIC2 IP
10% Input
Extract + GST
Extract + GST-RB
Extract + GST-RBΔ21
160 kD
105 kD
75 kD
BRF
1
2
3
4

FIG. 4.8 Null mutant forms of RB are not able to disrupt the interaction between TFIIIB and pol III.

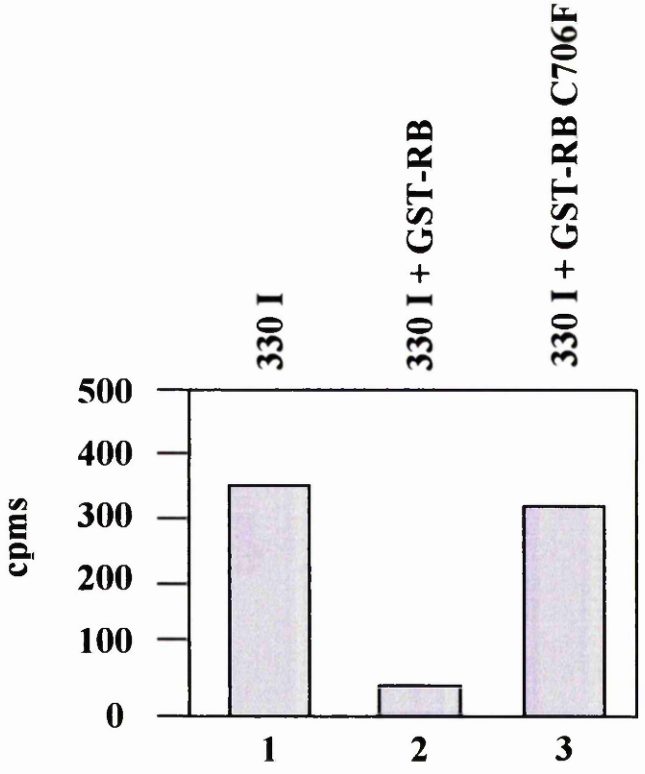
(A) HeLa cell extract (150 μg) was mixed with 200ng of GST alone (lane 1), GST-RB (379-928) (lane 2) or GST-RB (379-928)Δ21 (lane 3) and immunoprecipitated with the BRF antiserum 330. The presence of pol III was detected by a random polymerisation assay.

(B) HeLa cell extract (150 μg) mixed with 200ng of GST alone (lane 1), 200ng of GST-RB (379-928) (lane 2) or 200ng of GST-RB (379-928)C706F (lane 3) was immunoprecipitated with BRF antiserum 330. Pol III activity was detected by random polymerisation assay.

A


330 I + GST
330 I + GST-RB
330 I + GST-RB Δ21
cpms
1000
800
600
400
200
0
1
2
3

B


330 I
330 I + GST-RB
330 I + GST-RB C706F
cpms
500
400
300
200
100
0
1
2
3

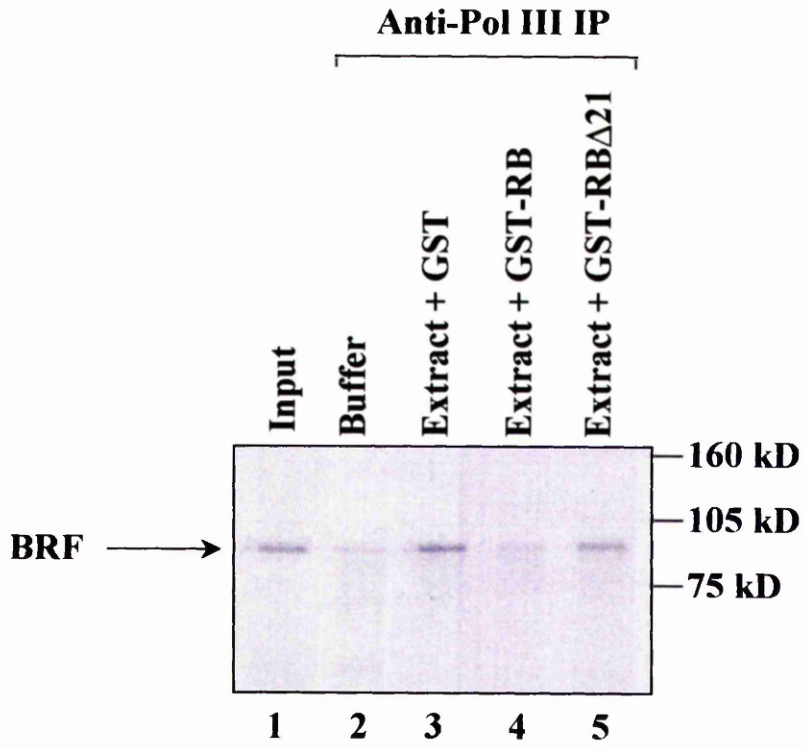
that the ability of RB to disrupt the interaction between TFIIIB and pol III is dependent upon wild type RB function, as a RB null mutant was unable to disrupt the interaction between TFIIIB and pol III.

This observation was further confirmed by using an alternative RB null mutant, namely GST-RB (379-928)C706F. This RB mutant has a point mutation at residue 706, at which the cysteine residue had been mutated to phenylalanine (Kaye et al., 1990). One consequence of this substitution is that the mutant is not able to repress pol III transcription (White et al., 1996). As is seen in fig. 4.8.B, the presence of GST-RB (379-928)C706F does not abolish the interaction between TFIIIB and pol III (lane 3). Again the same pattern is observed for both GST alone and GST-RB (379-928) (lanes 1 and 2 respectively), that is GST alone does not abolish the interaction between TFIIIB and pol III, whereas GST-RB (379-928) does.

Additional immunoprecipitation reactions were carried out to support these findings. Reticulocyte lysate containing in vitro-translated BRF was mixed with buffer or HeLa extract with the addition of GST, GST-RB (379-928) or GST-RB (379-928)Δ21. The mixtures were immunoprecipitated with the pol III antiserum BN51 113. The precipitate was washed, resolved by SDS-PAGE and visualised by autoradiography (fig. 4.9). As can be seen, in the presence of buffer a low, background amount of BRF is immunoprecipitated (lane 2). In the presence of HeLa extract mixed with purified GST, the amount of BRF immunoprecipitated is greatly increased (lane 3). Upon the addition of GST-RB (379-928) into the reaction, the

FIG. 4.9 The RB null mutant Δ21 cannot compromise the association between pol III and TFIIIB.

Reticulocyte lysate containing in vitro-translated BRF (15μl) was mixed with 20μl buffer (lane 2), or 150μg HeLa extract (lanes 3 to 5), with the addition of 200ng of GST (lane 3), 200ng of purified GST-RB (379-928) (lane 4), or 200ng of purified GST-RB (379-928)Δ21 (lane 5), then immunoprecipitated with the pol III antiserum BN51 113. The precipitated material was resolved by SDS-7.8% polyacrylamide gel electrophoresis and visualised by autoradiography. Lane 1 shows 10% of the input reticulocyte lysate containing in vitro-translated BRF.


Anti-Pol III IP
Input
Buffer
Extract + GST
Extract + GST-RB
Extract + GST-RBΔ21
160 kD
105 kD
75 kD
BRF
1
2
3
4
5

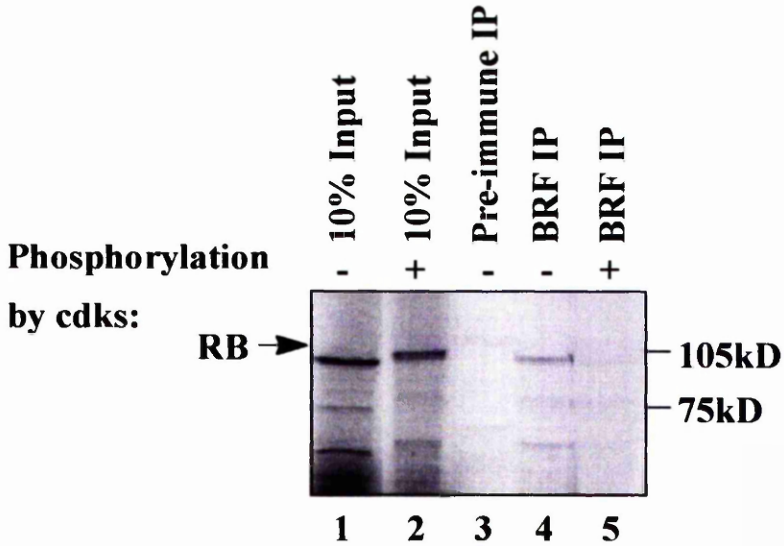
amount of BRF immunoprecipitated is greatly reduced (lane 4); in contrast, the addition of GST-RB (379-928)Δ21 (lane 5) did not affect the amount of BRF immunoprecipitated. These observations consolidate the previously described data wherein RB disrupts the interaction between TFIIIB and pol III. This effect is specific to the wild type RB, as RB null mutants are not capable of disrupting this interaction.

4.2.7 Phosphorylation of RB prevents interaction with the BRF subunit of TFIIIB

As shown above, the ability of RB to interact with BRF can be diminished by transforming proteins or by inactivating mutations; both these forms of RB inactivation are prevalent in cancerous cells (Weinberg, 1995). However, another form of RB inactivation in cancerous cells exists, namely hyperphosphorylation (Hunter and Pines, 1994; Pines, 1995; Sherr, 1994; Weinberg, 1995). Hence, it was decided to determine if phosphorylating RB affected its interaction with BRF. RB was expressed and radiolabelled in vitro by reticulocyte lysate. This RB was then phosphorylated by cdk 4/cyclin D1, cdk 2/cyclin A, and cdk 2/cyclin E. HeLa extract was then mixed with lysate expressing either hyperphosphorylated or hypophosphorylated RB and the mixture immunoprecipitated with BRF antiserum and its corresponding preimmune. The coprecipitate was resolved by SDS-PAGE and visualised by autoradiography (fig. 4.10). The mobility of the RB incubated with cdks

FIG. 4.10 Hypophosphorylated RB but not hyperphosphorylated RB interacts with BRF.

Reticulocyte lysate containing in vitro translated RB, (lanes 3 and 4), or reticulocyte lysate containing in-vitro translated RB which had been phosphorylated (lane 5) (15μl of each) was mixed with 150μg HeLa extract, and immunoprecipitated with BRF antiserum 330 (lanes 4 and 5) and its corresponding preimmune (lane 3). The coprecipitate was resolved on a SDS-7.8% polyacrylamide gel and visualised by autoradiography. Lanes 1 and 2 show 10% of the input of the reticulocyte lysate containing in vitro-translated RB, unphosphorylated (lane 1) and phosphorylated (lane 2).


Phosphorylation
by cdks:
- 10% Input
+ 10% Input
- Pre-immune IP
- BRF IP
+ BRF IP
RB
105kD
75kD
1
2
3
4
5

is slower than that of the RB not incubated with cdks, which suggests that RB has been modified, namely by phosphorylation (lane 2 versus lane 1). In its hypophosphorylated form, RB is able to interact with BRF (lane 4). However, when RB is phosphorylated this binding is not observed (lane 5). These immunoprecipitation data indicate that whilst RB is hypophosphorylated it is capable of interacting with BRF; however, when RB becomes hyperphosphorylated the ability of RB to interact with BRF is abolished.

The data described above demonstrate that the various ways in which RB is inactivated in cancerous cells, such as sequestration by a transforming protein, mutations, or hyperphosphorylation, can all compromise the ability of RB to interact with BRF.

4.3 Discussion

There are various ways the level of pol III transcription can be regulated in either a positive or negative fashion. For example, as a cell enters mitosis pol III transcription has been shown to be repressed in both Xenopus egg extract (Hartl et al., 1993) and humans (White et al., 1995a). The level of pol III transcription can also be altered in response to viral infection, as seen in cells infected with poliovirus, which display a reduced ability to transcribe pol III genes (Fradkin et al., 1987). An

alternative means of regulating pol III transcription is RB-mediated repression (White et al., 1996).

In a broad range of cancers and transformed cell lines RB has been inactivated either by mutational inactivation, hyperphosphorylation or sequestration by viral oncoproteins (Weinberg, 1995). The introduction of wild-type RB into cells lacking functional RB has been reported to induce G1 cell cycle arrest, hence RB is believed to control cell cycle progression by regulating G1/S phase transition (Pardee, 1989). Until recently it was proposed that the ability of RB to regulate cell cycle progression was by binding the pol II transcription factor E2F, thereby making E2F unavailable to express genes which promote DNA replication and cell cycle regulation (La Thangue, 1994). This model however does not provide a means for explaining how the loss of RB allows tumour cells to increase their biosynthetic capacity, without which an increased rate of chromosome duplication would ultimately be lethal (Nasmyth, 1996). The discovery that RB also regulates pol I and pol III transcription and subsequently the expression of rRNA and tRNA genes, respectively (Cavanaugh et al., 1995; White et al., 1996), potentially provides an explanation as to how RB also regulates the biosynthetic capacity of a cell. The target of RB mediated repression of pol III transcription has been demonstrated as being the BRF subunit of TFIIIB (Larminie et al., 1997). Hence it was decided to determine if the ability of RB to regulate cell cycle progression correlated with its ability to regulate pol III transcription by binding to BRF. This was determined by investigating if the loss of RB function with regard to cell cycle regulation is also accompanied by the diminished ability of RB to interact with BRF.

It has been well documented that cells transformed with SV40 display elevated levels of pol III transcription (Carey et al., 1986b; Scott et al., 1983; Singh et al., 1985; White et al., 1990). This is due to an increase in the abundance of TFIIICα and TFIIICβ, and also the release of TFIIIB from RB, upon the sequestration of RB by viral large T antigen (Larminie et al., 1999). In untransformed murine fibroblasts, TFIIIB is negatively controlled by physically associating with RB. In cells transformed by SV40, the amount of TFIIIB associated with RB is dramatically reduced (fig. 4.2). Moreover, a specific increase in TFIIIB activity is observed (Larminie et al., 1999). These two events could be explained by the partial release of BRF from RB, upon viral transformation, resulting in an increase in TFIIIB activity (Larminie et al., 1999). It could be that viral transformation altered the levels of TFIIIB subunits. However, western analysis demonstrated that the abundance of two TFIIIB subunits, TBP and BRF, varied little between A31 extracts and Cl38 and Cl49 extracts (Larminie et al., 1999). Human TFIIIB is not completely characterised; it is believed to contain one or more subunit(s) in addition to TBP and BRF (Hernandez, 1993; Rigby, 1993). Hence, the possibility remains that following SV40 transformation the abundance of a currently uncharacterised subunit increases. Damania et. al. reported an association between T antigen and the BRF subunit of TFIIIB (Damania et al., 1998). Immunoprecipitations were carried out to test for interaction between T antigen and BRF in Cl38 and Cl49 cells, but no association was detected. Instead, it appears that SV40 activates TFIIIB by neutralising RB.

An alternative way by which RB becomes inactivated is by mutation of the RB gene. A number of studies have identified and isolated mutated RB from various tumourgenic cell lines (Weinberg, 1995). Observations reported here suggest that the inability of BRF to interact with mutant RB is not specific to a particular mutant form of RB. For example, the ability of BRF to interact with mutated RB present in SAOS2 cells (Shew et al., 1990) and C33A cells (Scheffner et al., 1991) is greatly reduced compared to cell lines containing wild-type RB, namely U2OS and HeLa cells respectively. Moreover, as mutant forms of RB are unable to bind BRF, it follows they will not perform the functions displayed by wild-type RB, in particular compromising the interactions between TFIIIB and TFIIIC and between TFIIIB and pol III. Indeed, this was shown to be the case. Two mutant forms of RB, isolated from small cell lung carcinoma cell lines, which contain either a point or deletion mutation, GST-RB (379-928)C706F (Kaye et al., 1990) or GST-RB (379-928)Δ21 (Horowitz et al., 1990) respectively, were unable to disrupt the interactions between TFIIIB and the other components of the initiation complex, namely TFIIIC and pol III. These findings demonstrate that a variety of mutant forms of RB are unable to bind BRF. Those further investigated were unable to prevent TFIIIB from interacting with the other components of the pre-initiation complex hence it would follow that they would not be able to repress pol III transcription, as has previously been shown.

The third way by which RB can become inactivated is through phosphorylation (Hunter and Pines, 1994; Pines, 1995; Sherr, 1994; Weinberg, 1995). In normal cells, RB is silenced by cyclin-dependent kinases, after which it can no

longer restrain cell cycle progression (Weinberg, 1995). In a vast number of tumour cells, RB is physiologically inactivated; hence it can no longer act as a cell cycle brake, therefore tumour cells can continuously cycle (Weinberg, 1995). RB can be maintained in a phosphorylated state by deregulation of the RB pathway (Weinberg, 1995). As normal cells pass through the cell cycle, the phosphorylation state of RB cells oscillates (Weinberg, 1995), as does the level of pol III transcription (White et al., 1995b). Hence, it was of interest to determine the effect phosphorylation of RB would have on its interaction with BRF. As shown, upon phosphorylation RB is unable to bind to BRF. Hence, in cancer cells within which RB is hyperphosphorylated, TFIIIB will be released from RB-mediated repression, and will be able to promote pol III transcription.

The work presented here demonstrates that the various ways by which RB becomes inactivated with regard to its ability to inhibit cell growth also prevent RB from interacting with BRF. Thus, a potential correlation exists between the ability of RB to bind BRF and to induce cell growth arrest.

Chapter 5

Characterisation of the interaction between the tumour suppressor protein RB and the pol I transcription factor UBF

5 Characterisation of the interaction between the tumour suppressor protein RB and the pol I transcription factor UBF

5.1 Introduction

In order for pol I to be recruited to the transcription start sites of class I genes, two transcription factors are required, namely SL1 and UBF. The human rRNA promoter consists of two control sequences, the core and an upstream control element (UCE). UBF can recognise both of these promoter sequences. SL1, however, has relatively low affinity for the human rRNA promoter and is recruited to the template by protein-protein interactions with UBF. This results in an extended region of the promoter being protected, as shown by DNase footprinting (Bell et al., 1988; Hempel et al., 1996; Learned et al., 1986).

The structural and functional properties of UBF and SL1 have been investigated, in particular their involvement in the regulation of pol I transcription. Pol II and pol III both utilise a transcription factor complex containing TBP and pol I is no exception. In this instance the complex is SL1, which in addition to TBP contains three TAFs (Comai et al., 1992). Each TAF can bind to the other two and TBP (Comai et al., 1994). The properties of the other pol I transcription factor have also been elucidated. *Xenopus* (x) UBF has a dimerisation domain at the amino terminus, and an acidic region at the carboxy-terminus. Between these two regions lie 5 high mobility group (HMG) boxes, which are responsible for binding DNA (McStay et al., 1991a). Human (h) UBF has a similar structure, but contains 6 HMG

boxes (Jantzen et al., 1992). In all mammalian systems studied to date two forms of UBF have been found, UBF1 and UBF2 (O'Mahony and Rothblum, 1991). The difference between these forms in humans, is a deletion of 35 amino acids from HMG box 2 of UBF2.

The level of pol I transcription is regulated throughout the cell cycle and also by various proteins. For example, the level of pol I transcription, like pol III, is high in S and G2 phase, low in mitosis, then recovers in G1. The repression of pol I transcription during mitosis is due to cdc2/cyclin B-directed phosphorylation of the TBP-containing complex SL1, which results in dissociation of SL1 from UBF (Heix et al., 1998; Kuhn et al., 1998). Moreover, UBF would also appear to be inactivated by phosphorylation during mitosis; during G1, UBF is reactivated by dephosphorylaiton, correlating with the onset of pol I transcription (Klein and Grummt, 1999). Alternatively, repression of pol I transcription can be mediated by cellular proteins such as p53 (Zhai and Comai, 2000) and RB (Cavanaugh et al., 1995; Voit et al., 1997).

In addition to being down-regulated, pol I transcription can also be enhanced, as illustrated upon the discovery that SV40 large T antigen binds to SL1 and is capable of activating pol I transcription (Zhai et al., 1997). Furthermore, it is believed that UBF can be phosphorylated by a SV40-associated kinase, giving rise to a stable association between SL1 and UBF, thereby promoting pol I transcription (Zhai and

Comai, 1999). Other kinases, such as the G1-specific kinases, cdk4-cyclin D1 and cdk2-cyclin E can also phosphorylate and activate UBF (Voit et al., 1999).

As mentioned previously, RB has been shown to be capable of repressing pol I transcription. The ability of RB to regulate pol I transcription was first demonstrated by Cavanaugh and co-workers (Cavanaugh et al., 1995) who showed that RB represses pol I transcription by interacting with UBF (Upstream Binding Factor). They showed that upon differentiation of the cell line U937, there is an accumulation of RB protein in the nucleoli, the site of pol I transcription, and a decrease in rDNA synthesis (Cavanaugh et al., 1995). These observations suggest a correlation between the localisation of RB to the nucleoli and the inhibition of pol I transcription. It was also shown that upon addition of GST-RB to an in vitro transcription system, pol I transcription decreased. In contrast, a GST-RB construct containing a point mutation at residue 706 did not decrease pol I transcription, therefore illustrating the requirement for an intact pocket domain for RB-mediated pol I repression (Cavanaugh et al., 1995). Collectively, these observations suggest that RB represses pol I transcription, and this effect is exerted by the ability of RB to target UBF.

The mechanism whereby RB represses pol I transcription was further examined, by Voit and co-workers (Voit et al., 1997). They confirmed the previous observations of Cavanaugh and co-workers of RB repressing pol I transcription, and the ability of UBF and RB to interact (Voit et al., 1997). The UBF-binding site in RB was examined by two independent means. Firstly it was shown that the addition of an E7 peptide did not relieve RB mediated pol I repression; the conclusion from this was

that the UBF-binding domain must reside outside the E7-binding domain. The second experiment employed various GST-RB fusion protein constructs; each was assayed for their ability to repress pol I transcription and interact with UBF. These investigations showed that whilst GST-RB (379-928) was able to repress pol I transcription and bind UBF, the ability of GST-RB (379-792) to do so was greatly reduced (Voit et al., 1997). From these observations, Voit and co-workers concluded that the UBF-binding domain in RB resides in the C-terminus of RB, namely residues 763-928 (Voit et al., 1997). This contrasted with the conclusion of Cavanaugh et al., (Cavanaugh et al., 1995) who had suggested that the pocket domain was important for binding to UBF.

Further investigations allowed the mechanism whereby RB represses pol I transcription to be determined. GST-pull down assays, immunoprecipitation data and bandshift assays identified the RB-binding domain in hUBF as being HMG boxes 1 and 2 (Voit et al., 1997). This work also demonstrated that the presence of RB does not disrupt the interaction between UBF and the other pol I transcription factor SL1, nor does it disrupt the interaction between UBF and pol I (Voit et al., 1997). Instead, the presence of RB greatly perturbs the ability of UBF to interact with DNA (Voit et al., 1997).

The work presented below confirms that there is an interaction between endogenous RB and endogenous UBF. The human (h) and *Xenopus* (x) UBF-binding domain in RB has been identified, as has the RB-binding domain in xUBF. Lastly, an interaction between the other pol I transcription factor SL1 and RB has been shown.

5.2 Results

5.2.1 Endogenous UBF interacts with endogenous RB

In order to investigate if RB and UBF physically associate with one another, immunoprecipitations were undertaken. Extract prepared from HeLa cells was immunoprecipitated using anti-RB antibodies immobilised on protein A beads. The protein retained on the beads was washed extensively, resolved by SDS-PAGE, and the presence of UBF was probed for by western analysis (fig. 5.1). The protein A alone did not bring down UBF (lane 2). However, the protein A beads coupled to anti-RB antibody did coprecipitate UBF (lane 3). These immunoprecipitation data suggest that endogenous RB and UBF interact with one another.

5.2.2 Identification of the UBF-binding domain in RB

Having shown that UBF binds RB, it was of interest to determine the sites of interaction. To do this, GST-RB (379-928), GST-RB (379-792), and GST-RB (763-928) were expressed in bacteria and immobilised on glutathione-agarose beads. These beads were incubated with reticulocyte lysate containing radiolabelled hUBF. After incubation, the beads were washed and resolved by SDS-PAGE and visualised by autoradiography (fig. 5.2.A). The autoradiogram shows that GST alone brings down a background amount of hUBF (lane 2), whereas the amount brought down by GST-RB (379-928) is much greater (lane 3). Both GST-RB (379-792) (lane 4) and

FIG. 5.1 Endogenous UBF interacts with endogenous RB.

HeLa cell extract (150μg) was immunoprecipitated with beads alone (lane 2) and anti-RB antibody G3-245 (lane 3); in addition, beads alone were incubated with buffer (lane 1). The precipitated material was resolved by SDS-7.8% polyacrylamide gel electrophoresis. The presence of UBF was visualised by western analysis by probing with anti-UBF antibody.

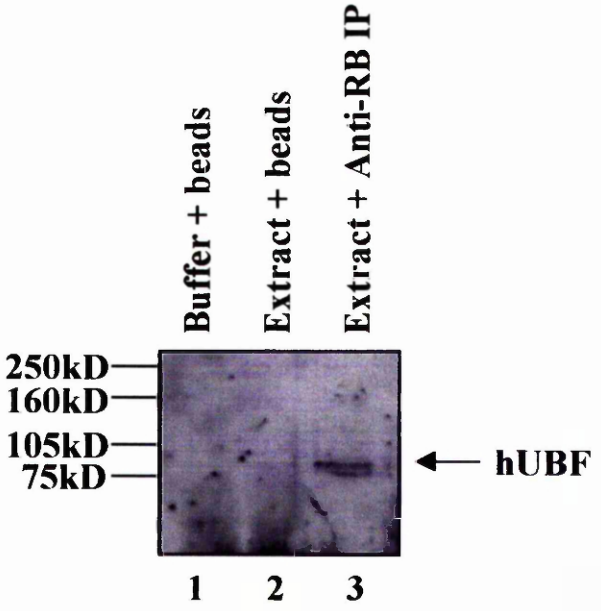
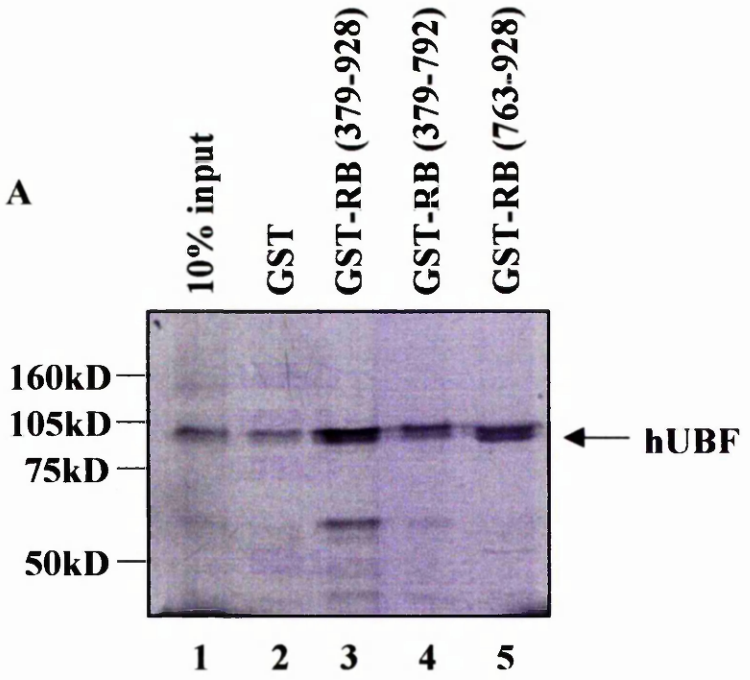
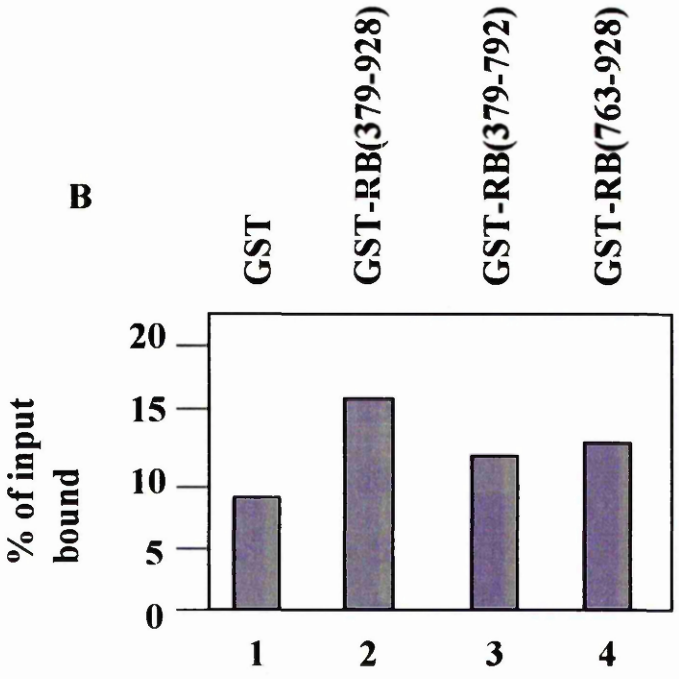

Buffer + beads
Extract + beads
Extract + Anti-RB IP
250kD
160kD
105kD
75kD
hUBF
1
2
3

FIG. 5.2 Identification of the hUBF-binding domain in RB.

(A) Reticulocyte lysate containing hUBF (15μl) was incubated with glutathione-agarose beads carrying equal amounts of GST (lane 2), GST-RB (379-928) (lane 3), GST-RB (379-792) (lane 4) or GST-RB (763-928) (lane 5). After extensive washing, the retained proteins were resolved on a SDS-7.8% polyacrylamide gel and visualised by autoradiography. Lane 1 shows 10% of the input reticulocyte lysate containing in vitro-translated hUBF.

(B) The results of the GST pull down assay were quantitated by a phosphorimager, and plotted as a percentage of input bound.


A
10% input
GST
GST-RB (379-928)
GST-RB (379-792)
GST-RB (763-928)
160kD
105kD
75kD
50kD
hUBF
1
2
3
4
5


B
GST
GST-RB(379-928)
GST-RB(379-792)
GST-RB(763-928)
% of input bound
20
15
10
5
0
1
2
3
4

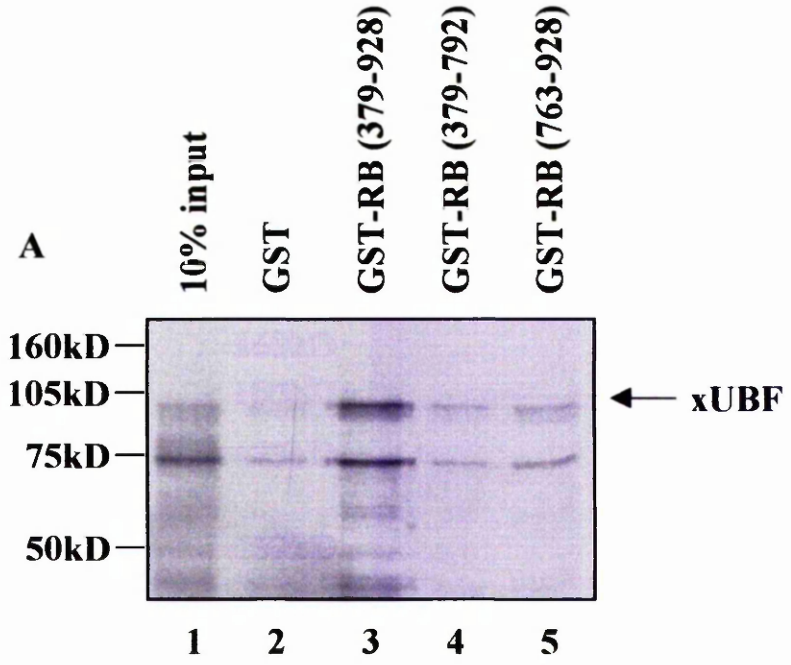
GST-RB (763-928) (lane 5) brought down more hUBF than GST alone, but less than GST-RB (379-928). These results were quantitated by phosphorimager and plotted on a graph as a percentage of input (fig. 5.2.B). From the graph it is evident that the binding domain for hUBF in RB resides in the large pocket domain.

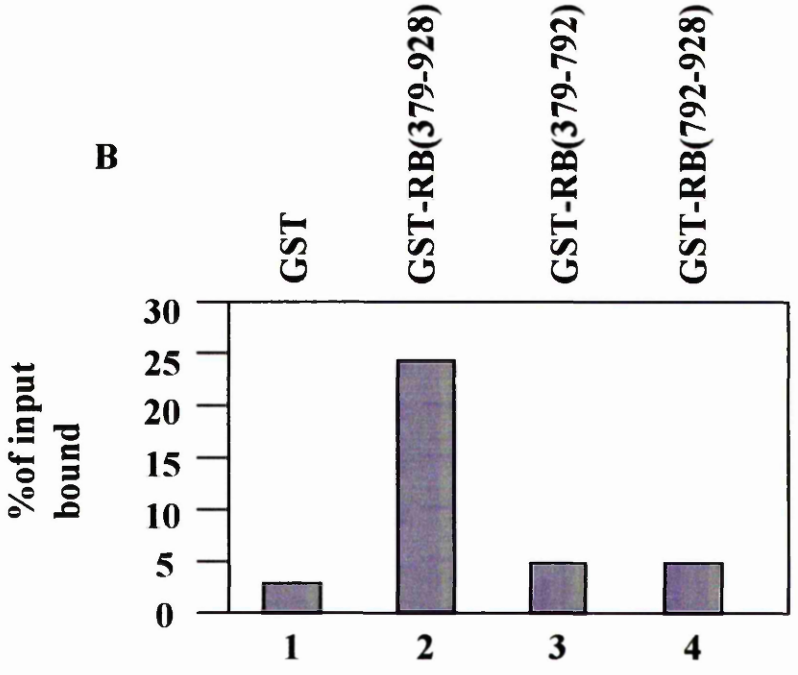
The UBF-binding domain in RB was further characterised by utilising the *Xenopus* protein xUBF. Once again a variety of GST fusion proteins (GST, GST-RB (379-928), GST-RB (379-792), and GST-RB (763-928)) were expressed and immobilised on glutathione-agarose beads. Each of these beads was incubated with reticuloctye lysate containing in vitro translated and radiolabelled xUBF. After incubation, the beads were washed and the retained protein resolved by SDS-PAGE and visualised by autoradiography (fig. 5.3.A). As can be seen, GST alone brings down very little xUBF (lane 2), whereas GST-RB (379-928) brings down a readily detectable amount of xUBF (lane 3). GST-RB (379-792) (lane 4) and GST-RB (763-928) (lane 5) bring down an amount of xUBF which is only slightly greater than that observed with GST alone (lane 2). The results were quantitated by phosphorimager and plotted on a graph as a percentage of input bound (fig. 5.3.B). It is clear that the large pocket domain of RB is also responsible for xUBF binding.

FIG. 5.3 Identification of the xUBF-binding domain in RB.

(A) Reticulocyte lysate containing xUBF was incubated with glutathione-agarose beads carrying equal amounts of GST (lane 2), GST-RB (379-928) (lane 3), GST-RB (379-792) (lane 4) or GST-RB (763-928) (lane 5). After extensive washing, the retained proteins were resolved on a SDS-7.8% polyacrylamide gel and visualised by autoradiography. Lane 1 shows 10% of the input reticulocyte lysate containing in vitro-translated xUBF.

(B) The results of the GST pull down assay were quantitated by a phosphorimager, and plotted as a percentage of input bound.


A
10% input
GST
GST-RB (379-928)
GST-RB (379-792)
GST-RB (763-928)
160kD
105kD
75kD
50kD
xUBF
1
2
3
4
5


B
GST
GST-RB(379-928)
GST-RB(379-792)
GST-RB(792-928)
%of input bound
30
25
20
15
10
5
0
1
2
3
4

5.2.3 The presence of a four amino acid deletion mutation in RB is sufficient to disrupt its interaction with UBF

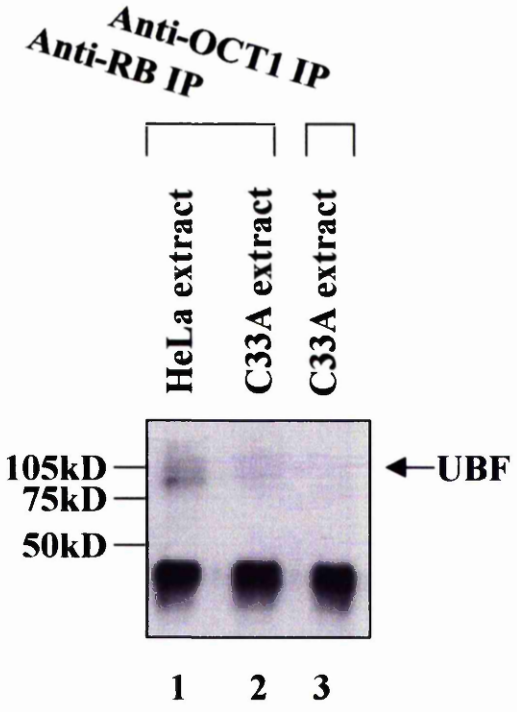
As UBF binding was found to require the large pocket of RB, it was decided to examine the effect of a four amino acid deletion within the pocket domain of RB. Cell extracts prepared from a cervical carcinoma cell line, C33A, which contains RB with a deletion of four amino acids (Scheffner et al., 1991), were immunoprecipitated with an anti-RB antibody alongside HeLa extract. The coprecipitate was resolved by SDS-PAGE and probed for UBF by western analysis (fig. 5.4.). The UBF coprecipitated from HeLa cell extract, which contains wild type RB, (lane 1), is a far greater amount than that coprecipitated from C33A cell containing a mutant form of RB (lane 2). The interaction between UBF and RB is specific, and not due to cross-reaction, as an irrelevant antibody used as a negative control, (anti-OCT-1, lane 3) did not coprecipitate UBF. These immunoprecipitation data suggest that the presence of a 4 amino acid deletion in the pocket domain of RB is sufficient to compromise the interaction between UBF and RB.

5.2.4 Identification of the RB-binding region in xUBF

The identification of the UBF-binding site in RB lead to investigating the reciprocal, namely the RB-binding domain in xUBF. GST alone and GST-RB (379-

FIG. 5.4 Endogenous UBF and RB interact in HeLa cell extract, but this interaction is compromised in C33A cell extract.

150μg HeLa cell extract (lane 1) and 150μg C33A cell extract (lanes 2 and 3), were immunoprecipitated with anti-RB antibody C-15 (lanes 1 and 2) or anti OCT-1 antibody C-21 (lane 3). The coprecipitated material was resolved by SDS-7.8% polyacrylamide gel electrophoresis and probed for UBF by western analysis. Lane 1 shows 10% of input.


Anti-OCT1 IP
Anti-RB IP
HeLa extract
C33A extract
C33A extract
105kD
75kD
50kD
UBF
1
2
3

928) were purified and immobilised on glutathione-agarose beads, and were incubated with reticulocyte lysate containing either wild type or truncated mutant forms of radiolabelled xUBF (see fig. 5.5 for a schematic representation of the xUBF truncated mutants). After incubation, the beads were washed, resolved by SDS-PAGE and visualised by autoradiography (fig. 5.6.A). As is shown by the autoradiogram, the ability of xUBFWT to bind RB is readily detectable (lane 3). In contrast, xUBFMT13 (lane 6) and xUBFMT14 (lane 9) bind to GST nearly as well as GST-RB. This suggests that HMG boxes 1 and 2, which are lacking in these truncated mutants respectively, may contribute to the specific recognition of RB. The ability of the xUBFMT15 (lane 9) to interact with RB has not been abolished; indeed, the interaction observed is comparable to that seen with xUBFWT. These observations suggest that the region deleted from this truncated mutant, namely HMG box 4, is not part of the RB-binding domain. The xUBF mutant xUBFMT18, which lacks all 3 aforementioned HMG boxes (lane 15), is not able to interact with RB. As the HMG box 4 was shown not to contribute to the RB-binding capabilities of xUBF, it can be concluded that HMG boxes 1 and 2 constitute the RB-binding domain in xUBF. These results were quantitated using a phosphorimager, and plotted on a graph as a fold increase compared to GST alone (fig. 5.6.B). As is evident from the graph, the ability of xUBFMT18 to interact with RB is abolished, whereas the abilities of xUBFMT13 and xUBFMT14 to bind RB selectivity were severely compromised. In contrast, the ability of xUBFMT15 to interact with RB is comparable to the wild type, therefore suggesting that the regions deleted from xUBFMT13, 14 and 18, namely HMG boxes 1 and 2, are responsible for binding RB.

FIG. 5.5 Schematic representation of xUBF truncated mutants.

An illustration of the various xUBF truncations used.


xUBFWT
HMG 1 HMG 2 HMG 4
Acid Tail


xUBFMT13


xUBFMT14


xUBFMT15


xUBFMT18

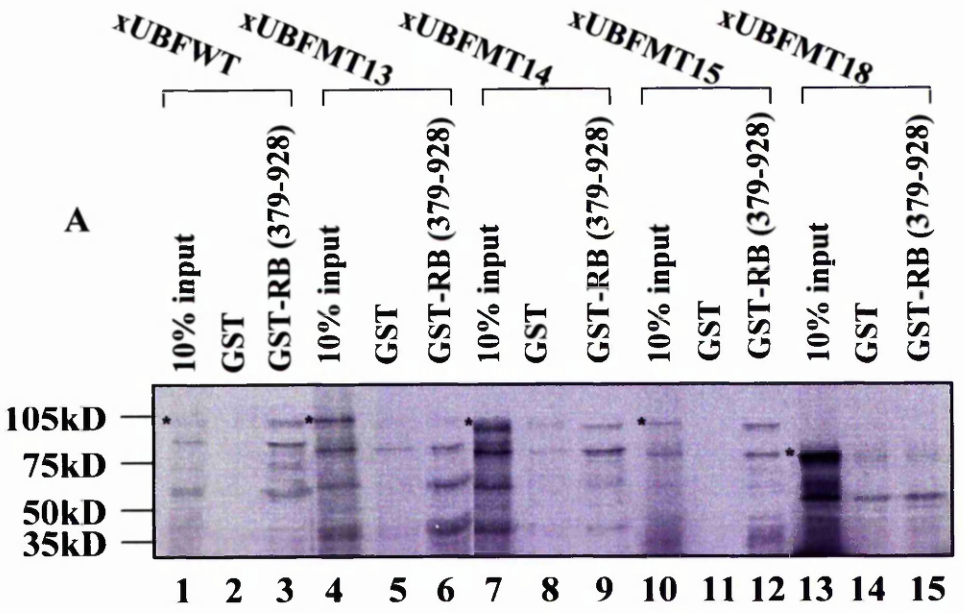

xUBFMT21

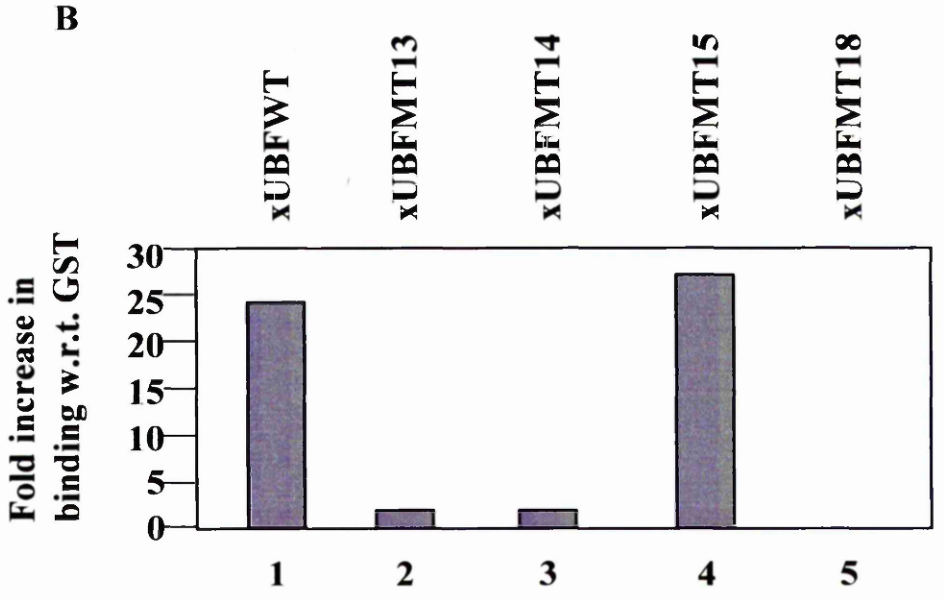

xUBFMTK

FIG. 5.6 Identification of the RB-binding region in xUBF.

(A) 15μl of reticulocyte lysate containing xUBFWT (lanes 2 and 3), xUBFMT13 (lanes 5 and 6), xUBFMT14 (lanes 8 and 9), xUBFMT15 (lanes 11 and 12) or xUBFMT18 (lanes 14 and 15) was incubated with glutathione-agarose beads carrying equal amounts of GST (lanes 2, 5, 8, 11 and 14) or GST-RB (379-928) (lanes 3, 6, 9, 12 and 15). After extensive washing, the retained proteins were resolved on a SDS-7.8% polyacrylamide gel and visualised by autoradiography. Lanes 1, 4, 7, 10 and 13 show 10% of input.

(B) The results of the GST pull down assay were quantitated by a phosphorimager, and plotted as a fold increase of input bound with respect to GST alone.


A
xUBFWT
xUBFMT13
xUBFMT14
xUBFMT15
xUBFMT18
10% input
GST
GST-RB (379-928)
105kD
75kD
50kD
35kD
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

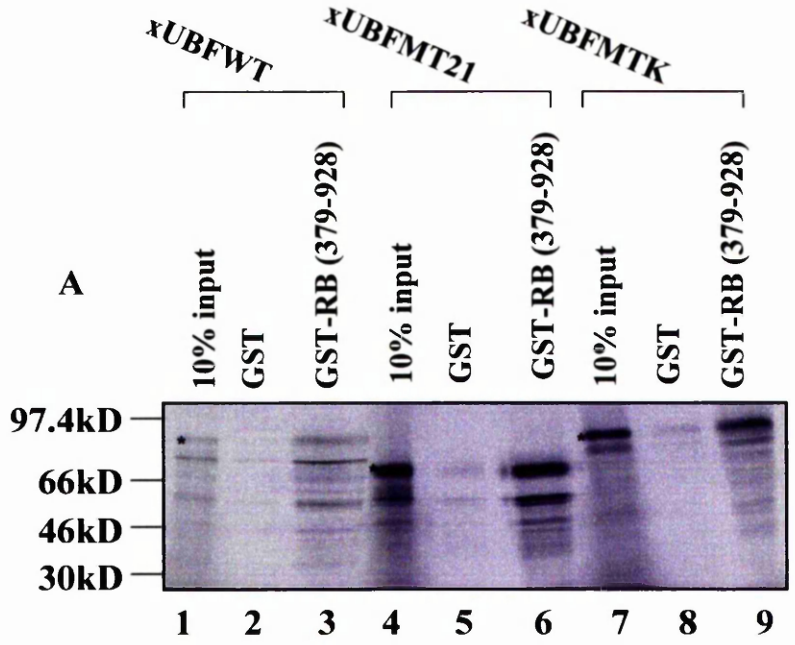

B
xUBFWT
xUBFMT13
xUBFMT14
xUBFMT15
xUBFMT18
Fold increase in binding w.r.t. GST
30
25
20
15
10
5
0
1 2 3 4 5

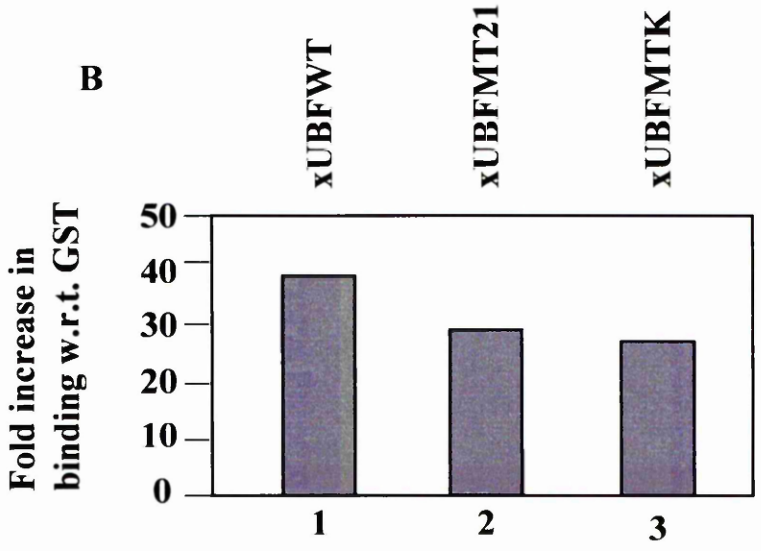
The xUBF mutants discussed above all contain mutations within the N-terminal portion of the protein. The contribution of the C-terminal domain towards the ability of xUBF to bind RB was also investigated by GST pull down assays. GST alone and GST-RB (379-928) were purified and immobilised on glutathione-agarose beads and incubated with various xUBF C-terminal truncations. After incubation, the proteins retained on the beads were resolved by SDS-PAGE and visualised by autoradiography (fig. 5.7.A). As shown, the ability of xUBFMT21 (lane 6) and xUBFMTK (lane 9) to bind RB is comparable to that observed with xUBFWT (lane 3). This shows that the ability of these truncations to interact with RB is not seriously compromised by their deleted domains, which suggests that the region between the HMG boxes and the acidic tail, as well as the acidic tail itself, do not contribute much to the interaction between xUBF and RB. These results were quantitated using the phosphorimager, and the results plotted as a fold increase relative to the level observed for GST alone (fig. 5.7.B). As is apparent from the graph, the ability of these xUBF truncated mutants to interact with RB is only slightly compromised, therefore suggesting that these deleted domains do not contribute much to the interaction between xUBF and RB. Collectively, these GST pull down assays demonstrate that the domains in xUBF which are responsible for the interaction between xUBF and RB are the HMG boxes 1 and 2 located in the N-terminal half of xUBF. In contrast, the ability of xUBFMT15 to interact with RB is comparable to the wild type, therefore suggesting that the regions deleted from xUBFMT13, 14 and 18, namely HMG boxes 1 and 2, are responsible for binding RB.

FIG. 5.7 Identification of the RB-binding region in C-terminal truncated xUBF.

(A) 15µl of reticulocyte lysate containing xUBFWT (lanes 2 and 3), xUBFMT21 (lanes 5 and 6), or xUBFMTK (lanes 8 and 9) was incubated with glutathione-agarose beads carrying equal amounts of GST (lanes 2, 5 and 8) or GST-RB (379-928) (lanes 3, 6 and 9). After extensive washing, the retained proteins were resolved on a SDS-7.8% polyacrylamide gel and visualised by autoradiography. Lanes 1, 4 and 7 show 10% of the input reticulocyte lysate containing in vitro-translated xUBFs.

(B) The results of the GST pull down assay were quantitated by a phosphorimager and plotted as a fold increase of input bound with respect to GST alone.


A
xUBFWT
xUBFMT21
xUBFMTK
10% input
GST
GST-RB (379-928)
10% input
GST
GST-RB (379-928)
10% input
GST
GST-RB (379-928)
97.4kD
66kD
46kD
30kD
1 2 3 4 5 6 7 8 9


B
xUBFWT
xUBFMT21
xUBFMTK
Fold increase in binding w.r.t. GST
50
40
30
20
10
0
1
2
3

5.2.5 Endogenous SLI interacts with endogenous RB

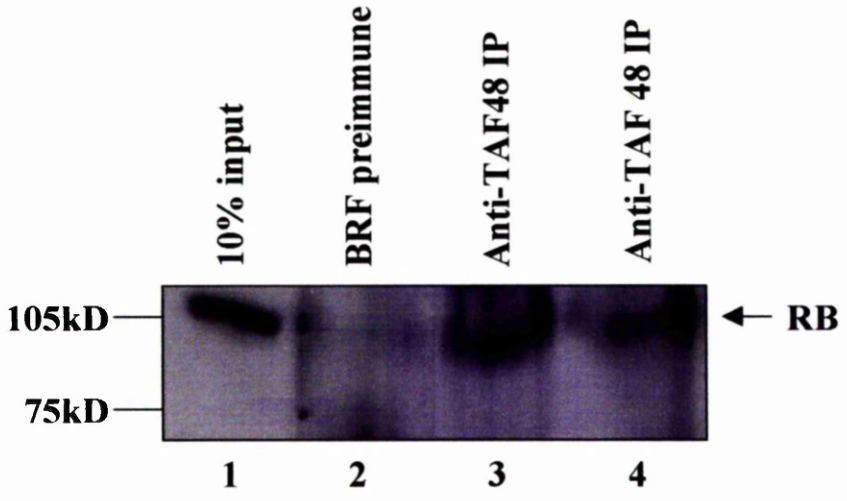
The tumour suppressor protein RB has been shown to interact with the pol I transcription factor UBF. Hence it was of interest to determine if RB can also be found associated with the other pol I transcription factor SL1. The potential physical association between RB and SL1 was investigated by immunoprecipitations. Extracts prepared from HeLa cells were incubated with anti-SL1 antibodies immobilised on protein A beads. After washing, the proteins retained on the beads were resolved by SDS-PAGE and the presence of RB determined by western analysis (fig 5.8). It can be observed that the anti-TAF48 antibodies coprecipitated RB (lanes 3 and 4). These interactions are specific, as the negative control did not coprecipitate RB (lane 2). These immunoprecipitation data suggest that cellular SL1 can be found in complex with endogenous RB.

5.3 Discussion

Pol I transciption can be regulated in a positive or negative fashion by a variety of means. Of particular interest with regard to these studies are the effects of RB upon pol I transcription, and the proteins which it targets in order to produce these effects.

FIG. 5.8 Endogenous SL1 interacts with endogenous RB.

HeLa cell extract (150μg) was immunoprecipitated with the anti-TAF48 antibody C-19 (lane 3), the anti-TAF48 antibody M-19 (lane 4), and the BRF preimmune (lane 2). The precipitated material was resolved by SDS-7.8% polyacrylamide gel electrophoresis. The presence of RB was visualised by western analysis by probing with anti-RB antibody IF8. Lane 1 shows 10% of HeLa input.


10% input
BRF preimmune
Anti-TAF48 IP
Anti-TAF 48 IP
105kD
75kD
RB
1
2
3
4

As shown above and by work undertaken by Cavanaugh and colleagues using immunoprecipitation analyses, endogenous UBF and RB can interact (Cavanaugh et al., 1995). With regard to the ability of an E7 peptide to disrupt the interaction between UBF and RB, conflicting data have been reported. Cavanaugh et. al. demonstrated that the addition of an E7 peptide greatly reduced the ability of UBF to interact with RB in immunoprecipitation analysis and GST-pull down assays. However, Voit et. al. reported that in a reconstituted transcription system the E7 peptide did not relieve RB-mediated repression, implying that the addition of this peptide did not alleviate the interaction between RB and UBF (Voit et al., 1997). The E7 protein binds to a domain in RB referred to as the small pocket; as its addition did not disrupt the UBF/RB interaction, they concluded that the UBF-binding domain in RB resides outside the small pocket, namely towards the C-terminus of RB (Voit et al., 1997).

In order to define the UBF-binding domain within RB, a variety of RB constructs were utilised for in vitro interaction analysis. These studies suggest that the UBF-binding domain in RB is the large pocket domain for both hUBF and xUBF.

The identification of the UBF-binding domain in RB is of interest as it may allow for a correlation between the ability of RB to repress pol I transcription by interacting with UBF, and the ability of RB to prevent cell growth. The sequestration of E2F by RB makes E2F unavailable to promote the expression of genes required for DNA replication (e.g. dihydrofolate reductase and thymidine kinase) and cell cycle regulation, (e.g. cyclin E, cyclin A, cdc2, p107 and E2F). This was believed to be the

mechanism of RB-mediated cell cycle arrest (Weinberg, 1995). This model, however, does not explain the increased biosynthesis observed in tumours that lack functional RB. More recently, it has been proposed that RB functions not only by controlling the expression of genes required for DNA synthesis and cell cycle regulation, but also by controlling the expression of genes required for promoting cell growth (Nasmyth, 1996). The designation of the UBF-binding domain in RB by Voit et al (1997) is inconsistent with a correlation between the ability of RB to arrest cell growth and its ability to regulate rRNA synthesis. The data presented by Cavanaugh et al (1995) defining the large pocket domain as the site recognised by UBF, and by Hannan et al. (2000a) showing a functional A/B pocket is required for the RB/UBF interaction, provide a direct correlation between the ability of RB to bind UBF and repress pol I transcription, and its ability to inhibit cell growth, inasmuch as both functions reside in the large domain of RB.

To delineate the regions in xUBF responsible for binding RB, a variety of xUBF constructs were utilised (fig. 5.1) (McStay et al., 1991a). Many proteins which bind RB have been shown to bear the LXCXE motif, such as adenovirus E1A (Whyte et al., 1988), SV40 large T antigen (DeCaprio et al., 1988), and HPV 16 E7 (Dyson et al., 1989). Transcription factors that bind RB do not necessarily display this motif. For example, the pol II transcription factor E2F does not bear an LXCXE motif (La Thangue, 1994), neither does the pol III transcription factor BRF (Larminie and White, 1998). Nevertheless, the first step in elucidating the RB-binding domain in UBF was to determine if UBF had a LXCXE motif. In hUBF, residues 307 to 311, which reside in HMG box 3, contain a LXCXE motif (McStay et al., 1991a). In

xUBF residues 307 to 311, which are encompassed by HMG domain 4, contain an altered LXCXE motif, in which the L residue is replaced by M (McStay et al., 1991a). It was previously reported that the RB-binding domain in hUBF consists of HMG boxes 1 and 2 (Voit et al., 1997). As shown above, not only does xUBF bind RB, but also the binding domain for RB in xUBF mirrors that observed for hUBF, namely HMG boxes 1 and 2. Neither of these HMG boxes in hUBF or xUBF include the LXCXE motif, therefore suggesting that this motif present in these proteins does not contribute to the interaction with RB.

The region of UBF, which is sufficient for DNA sequence specificity is HMG box 1 (Jantzen et al., 1992). It is therefore possible that RB represses pol I transcription by binding the HMG boxes 1 and 2 and thereby preventing them from binding DNA. The addition of RB has been shown to prevent UBF from binding cruciform DNA in bandshift assays, and ribosomal DNA in DNase footprinting experiments (Voit et al., 1997). These experiments demonstrate that the binding of RB to UBF prevents UBF from associating with DNA. Indeed, the addition of RB did not disrupt the interaction between UBF and pol I; it also did not affect the interaction between UBF and a subunit of SL1, TAF95 (Voit et al., 1997). The SL1 binding site in UBF has been mapped to multiple sites in the C-terminal half of UBF (Jantzen et al., 1992), which is physically distinct from the RB-binding domain. Hence, it is feasible that the interaction between UBF and SL1 is not disrupted by RB. As discussed above, the TAF48 subunit of SL1 was able to coprecipitate with RB; whether this was via association with UBF, or due to the ability of RB to target SL1 independently from UBF cannot be concluded from this experiment. The available

data therefore suggest that RB represses pol I transcription by binding UBF and preventing it from binding DNA, but without disrupting interactions with the other pol I transcription factor SL1 or pol I itself. This mechanism of RB mediated repression of transcription is different from that observed for pol III, in which RB targets TFIIIB and prevents it from binding other transcription factors (Sutcliffe et al., 2000). It also differs from that observed for pol II, wherein RB binds E2F, blocking its interaction with basal transcription factors (Ross et al, 1999).

It would be of interest to determine if RB could target SL1 independently of UBF, and the consequences of such an interaction for pol I transcription. This might lead to an alternative model regarding RB-mediated regulation of pol I activity.

Chapter 6

The pocket proteins p107 and p130 interact with both BRF and UBF

6 The pocket proteins p107 and p130 interact with both the BRF and UBF

6 Introduction

The product of the *Rb* susceptibility gene, RB, belongs to a family of proteins called the pocket proteins. The two other pocket proteins, p107 and p130, are related in sequence and function to RB (Ewen et al., 1991; Hannon et al., 1993; Li et al., 1993; Mayol et al., 1993; Zhu et al., 1993).

p107 and p130 are 30-35 % identical to RB, but show greater homology between each other, namely 50% amino acid identity (Grana et al., 1998; Mulligan and Jacks, 1998). These proteins display similar functions to RB, some are identical, whilst others appear to be related. Like RB, p107 and p130 are able to bind the products of DNA tumour viruses (Dyson, 1998; Herwig and Strauss, 1997; Mulligan and Jacks, 1998; Taya, 1997), and mediate arrest of cell cycle progression upon overexpression in particular cell types (Claudio et al., 1994; Qin et al., 1992; Zhu et al., 1993). They are also able to complex with E2F. However, whereas RB-E2F complexes are found in G1 (Hiebert et al., 1992), complex formation between E2F and the remaining pocket proteins occurs at other points during the cell cycle. For example, the binding of p107 to E2F is found at the G1-S boundary (Schwarz et al., 1993), whilst p130 coupled to E2F is detected in G0-G1 (Cobrinik et al., 1993). In addition, the E2F family member for which p107 and p130 have a preference to bind is different from that of RB. p107 and p130 prefer E2F4 and 5 (Beijersbergen et al., 1994; Ginsberg et al., 1994; Hijmans et al., 1995; Vairo et al., 1995), whilst RB

prefers E2F1, 2 and 3 (Lees et al., 1993). Another interesting difference is the composition of the pocket protein-E2F complex; RB complexes to E2F alone, in contrast E2F4 and 5 complex to p107 and p130 in the presence of cyclin A-cdk2 or cyclin E-cdk2 (Cobrinik et al., 1993; Li et al., 1993; Shirodkar et al., 1992). Hence, whilst p107 and p130 are able to mimic some functions of RB, such as binding E2F family members and cell cycle arrest, they clearly also display some peculiarities of function.

There is considerable redundancy between the various pocket proteins and this is well illustrated by studies characterising the phenotype of knock-out mice. Animals which lack either p107 or p130 develop normally, but animals which lack both of these proteins die within hours of being born (Cobrinik et al., 1996). In contrast, animals lacking RB die at midgestation, displaying defects in neurogenesis and erythropoiesis (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Thus it would appear that p107 and p130 are not able to undertake all the functions performed by RB. Furthermore, whilst RB has been shown to be a tumour suppressor suffering from mutational inactivation in a vast cross-section of tumours, the same can not be said for the other pocket proteins (Weinberg, 1995). No examples of naturally occurring mutations of p107 have been found in human cancers. In the instance of p130, the chromosome area to which it is mapped is reported to be deleted in a variety of cancers. Moreover, one mutant form of p130 has been identified in a small-cell lung cancer cell line (Helin et al., 1997).

As p107 and p130 are able to undertake some of the functions of RB, it was decided to determine if they were also able to assume other activities, such as binding the factors RB targets in order to repress pol I and pol III transcription. The work presented below demonstrates that like RB, p107 and p130 are able to interact with BRF, and that this interaction occurs at physiological ratios. It also shows that p107 and p130, as has been demonstrated for RB, are able to interact with the pol I transcription factor UBF.

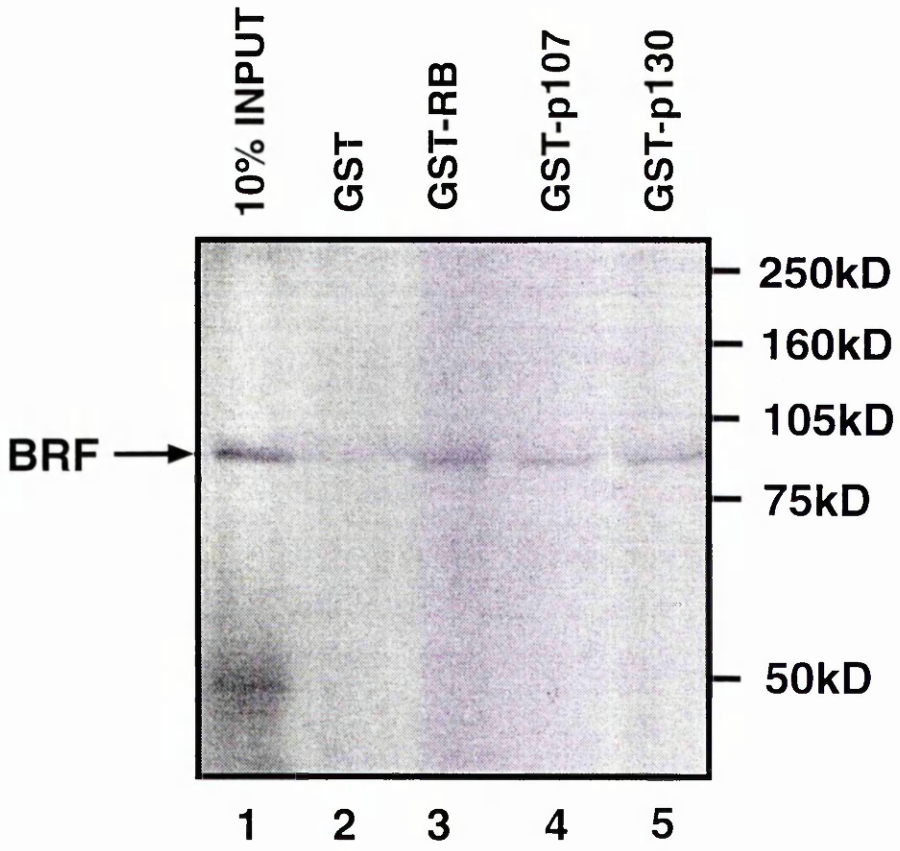
6.2 Results

6.2.1 Recombinant p107 and p130 interact with the BRF subunit of TFIIIB

Since RB has been shown to interact with the BRF subunit of TFIIIB, it was of interest to determine if the other members of the pocket protein family, p107 and p130, could do the same. In order to investigate if recombinant p107 and p130 could interact with BRF, GST alone, GST-RB (379-928), GST-p107 and GST-p130 were purified and immobilised on glutathione-agarose beads. Reticulocyte lysate expressing radiolabelled BRF was added to each of these beads and the mixtures incubated. After incubation, the beads were washed, and the protein retained by the beads was visualised by SDS-PAGE and autoradiography (fig. 6.1). As can be seen from the autoradiogram, both GST-p107 (lane 4) and GST-p130 (lane 5) retained BRF with an efficiency comparable with that observed for GST-RB (lane 3). GST alone (lane 2) retained only a small background amount of BRF. Hence, the retention

FIG. 6.1 Recombinant p107 and p130 interact with the BRF subunit of TFIIIB.

Reticulocyte lysate containing radiolabelled BRF (15μl) was incubated in the presence of glutathione beads carrying equal amounts of GST alone (lane 2) GST-RB (379-928) (lane 3) GST-107 (lane 4) and GST-p130 (lane 5). Proteins retained after extensive washing were resolved on a SDS-7.8% polyacrylamide gel and visualised by autoradiography. Lane 1 shows 10% of the input of reticulocyte lysate containing in vitro-translated BRF.


10% INPUT
GST
GST-RB
GST-p107
GST-p130
BRF
250kD
160kD
105kD
75kD
50kD
1
2
3
4
5

of BRF is specific to the pocket protein portion of the fusion protein. These data suggest that both p107 and p130 can interact with BRF.

6.2.2 Endogenous p107 and p130 can interact with the BRF subunit of TFIIIB

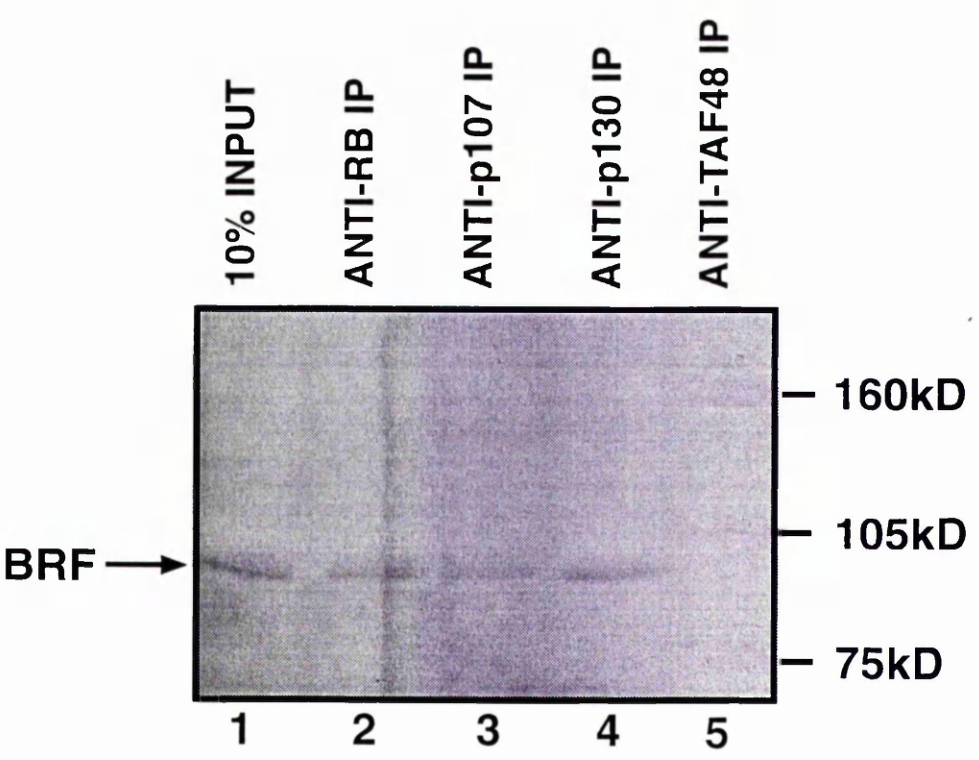
Immunoprecipitations were undertaken in order to determine if endogenous p107 and p130 could interact with BRF. BRF expressed in vitro using reticulocyte lysate was mixed with HeLa cell extract and the mixture immunoprecipitated with a variety of antibodies immobilised on protein A beads. The precipitated material was examined for the presence of BRF by SDS-PAGE and autoradiography (fig. 6.2). As is shown, the antiserum specific for RB (lane 2), p107 (lane 3) and p130 (lane 4) each coprecipitated readily detectable amounts of BRF. This effect was demonstrated to be specific, as the control antiserum against TAF48 (lane 5) was not observed to coprecipitate BRF.

6.2.3 Endogenous p107 and p130 interact with endogenous BRF

To test for association of p107 and p130 with TFIIIB at physiological ratios, an immunoprecipitation was undertaken to determine if endogenous p107 and p130 could interact with endogenous BRF. HeLa cell extract was subjected to immunoprecipitation using a range of antibodies immobilised on protein A beads. The coprecipitated material was resolved by SDS-PAGE and the presence of BRF

FIG. 6.2 Endogenous p107 and p130 interact with the BRF subunit of TFIIIB.

Reticulocyte lysate (15μl) containing in vitro-translated BRF was mixed with HeLa cell extract (150μg) and the mixture was immunoprecipitated with anti-RB antibody C-15 (lane 2), anti-p107 antibody C-18 (lane 3), anti-p130 antibody C-20 (lane 4) and anti-TAF48 antibody C-19 (lane 5). The material retained after extensive washing was resolved by SDS-7.8% polyacrylamide gel electrophoresis and visualised by autoradiography. Lane 1 shows 10% input of the reticulocyte lysate containing radiolabelled BRF.


10% INPUT
ANTI-RB IP
ANTI-p107 IP
ANTI-p130 IP
ANTI-TAF48 IP
BRF
160kD
105kD
75kD
1
2
3
4
5

determined by western analysis (fig. 6.3.A). This figure demonstrates that antibodies raised against either RB (lane 3), p107 (lane 1) or p130 (lane 5) were all able to coprecipitate BRF. Once again, the antibody against the TAF48 subunit of SL1 (lanes 2 and 4) was unable to coprecipitate BRF, thus illustrating the specificity of the interaction. From these immunoprecipitation data it can be concluded that endogenous p107 and p130 interact with endogenous BRF.

The reciprocal experiments were also undertaken, immunoprecipitating with antiserum against BRF or the corresponding preimmune as a negative control. The coprecipitated material was analysed for the presence of pocket proteins by western blotting and probing for p107 (fig. 6.3.B) or p130 (fig. 6.3.C). As shown, both p107 and p130 are coprecipitated with the BRF antiserum, but not with the preimmune control. These immunoprecipitation data confirm the previous observations that p107 and p130 form a physical association with BRF at physiological ratios.

6.2.4 Recombinant p130 interacts with hUBF

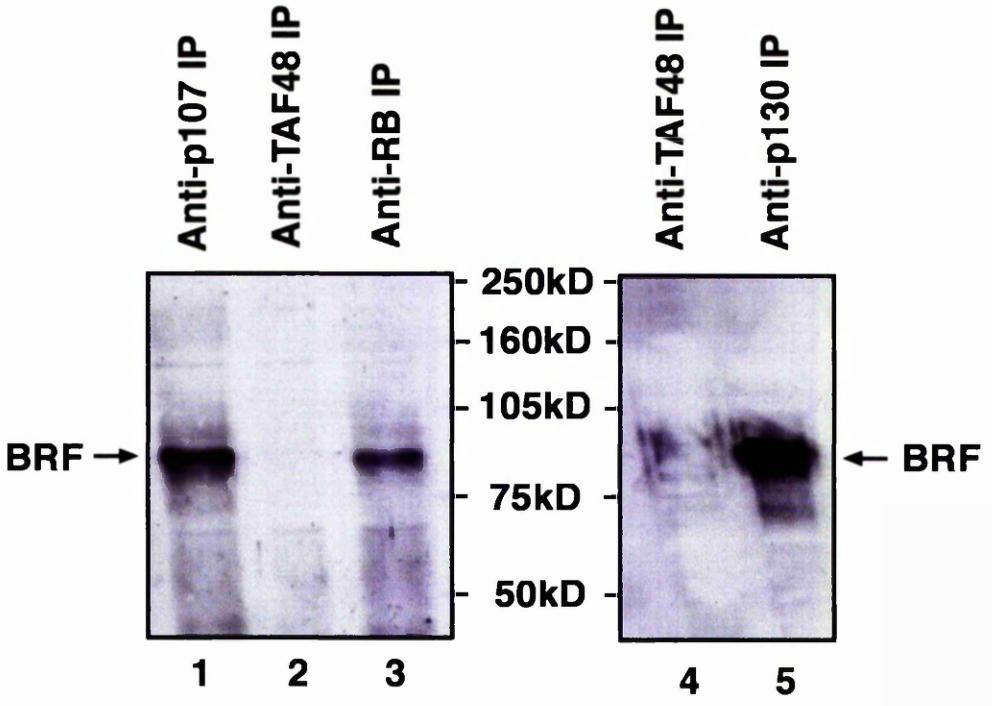
It has been demonstrated that the two pocket proteins p107 and p130 are able, like RB, to interact with the BRF subunit of TFIIIB. Hence it was decided to determine if p107 and p130 were also able to undertake another characteristic of RB, namely to interact with the pol I transcription factor UBF. The initial step in these investigations was to determine if recombinant p130 could interact with hUBF. This was undertaken by purifying GST alone, GST-RB (379-928) and GST-p130 and

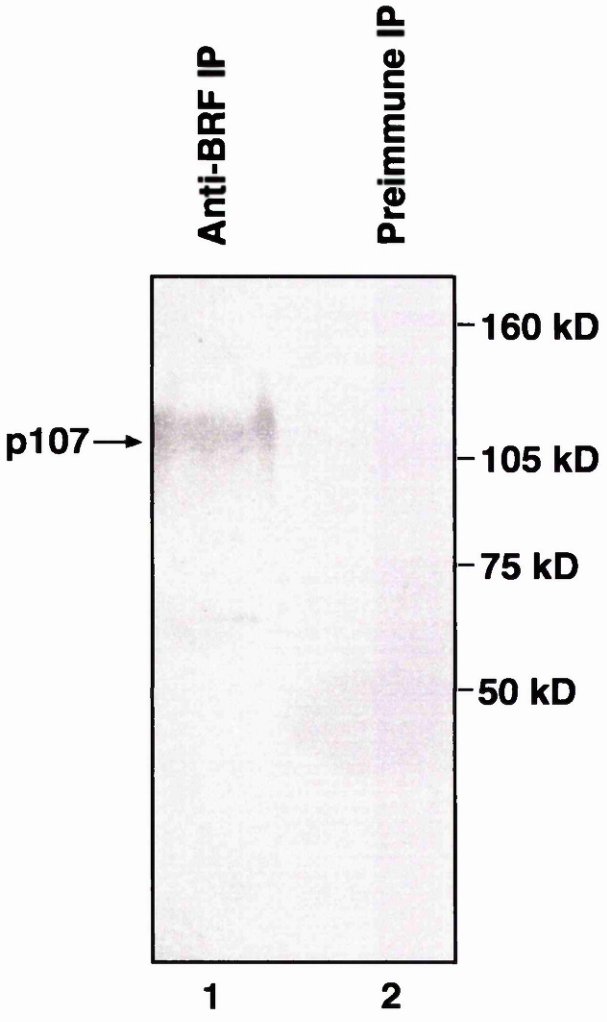
FIG. 6.3 Endogenous p107 and p130 interact with endogenous BRF.

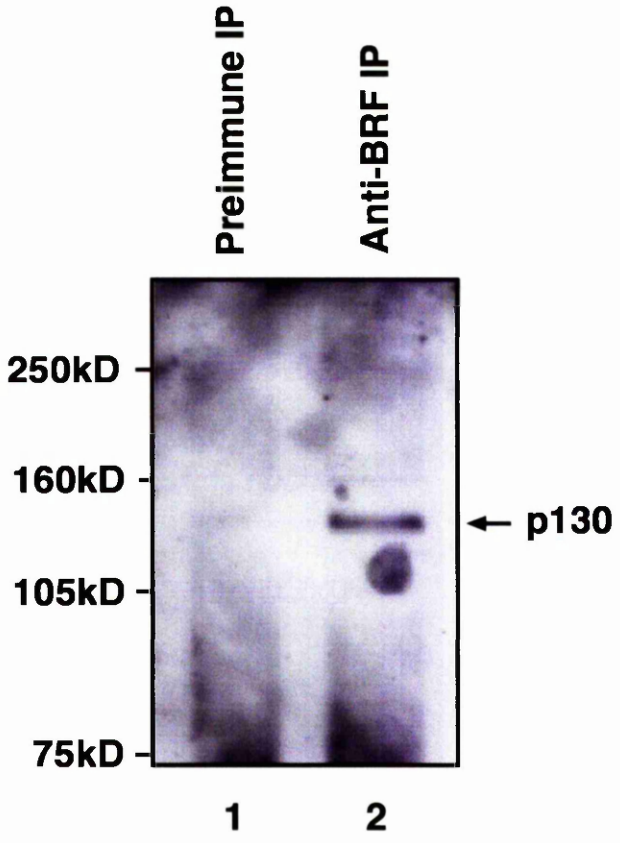
(A) HeLa cell extract (150 μg) was immunoprecipitated by anti-p107 antibody (lane 1), anti-RB antibody (lane 3), anti-p130 antibody (lane 5) and anti-TAF48 antibody (lanes 2 and 4). The coprecipitated material was resolved by SDS-7.8% polyacrylamide gel electrophoresis and probed for BRF by western blotting using the anti-BRF antiserum 128.

(B) HeLa cell extract (150 μg) was immunoprecipitated with anti-BRF antiserum 128 (lane 1) or preimmune serum (lane 2). The coprecipitated material was resolved on a SDS-7.8% polyacrylamide gel and analysed by western blotting with the anti-p107 antibody SD9.

(C) HeLa cell extract (150 μg) was immunoprecipitated with anti-BRF antiserum 128 (lane 2) or preimmune serum (lane 1). The coprecipitated material was resolved on a SDS-7.8% polyacrylamide gel and analysed by western blotting with the anti-Rb2 antibody against p130.


Anti-p107 IP
Anti-TAF48 IP
Anti-RB IP
Anti-TAF48 IP
Anti-p130 IP
250kD
160kD
105kD
75kD
50kD
BRF
BRF
1
2
3
4
5


Anti-BRF IP
Preimmune IP
160 kD
105 kD
75 kD
50 kD
p107
1
2


Preimmune IP
Anti-BRF IP
250kD
160kD
105kD
75kD
p130
1
2

immobilising them on glutathione-agarose beads. These beads were incubated with radiolabelled hUBF expressed in vitro by reticulocyte lysate. After incubation, the beads were washed and the retained protein analysed by SDS-PAGE and autoradiography (fig. 6.4.). As is illustrated, GST-RB retains readily detectable amounts of hUBF (lane 3). GST-p130 does not retain as much hUBF as GST-RB (compare lanes 4 and 3), although it does retain more than GST alone (compare lanes 4 and 2). This pull down suggests that whilst GST-p130 may not retain hUBF to the same efficiency as GST-RB, there does appear to be an interaction which warrants further investigation.

6.2.5 Endogenous p107 and p130 interact with endogenous UBF

Immunoprecipitations were undertaken to establish an interaction between endogenous p130 and UBF. HeLa cell extract was immunoprecipitated with a range of antibodies immobilised on protein A beads. The coprecipitated material was examined for the presence of UBF by western blotting and probing for UBF (fig. 6.5). The antisera against p130 (lane 5) and RB (lane 4) coprecipitate UBF, therefore illustrating a physical association between p130 and UBF at physiological ratios. The interaction was shown to be specific, as an irrelevant antiserum against the transcription factor OCT-1 (lane 6) was not able to precipitate UBF.

The ability of p107 to interact with UBF was also investigated by immunoprecipitating HeLa cell extract with a variety of antibodies immobilised on

FIG. 6.4 Recombinant p130 interacts with the pol I transcription factor UBF.

Reticulocyte lysate containing radiolabelled UBF was incubated in the presence of glutathione beads carrying equal amounts of GST alone (lane 2), GST-RB (379-928) (lane 3) and GST-p130 (lane 4). Proteins retained after extensive washing were resolved by SDS-7.8% polyacrylamide gel electrophoresis and visualised by autoradiography. Lane 1 shows 10% of the input of reticulocyte lysate containing in vitro-translated UBF.

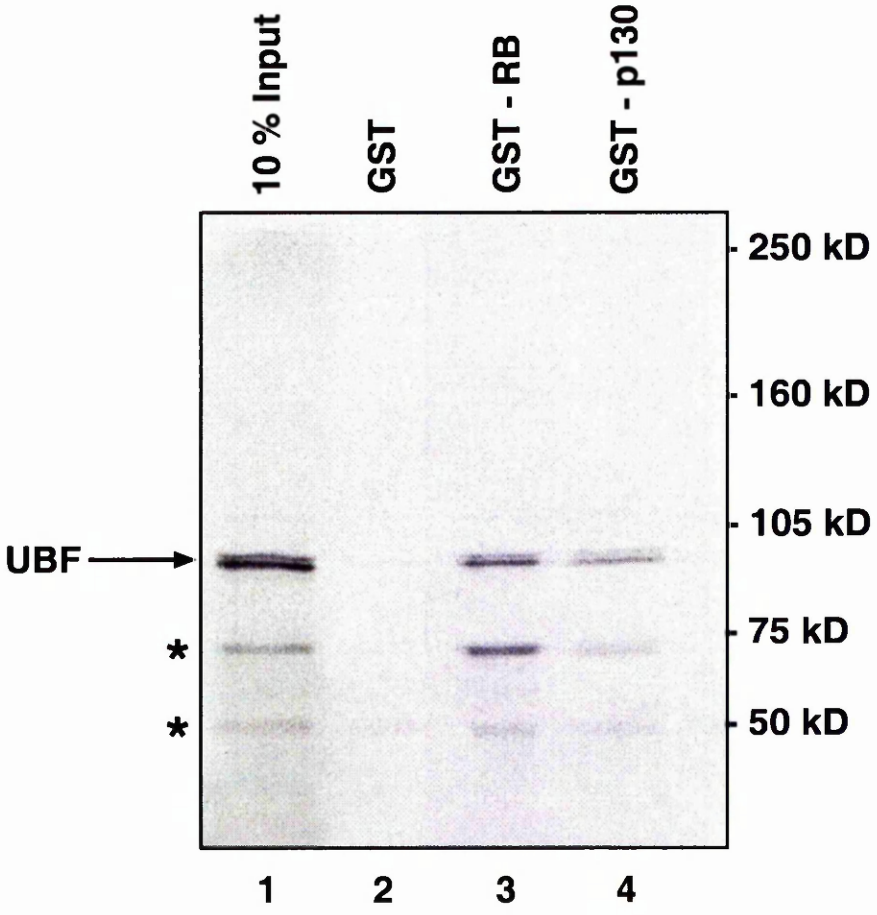
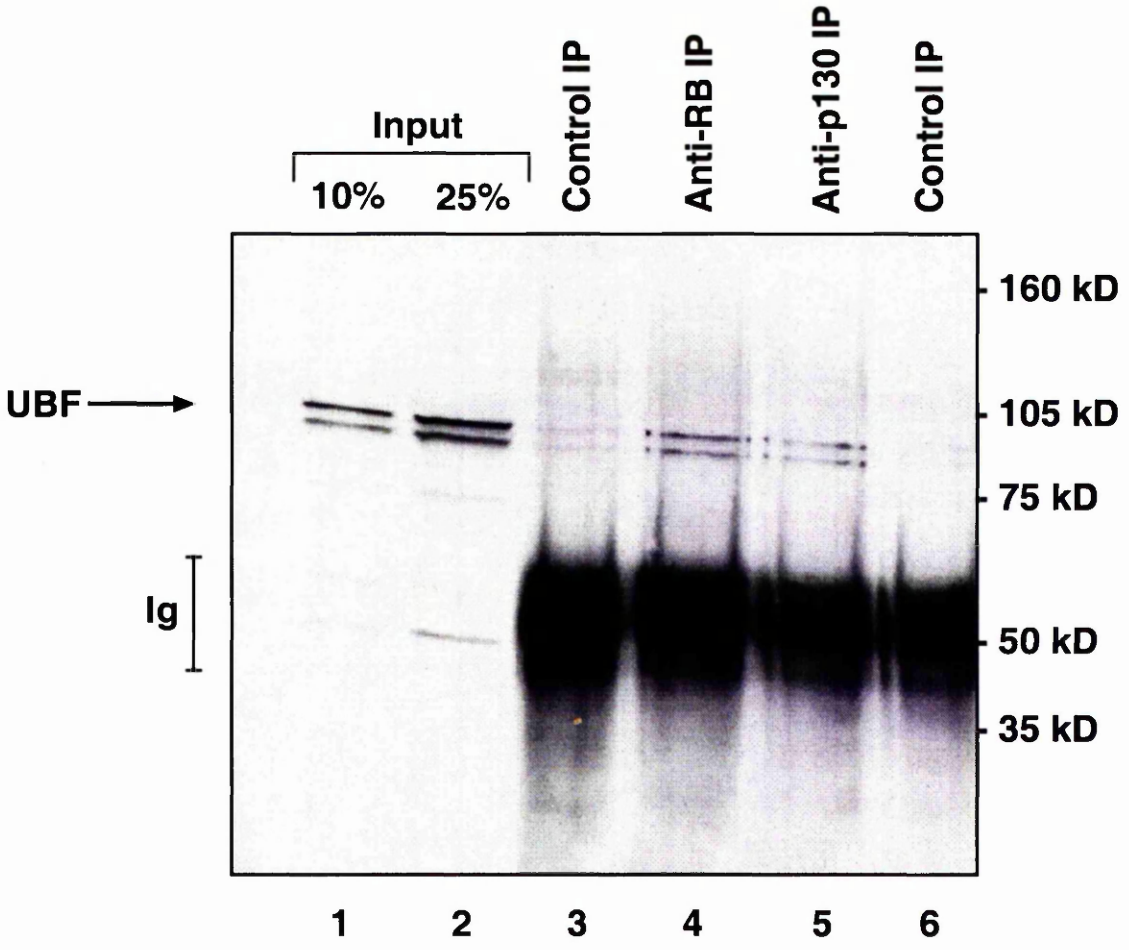

10 % Input
GST
GST - RB
GST - p130
250 kD
160 kD
105 kD
75 kD
50 kD
UBF
*
*
1
2
3
4

FIG. 6.5 Endogenous p130 interacts with endogenous UBF.

HeLa cell extract (150 μg) was immunoprecipitated using anti-OCT-1 antibody C-21 (lane 6), anti-RB antibody C-15 (lane 4), anti-p130 antibody C-20 (lane 5) or beads alone (lane 3). The coprecipitated material was resolved on a SDS-7.8% polyacrylamide gel and probed for UBF by western blotting using the anti-UBF antiserum 131. Lanes 1 and 2 show 10% and 25%, respectively, of the input of HeLa cell extract.


Input
10%
25%
Control IP
Anti-RB IP
Anti-p130 IP
Control IP
UBF
Ig
160 kD
105 kD
75 kD
50 kD
35 kD
1
2
3
4
5
6

protein A beads. The coprecipitated material was analysed for UBF by western blotting (fig. 6.6). The antiserum against p107 (lane 3) coprecipitates UBF, therefore suggesting that at physiological ratios p107 and UBF interact with one another. This interaction is specific, as antiserum against OCT-1 (lane 4) does not coprecipitate UBF.

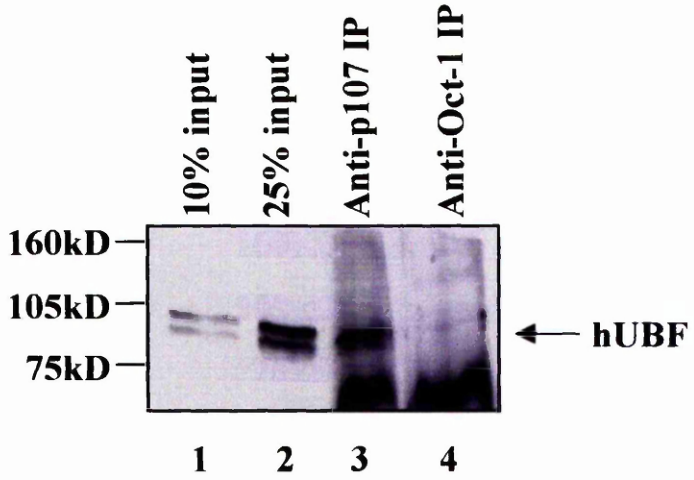
6.3 Discussion

RB has been shown to target specific pol I and pol III transcription factors, namely UBF (Cavanaugh et al., 1995) and BRF, respectively. Like RB, p107 and p130 have been shown to bind to the pol II transcription factor E2F (Grana et al., 1998), hence their ability to bind BRF and UBF was investigated.

The pocket proteins p107 and p130 were both shown to bind BRF. This interaction was demonstrated to occur with endogenous proteins by coimmunoprecipitation and cofractionation data (Sutcliffe et al., 1999). The binding of p107 and p130 to BRF was shown to be of functional significance, as the overexpression of both p107 and p130 in vivo inhibited pol III transcription (Sutcliffe et al., 1999). In addition, the inclusion of either p107 or p130 in a reconstituted in vitro transcription system repressed pol III transcription from a range of promoter-types (Sutcliffe et al., 1999). The E7 oncoprotein of human papillomavirus type 16 has been shown to bind RB and prevent it from binding other cellular targets. In

FIG. 6.6 Endogenous p107 interacts with endogenous UBF.

HeLa cell extract (150 μg) was immunoprecipitated using anti-OCT-1 antibody C-21 (lane 4), and anti-p107 antibody C-18 (lane 3). The coprecipitated material was resolved by SDS-7.8% polyacrylamide gel electrophoresis and probed for UBF by western blotting using the anti-UBF antiserum 131. Lanes 1 and 2 show 10% and 25% of the input of HeLa cell extract, respectively.


10% input
25% input
Anti-p107 IP
Anti-Oct-1 IP
160kD
105kD
75kD
hUBF
1
2
3
4

addition to relieving RB-mediated pol III repression, E7 was shown to overcome pol III repression imposed by p107 and p130, as demonstrated by overexpressing wild type E7 in SAOS2 cells which lack a functional RB protein (Sutcliffe et al., 1999). Further evidence regarding the ability of p107 and p130 to regulate pol III activity was observed using mouse embryonic fibroblasts from p107 and p130 double knockout mice. Northern blot analysis showed that the p107 and p130 null cells displayed slightly elevated B2 transcript expression. Moreover, upon serum withdrawal the level of B2 RNA expression in the p107 and p130 null cells was approximately twice that observed in the heterozygous counterpart (Sutcliffe et al., 1999).

These observations provide additional evidence that p107 and p130 make a significant contribution to the control of pol III transcription in vivo. A model of how RB mediates repression of pol III transcription has been proposed. This model postulates that upon binding the general factor TFIIIB, RB does not disrupt the multi-subunit complex of TFIIIB, but instead prevents it from interacting with other members of the initiation complex, namely TFIIIC and pol III (Sutcliffe et al, 2000). As p107 and p130 are both capable of repressing pol III transcription (Sutcliffe et al., 1999), and display significant homology to RB (Grana et al., 1998; Mulligan and Jacks, 1998), it would be reasonable to speculate that p107 and p130 repress pol III transcription in a similar fashion to RB.

The ability of p107 and p130 to bind TFIIIB and regulate pol III transcription instigated investigations regarding the ability of p107 and p130 to perform another RB

function, namely the targeting of UBF and regulation of pol I transcription. p130 has been demonstrated by pull-down assays and immunoprecipitations to form a physical association with UBF, consistent with previous observations (Hannan et al., 2000a). Furthermore, p130 has been shown to repress pol I activity (I. Grummt; personal communication), probably via its interaction with UBF. In addition, these studies have shown a physical association between p107 and UBF. This conflicts with the findings of Voit et. al. (1997) and Hannan et al. (2000a) who have previously demonstrated that p107 is not capable of binding UBF (Hannan et al., 2000a) or inhibiting pol I transcription in vitro (Voit et al., 1997). Therefore, the question remains as to what, if any, is the functional significance of p107 binding to UBF

The ability of RB, p107 and p130 to arrest cell cycle progression is suggested to be due to these proteins sharing particular cellular targets. Recent studies have demonstrated that like RB, p107 and p130 are able to repress pol III activity and probably also pol I transcription. p107 and p130 are therefore able to control cellular biosynthesis by regulating the expression of tRNA and rRNA genes.

Chapter 7

Conclusions

7 Conclusions

In 1996 White et al (White et al., 1996) showed that RB was able to act as a general repressor of pol III transcription. In addition, they showed that the large pocket domain of RB was responsible for repressing pol III transcription, and that mutations within the domain alleviated this inhibitory effect (White et al., 1996).

Further studies were undertaken by Larminie et al (Larminie et al., 1997) in order to elucidate the target for RB within the pol III transcription machinery. It was reasoned that as RB is a general repressor of pol III transcription, it most likely targets a general component of the pol III apparatus. Indeed, this hypothesis was correct, as it was shown that RB specifically targets the general transcription factor TFIIIB (Larminie et al., 1997).

TFIIIB is a multisubunit complex, which contains TBP (Hernandez, 1993; Rigby, 1993) and TAFs. One of these TAFs has been shown to be homologous to the pol II transcription factor TFIIB, and is called BRF (Buratowski and Zhou, 1992; Colbert and Hahn, 1992; Khoo et al., 1994; Lopez-de-Leon et al., 1992; Mital et al., 1996; Wang and Roeder, 1995). The next question to be answered was which subunit of TFIIIB does RB target in order to repress pol III transcription. Previous work had demonstrated that TBP did not interact with RB (Weintraub et al., 1995). In addition, purified TBP could not rescue RB-mediated pol III repression (Larminie et al., 1997). Therefore, the ability of RB to interact with the other characterised subunit of TFIIIB, that is BRF, was investigated.

The work presented here clearly demonstrates that there is an interaction between RB and BRF, and thereby identifies BRF as a subunit of TFIIIB recognised by RB. Since it is feasible that mammalian TFIIIB contains additional subunits yet to be identified, it is possible that RB also recognises one of these.

In addition to establishing a physical association between BRF and RB, the binding interfaces on each protein have been mapped. The BRF-binding domain has been demonstrated to be the large pocket domain of RB. This finding is of great significance, as it provides a direct relationship between the large pocket of RB repressing pol III transcription (Larminie et al., 1997; White et al., 1996) and binding BRF. This suggests that by binding BRF, the large pocket domain of RB is able to mediate repression of pol III transcription. Moreover, the ability of RB to restrain cell growth (Qin et al., 1992) and bind BRF are both attributable to the large pocket domain. These observations suggest that the ability of RB to bind BRF and repress pol III transcription may contribute to its ability to inhibit cellular growth.

The reciprocal binding interface was also determined; namely the RB-binding region in BRF was shown to be the direct repeats in the TFIIB homologous amino-terminal half. This interesting discovery has several implications; firstly, the region in BRF that binds tightly to TBP is in the carboxy-terminal half, in both yeast (Kassavetis, 1998; Khoo et al., 1994) and human (fig 3.17). RB and TBP recognise distinct regions in BRF. Hence, it was expected that the binding of the former should not affect the association of the latter. Indeed, in the presence of RB the interaction between BRF and TBP is not abolished. The second implication of this work regards

the direct repeats recognised by RB. BRF is the subunit of TFIIIB which forms contacts with both TFIIIC (Kassavetis et al., 1991; Kassavetis et al., 1992) and pol III (Khoo et al., 1994). Moreover, the location of these contacts has been mapped to the direct repeats (Kassavetis, 1998; Khoo et al., 1994). Hence, it was expected that upon binding BRF, RB would prevent TFIIIB from associating with TFIIIC, pol III or both. Work shown here indicates that the presence of RB abolishes the interaction between TFIIIB and TFIIIC, and between TFIIIB and pol III.

As mentioned previously, an intact large pocket domain of RB mediates binding and repression of E2F and cell growth suppression activities (Qin et al., 1992). Mutations in the RB pocket domain occur naturally in tumours, resulting in inactivation of these functions (Horowitz et al., 1990). Indeed, naturally occurring deletions of the RB pocket (deletion of exon 21 and 22 (Horowitz et al., 1990)) alleviate the ability of RB to repress pol III transcription (White et al., 1996). Moreover, a point mutation in the pocket domain which causes a substitution at residue 706 from cysteine to phenylalanine (Kaye et al., 1990) abolishes the ability of RB to repress pol III transcription (White et al., 1996). Hence, it was decided to determine if these naturally occurring RB mutants were unable to repress pol III transcription as they are unable to bind BRF and prevent TFIIIB from interacting with both TFIIIC and pol III.

The work presented here indicates that a naturally occurring RB mutant (deletion of exon 21 (Horowitz et al., 1990)) is unable to interact with BRF. Subsequent investigations demonstrated that this mutant is also unable to disrupt the

association between TFIIIB and TFIIIC, and between TFIIIB and pol III. Furthermore, another naturally occurring RB mutant, which contains a point mutation in the pocket domain (Kaye et al., 1990), is also unable to abolish the interaction between TFIIIB and pol III. Similar findings were observed in tumourigenic cell lines in which the large pocket of RB was mutated. The deletion of exons 21-27 (Shew et al., 1990), or of four amino acids at the beginning of exon 20 (Scheffner, et al., 1991) abrogated the ability of RB to interact with BRF. These findings demonstrate that the presence of mutations within the pocket domain abolish the ability of RB to bind and repress TFIIIB. RB is a recognised tumour suppressor protein and as such it is able to restrain cell growth (Weinberg, 1995). Hence, these findings suggest that RB-mediated growth control may entail inhibition of polymerase III transcription.

In addition to mutational inactivation there are two other ways, found in cancerous cells, which inactivate RB: sequestration by viral oncoproteins and hyperphosphorylation. Previous work has demonstrated that cell lines transformed by Simian virus 40 display elevated pol III transcription levels in comparison to the untransformed parental cell line (White et al., 1990). Work shown here demonstrates that the association between BRF and RB is reduced in a cell line transformed with SV40 compared to the untransformed parental cell line. This suggests that upon transformation, the sequestration of RB by a viral oncoprotein compromises the association between BRF and RB, so that BRF is released and is now able to promote pol III transcription (Larminie et al., 1999). It must be highlighted, however, that the oncoprotein binding and inactivation of RB is not the sole mechanism whereby SV40

transformation up-regulates pol III transcription; in addition, abnormally high levels of TFIIIC2 expression are observed (Larminie et al., 1999).

The third mechanism of RB inactivation observed in human cancer is hyperphosphorylation (Weinberg, 1995). The phosphorylation status of RB oscillates throughout the cell cycle, the majority of RB becoming phosphorylated during the last third of the G1 phase (Weinberg, 1995). Such phosphorylation prevents RB from interacting with a variety of cellular proteins, such as E2F (Chellappan et al., 1991). As RB has now been shown able to repress all three nuclear RNA polymerases (Cavanaugh et al., 1995; Kouzarides, 1995; White et al., 1996), it was of interest to determine if hyperphosphorylation of RB abolished its ability to interact with BRF, as it does for E2F. Data shown here illustrate that hyperphosphorylation of RB disrupts its ability to interact with BRF. This observation provides a strong correlation between the inactivation of RB during late G1, and the up-regulation of pol III transcription observed at the G1/S transition (White et al., 1995b). It suggests that the release of BRF from RB upon hyperphosphorylation of the latter promotes the elevated levels of pol III transcription observed. The pathway by which RB is phosphorylated is compromised in a variety of human cancers, such as breast and esophageal carcinomas. Disruption of this pathway ultimately gives rise to an inactive hyperphosphorylated form of RB (Weinberg, 1995). As this form is prevalent in tumourigenic cells, and is unable to bind BRF, this would suggest that the inability of RB to bind and repress TFIIIB via BRF contributes to the unrestrained growth associated with these cells. Therefore, these results demonstrate that the three ways

RB is inactivated in human cancers can all compromise its ability to interact with BRF; hence, TFIIIB is not repressed and is able to promote pol III transcription.

As mentioned previously, in 1995 Cavanaugh *et al* showed that RB was able to repress pol I transcription, by targeting UBF (Cavanaugh et al., 1995). These studies confirm the previous observation of a physical association between RB and both UBF splice variants (Cavanaugh et al., 1995; Voit et al., 1997). They also identify the binding regions involved in this interaction. The large pocket domain of RB is bound by UBF; this agrees with former investigations that the large pocket is required to be intact for this interaction (Cavanaugh et al., 1995). This domain of RB has also been identified as being responsible for growth suppression (Qin et al., 1992). Hence, the finding that this domain is also responsible for binding and repressing UBF suggests that repression of pol I transcription might contribute to this effect. The RB-binding domain in UBF was determined as HMG boxes 1 and 2, thereby substantiating previous work (Voit et al., 1997).

In addition to binding UBF, RB was also shown to bind the other pol I transcription factor SL1. This is consistent with the model for RB-mediated pol I repression, whereby RB binds UBF and prevents it from binding DNA, but not the other members of the pre-initiation complex, namely SL1 and pol I (Voit et al., 1997).

RB belongs to a family of proteins called the pocket proteins; the other two members, p107 and p130, display similar but not identical sequence homologies and

functional properties (Grana et al., 1998; Mulligan and Jacks, 1998). Hence, it was of interest to determine if, like RB, p107 and p130 were both able to interact with the pol I and pol III transcription factors which RB targets to mediate its effect. As shown here, p107 and p130 were both able to interact with BRF and UBF. Moreover, p107 and p130 are both able to repress pol III transcription (Sutcliffe et al., 1999). Recently, p130 has been shown to repress pol I transcription (I. Grummt, personal communication), but it is less clear whether p107 is also able to repress pol I transcription.

In summary, RB is able to interact with both BRF and UBF. For both these interactions, the large domain of RB needs to be intact. This provides a correlation between repression of pol I and pol III and the ability of RB to mediate growth suppression. RB represses pol III transcription by binding BRF, and preventing TFIIIB from associating with TFIIIC or pol III. Inactivation of RB by hyperphosphorylation, viral oncoprotein sequestration or mutations disrupts the ability of RB to interact with BRF. Furthermore, inactivating mutations compromise the ability of RB to disrupt TFIIIB from associating with TFIIIC and pol III. RB also represses pol I transcription by binding the HMG boxes 1 and 2 of UBF. This agrees with previous observations whereby RB represses pol I transcription by binding UBF and preventing it from contacting DNA, but not disrupting it from associating with SL1 or pol I. Lastly, the pocket proteins, p107 and p130 are both able to mimic particular functions of RB, as they both interact with BRF and UBF.

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