Studies on the mechanism of function of Retinoblastoma Binding Protein-1 (RBP1).

By

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Abstract

Proteins from the pRb family have all been demonstrated to actively repress transcription of E2F target genes. Previous studies have shown that they recruit both HDAC-dependent and –independent repression functions. RBP1 has been found to participate in the recruitment of HDAC to pRb through interaction via its R2 domain with the Sin3 HDAC complex. RBP1 also bring HDAC-independent repression to pRb via its R1 domain. However, the repression mechanism of R1 remains elusive. Preliminary studies suggested that HAT activity might be associated with the ARID domain portion of R1. The present study demonstrates that there is no HAT activity associated with RBP1. Attempts were made to isolate proteins binding to RBP1 by their ability to associate with different RBP1 domains fused to GST or by their association with RBP1 in co-immunoprecipitations using the LY11 antibody. Analysis of interacting proteins in both systems was complicated by the presence of many non-specific interactors that may prevent identification of specific binding proteins by peptide mapping or protein sequencing. An inducible stable cell line expressing C-terminally HA-tagged RBP1 was established to allow both analysis of proteins binding to RBP1 and the effects of RBP1 overexpression on the cell cycle. Finally, gel filtration studies demonstrated that all of RBP1 co-fractionates with SAP30, but not with hypophosphorylated pRb, and suggests that there is no stable complex formed at the R1 domain of RBP1.

Résumé

Les protéines de la famille du rétinoblastome (RB) sont toutes responsables de la répression active des gènes sous le contrôle du facteur de transcription E2F. Des études antérieures ont démontrées que pRb, p107 et p130 recrutent des co-répresseurs dépendant et indépendant des histones déacetylases. Grâce à une interaction entre son domaine de répression R2 et le complexe d'histone déacetylase Sin3, la protéine RBP1 est partiellement responsable du recrutement des histones déacetylases par pRb. Le domaine R1 de RBP1 recrute aussi des co-répresseur indépendants des histones déacetylases. Le méchanisme de répression par le domaine R1 reste, par contre, indéterminé. Des résultats préliminaires ont suggéré que le domaine ARID de RBP1 pourrait être associé à des histones acétylases. Cette étude démontre qu'il n'y a pas d'histone acétylase associée avec RBP1. Il a été tenté d'isoler des protéines interagissant avec RBP1 grâce à leur habilité à interagir avec différents domaines de RBP1 fusionnés à GST et grâce à leur association en co-immunoprécipitation avec l'anticorps LY11. L'analyse des protéines associées avec RBP1 a été compliquée par la présence de plusieurs protéines interagissant de façon non spécifique avec les protéines de fusion GST ou avec l'anticorps LY11. Une lignée cellulaire surexprimant de manière inductible la protéine RBP1 a été établie. Cette lignée cellulaire pourra être utilisée pour étudier les protéines associées avec RBP1 et les fonctions cellulaires de RBP1. Finalement, la séparation de complexes contenant RBP1 par filtration sur gel a permis de découvrir que RBP1 est majoritairement présente dans des complexes contenant SAP30, mais pas pRb hypophosphorylé, et suggère que le domaine R1 ne forme pas de complexes stables avec des co-répresseurs.

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List of Abbreviations

aa	Amino acids
ALP	Aprotinin - Leupeptin - Pepstatin
ARID	AT-rich interaction domain
ATCC	American Type Culture Collection
BCAA	Breast cancer-associated antigen
BME	B-Mercaptoethanol
CB	Cyclin A binding domain of E2F1-3
CBP	Creb binding protein
cdk	Cyclin dependant kinase
Chromo domain	Chromatin organisation modifier domain
CKI	Cdk inhibitor
CNS	Central nervous system
CpG	Dinucleotide, Cytosine followed by Guanine
CS	Calf Serum
DBD	DNA binding domain
DHFR	Dihydrofolate reductase
DRI	Dead ringer
DTT	Dithiothreitol
eARID	extended ARID domain
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
EMSA	Electrophoretic mobility shift assay
FBS	Fetal bovine serum
g	gram
GST	Glutathione S Transferase
H-AcetylCoA	Tritiated acetyl coenzyme A
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)

HMG	High mobility group
HMT	Histone methyl transferase
HPV	Human Papilloma Virus
HRP	Horse radish peroxidase
IPTG	Isopropyl β-D-thiogalactopyranoside
IxCxE	Peptide where I stands for Isoleucine, C, for Cysteine and E, for Glutamic acid and x stands for any amino acid
kb	Kilobase
kDa	KiloDaltons
LxCxE	Peptide where L stands for leucine, C, for cysteine and E, for Glutamic acid and x stands for any amino acid
MB	Marked box
min	Minute
ml	Milliliter
mm	millimeter
mtrRNA	mitochondrial ribosomal RNA
NAD	Nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information
NLS	Nuclear localization signal
O/N	Overnight
PCNA	Proliferating cell nuclear antigen
PMSF	Phenylmethylsulfonyl fluoride
PP1	Protein phosphatase 1
PPBD	Pocket protein binding domain
PSG	Penicillin, streptomycin, glutamine
PVDF	Polyvinylidene difluoride
RBP1	Retinoblastoma-binding protein-1
RBP1L1	Retinoblastoma-binding protein-1-like-1
RPA2	Replication protein A
rpm	Revolution per minute
RRM2	Ribonucleotide reductase subunit M2

rRNA	Ribosomal RNA
RT	Room temperature
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
sec	Second
SMA	Spinal muscular Atrophy
ssDNA	single stranded DNA
SV40	Simian Virus 40
TAD	Transcription activation domain
Tag	Large T antigen from SV40
TBP	TATA-box binding protein
Tk	Thymidine kinase
topo.1	Topoisomerase 1
TRD	Transcription repression domain
Tris	Tris (hydroxymethyl) aminomethane
tRNA	Transfer RNA
TS	Thymidylate synthetase
TSA	Trichostatin A

Chapter 1: Introduction

Malignancy is the result of mutations in genes regulating processes such as cell cyle control, apoptosis and DNA repair. This review will focus on the control exerted by the pRb family of pocket proteins on cell cycle progression.

The cell cycle is divided into four phases: growth phase 1 (G_1), synthetic phase (S), growth phase 2 (G_2) and mitotic phase (M). Quiescent cells that have exited the cell cycle are said to be in G_0. Progression through the cell cycle is a highly regulated process and disruption of the different checkpoints results in overproliferation that can, if left unchecked, develop into malignancy. One important checkpoint in G_1 is the restriction point. This checkpoint controls the decision of the cell to either proliferate or leave the cell cycle and differentiate. Once this point is passed, cells complete the cell cycle even in the absence of growth stimulatory signals (Pardee, 1989).

1.1 Retinoblastoma and cloning of RB1

There exist many hereditary cancers, but none have provided more insights into oncogenesis than retinoblastoma. Retinoblastoma is a rare human paediatric ocular tumour that has an incidence of 1 in 15,000 live births (Bishop and Madson, 1975). Inheritable cases represent 40% of all diagnosed retinoblastoma tumours. The remaining cases are sporadic and are usually due to somatic mutations (Singh et al., 2000). One important observation that was made is that bilateral inheritable retinoblastoma tumours have an earlier age of onset than sporadic cases, which are often unilateral. This led Knudson to propose the "two hit hypothesis" for retinoblastoma genesis that states that two mutations are required, the first one being inherited or occurring somatically during early development and the second one occurring later somatically in the retina (Knudson, 1971). A few years later, a locus on chromosome 13 (13q14) was found to be deleted in patients suffering from hereditary or sporadic retinoblastoma (Balaban et al., 1982; Vogel, 1979; Yunis and Ramsay, 1978). Shortly after the locus responsible for retinoblastoma was found, the retinoblastoma susceptibility gene, RB1, was cloned (Friend et al., 1986; Fung et al., 1987; Lee et al., 1987). The RB1 gene contains 27 exons

spread over 200kb of genomic DNA and encodes a 928aa protein (Goodrich and Lee, 1993).

1.2 RB1 is mutated or deleted in other cancers

Patients having germline mutations in RB1 and suffering from retinoblastoma are often concomitantly affected by primary tumours to other organs (Eng et al., 1993). This led to the idea that RB1 could be implicated in the development of other cancers. Upon closer examination it was found that retinoblastoma is not the only malignancy in which deletion or mutation of the RB1 gene is present. The 13q14 locus was also found to be mutated or disrupted in other cancers including osteosarcoma, small cell lung cancer, breast carcinoma, bladder carcinoma and glioblastoma to name a few (Cairns et al., 1991; T'Ang et al., 1988; Toguchida et al., 1988; Venter et al., 1991; Yokota et al., 1988). In fact, it is believed that the pRb pathway is mutated in most, if not all, cancers through mutations, translocation or amplification of *cyclin D*, *cdk4*, $p16^{INK4a}$ or *Rb* (Palmero and Peters, 1996; Sellers and Kaelin, 1997). Because of its inactivation in so many tumours, RB1 became recognised as the prototype tumour suppressor gene. This was later confirmed by studies showing that reintroduction of *Rb* in retinoblastoma cells reversed the transformed phenotype (Huang et al., 1988).

1.3 Viral oncoproteins bind pRb

Studies on small DNA tumour viruses have shed a considerable amount of light on the cellular roles of pRb. In the late 80's, it was discovered that many viral oncoproteins interact with and inhibit pRb. The E7 protein from Human Papilloma Virus (HPV), the large T antigen (Tag) of Simian Virus 40 (SV40) and the E1A protein from human adenovirus all bind to the small pocket of pRb (DeCaprio et al., 1988; Dyson et al., 1989; Egan et al., 1989; Whyte et al., 1988). E7, E1A and Tag all share homology in a region that contains a LxCxE motif, which is a minimal consensus pRb binding

sequence (Figge et al., 1988). This particular pocket-binding motif is also present in many cellular proteins that interact with pRb, including RBP1, RBP2 and cyclin D1 (Defeo-Jones et al., 1991; Dowdy et al., 1993; Kim et al., 1994). Not all pRb binding proteins contain the LxCxE motif. E2Fs and c-abl for example have a distinct pRb binding motif (Flemington et al., 1993; Welch and Wang, 1993). The binding of viral oncoproteins to the pocket inhibits pRb function and is required for their transformation activity (Egan et al., 1989).

1.4 Cloning of p107 and p130

pRb is not the only protein that binds to the LxCxE motif of viral oncoproteins. Two other proteins have been cloned by their ability to interract with LxCxE motifs, p107 and p130 (Ewen et al., 1991; Li et al., 1993; Mayol et al., 1993). Both p107 and p130 are higly related to pRb. They share about 30% amino acid identity with pRb, the A and B boxes being the most conserved regions. However, p130 and p107 are more related to each other than to pRb, sharing 50% amino acid identity (Lipinski and Jacks, 1999; Sidle et al., 1996). Together with pRb, p107 and p130 form the pocket protein family.

1.5 Structure of pRb family

There is an increasing amount of evidence suggesting that the pocket of pRb family proteins is crucial to their cellular function. It is formed from two subdomains called the A and B boxes that are separated by the spacer region (Figure 1.1). The small pocket was first described as a structural and functional domain conserved between pRb and p107. This small pocket is responsible for LxCxE dependent binding. More precisely, LxCxE proteins bind to a shallow groove in the B box (Harbour and Dean, 2000; Lee et al., 1998; Morris and Dyson, 2001). Although the interaction with LxCxE takes place in the B box, the A box is necessary as it seems to ensure proper folding of the B box (Lee et al., 1998). The residues responsible for binding to the LxCxE motif are the

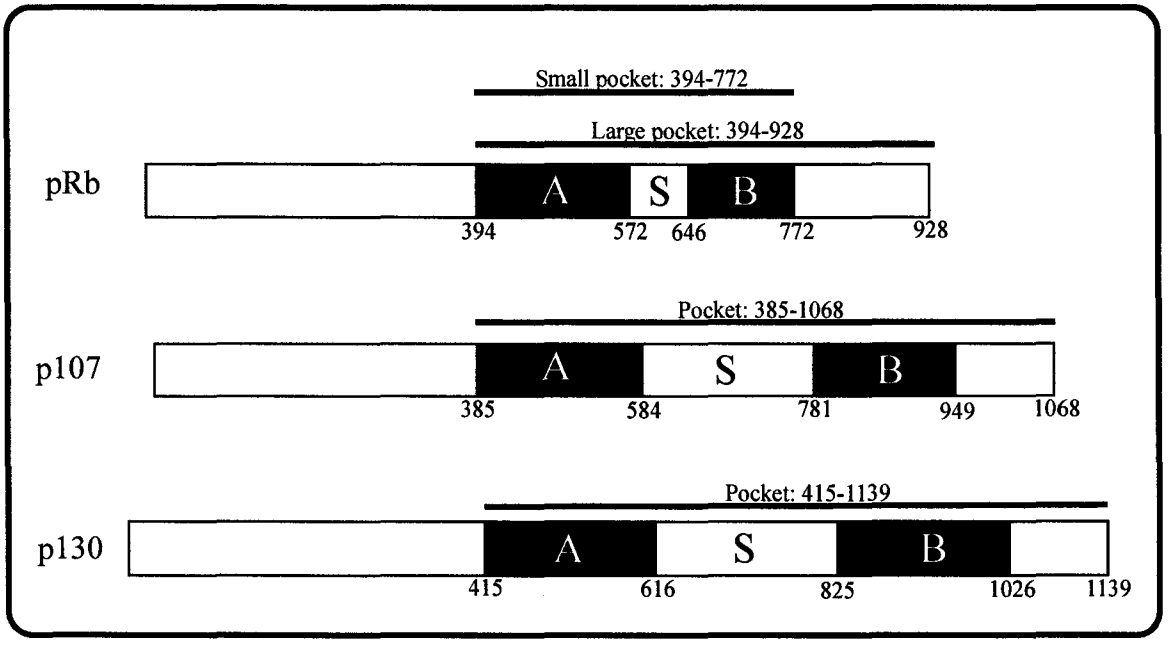


Figure 1.1: Structure of pRb family of pocket proteins. This figure is made to scale. All three pocket proteins, pRb, p107 and p130, have a pocket domain composed of the A and the B boxes separated by the spacer region (S). The spacer regions of p107 and p130 are much larger and share no homology to that of pRb. Embeded within p107 and p130 spacer is a binding site for cyclin E/A-cdk2. This site is abscent from the spacer of pRb. Adapted from (Sidle et al., 1996).

most conserved residues between pRb, p107 and p130 in various species and the majority of naturally occurring cancer-causing point mutations map to the A and B boxes (Hu et al., 1990; Huang et al., 1990; Lee et al., 1998).

The C-terminal domain of pRb is critical for growth suppression and it is thought to interact with and regulate the A/B pocket (Harbour et al., 1999). The C-terminus together with the small pocket forms the large or extended pocket that is required for binding of E2F (Kaelin, 1999; Kaelin, 1997). Although p107 and p130 are very similar to pRb in the pocket domain architecture, they somewhat differ in the spacer region. The spacer between the A and B boxes of p107 and p130 is much longer and shows little homology to the pRb spacer (Dyson, 1994; Lipinski and Jacks, 1999; Sidle et al., 1996). Embeded in the p107 and p130 spacer, but not in that of pRb, is a binding site for both cyclin E-cdk2 and cyclin A-cdk2 complexes (Ewen et al., 1992; Faha et al., 1992; Hannon et al., 1993).

1.6 Cell cycle expression of pRb family

The expression of pocket proteins varies during the cell cycle (Figure 1.2 Panel A). Although its mRNA level is relatively constant through out the cell cycle, p130 is mainly present in G_0 and its level decreases as cells enter G_1 (Smith et al., 1996; Smith et al., 1998). Upon entry into G_1, p130 becomes phosphorylated and it is exported to the cytoplasm to be targeted for degradation through the ubiquitin proteasome pathway (Dong et al., 1998; Smith et al., 1996; Smith et al., 1998). Expression of p107 is minimal in G_0 and increases upon entry into G_1. This increase is caused by the presence of E2F sites in the *p107* promoter which negatively regulate its transcription in G_0 and in early G_1 (Lavia and Jansen-Durr, 1999; Smith et al., 1998; Zhu et al., 1995). In G_1, p130 becomes phosphorylated and releases E2F4-5 which permits the expression of p107 (Smith et al., 1998). The Rb promoter also contains E2F sites and therefore its level of expression also increases, though somewhat less, upon entry into G_1 (Nevins, 1998; Shan et al., 1994a).

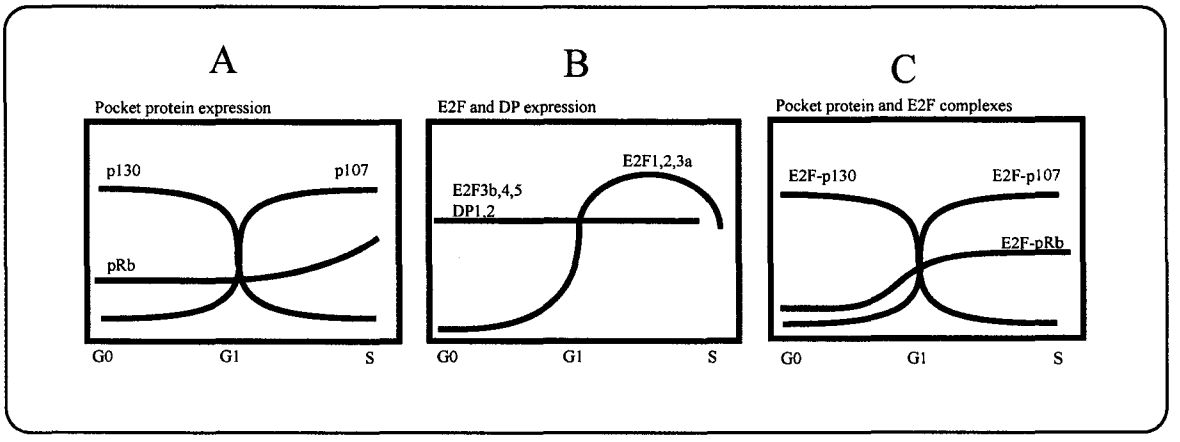


Figure 1.2: Pocket proteins/E2F/DP during cell cycle. Panel A: Pocket protein expression during cell cycle. Panel B: E2F and DP expression varies during transition from G_0 to S. Panel C: Major E2F-pocket protein complexes during G_0, G_1 and S. Adapted from (Nevins, 1998).

1.7 Knock out studies

One classic way to determine the function of a gene is to disrupt it in mice and to study the resulting phenotype of the knock out animal. This approach was used to study the functions of all three pocket proteins.

1.7.1 Disruption of *Rb*

Three groups knocked out the *Rb* gene in mice to discover its effect on development. *Rb* knock out ($Rb^{-/-}$) embryos die between days 14 and 15 from severe neuronal and haematopoietic abnormalities (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). This phenotype was specific for *Rb* inactivation because reintroduction of human *Rb* in knock out mice completely rescued their phenotype (Lee et al., 1992). *Rb* is therefore very important for early neuronal and haematopoeitic development. Although inheritance of a mutant *Rb* allele predisposes humans to retinoblastoma, $Rb^{+/-}$ mice did not present any retinal lesions and were perfectly viable and fertile although they had increased risk of pituitary and brain tumours (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). $Rb^{-/-}$ embryos also suffered from massive apoptosis in the central nervous system (CNS) (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). The apoptosis in the CNS was caused by failure of neuronal cells to terminally exit the cell cycle and differentiate. The uncontrolled proliferation of neuronal cells caused them to undergo apoptosis (Lee et al., 1994). In $Rb^{-/-}$ chimeric mice, it was discovered that the representation of $Rb^{-/-}$ cells in the retina was decreased suggesting deletion of *Rb* in the retina causes apoptosis (Maandag et al., 1994). It seems that *Rb* inactivation is not sufficient to cause retinoblastoma in mice.

1.7.2 Disruption of *p107* and *p130*

Knock out studies have also been conducted with both *p107* and *p130*. Disruption of these two genes has been shown to cause strain specific abnormalities. Disruption of *p107* or *p130* in 129/Sv:C57BL/6 or C57BL/6 genetic background did not cause any major developmental defect (Cobrinik et al., 1996; LeCouter et al., 1998; LeCouter et al., 1998; Lee et al., 1996). However, deletion of *p130* in BALB/cJ was embryonic lethal between embryonic day E11 to E13 and deletion of *p107* in BALB/cJ resulted in viable mice that had severely impaired growth (LeCouter et al., 1998; LeCouter et al., 1998). This suggests the existence of genes with epistatic relationships to *p107* and *p130*.

The knocking out of both *p107* and *p130* simultaneously caused neonatal lethality and limb developmental problems (Cobrinik et al., 1996). This suggests that complementation can occur between p107 and p130. In fact, it was found that in $p130^{-/-}$ cells, p130-E2F complexes present in quiescent cells were replaced by p107-E2F complexes (Cobrinik et al., 1996). In $p130^{-/-}$ cells, p130 mediated repression of the p107 promoter during quiescence is relieved allowing expression of *p107* in growth arrested cells (Smith et al., 1998).

1.7.3 Simultaneous disruption of *Rb* and *p107*

When *p107* and *Rb* were disrupted concomitantly in mice, the phenotype was even more dramatic than when *Rb* was disrupted alone. $p107^{-/-}$ $Rb^{-/-}$ embryos died two days earlier than $Rb^{-/-}$ embryos (Lee et al., 1996). In $Rb^{+/-}$ $p107^{-/-}$ mouse or chimera, no retinoblastoma tumours were detected although both showed dysplastic lesions on the retina (Lee et al., 1996; Robanus-Maandag et al., 1998). Inactivation of both *Rb* and *p107* is probably required for retinoblastoma in rodents, but, for a malignancy to fully develop, other events seem to be needed, probably the disruption of the apoptotic pathway that causes the death of $Rb^{-/-}$ cells in the retina of chimeric mice (Maandag et al., 1994; Robanus-Maandag et al., 1998). It is therefore not surprising that no case of spontaneous

retinoblastoma-like disease has ever been documented in mice since inactivation of the two alleles of both *Rb* and *p107* and inactivation of an apoptotic pathway are required for retinoblastoma genesis in mice (DiCiommo et al., 2000).

1.8 E2F transcription factor

Another way to learn about the function of a protein is to study its interacting partners. Proteins binding to pRb have been extensively studied and so far, more than a hundred have been recognised (Morris and Dyson, 2001). These interactors are involved in very diverse processes. Proteins involved in transcription repression, transcription activation, DNA replication, cell cycle control and growth suppression have all been found to interact with pRb. Using this approach, the main target for pRb mediated growth suppression was discovered. pRb controls cell proliferation through control of the E2F transcription factors.

E2F stands for E2 binding Factor. It is a transcription factor that was first described as an activator of the promoters of the E2 region of Adenoviruses whose activity is enhanced by E1A (Kovesdi et al., 1987; Yee et al., 1987). E1A activates the E2 region by allowing release of E2F transcription from inhibitory complexes with pocket proteins (Bagchi et al., 1990; Raychaudhuri et al., 1991). The E2F transcription factor is actually a heterodimer of one E2F protein and one DP protein (Huber et al., 1993; Lavia and Jansen-Durr, 1999; Muller and Helin, 2000). There are currently 6 known E2F genes (*e2f1-6*) and 2 known DP genes (*dp1-2*) in mammalian cells (Dyson, 1998; Helin, 1998; Kaelin, 1999; La Thangue, 1996). The first E2F was cloned as a pRb pocket binding protein (Helin et al., 1992; Kaelin et al., 1992; Shan et al., 1992) and the first DP was cloned as an E2F site specific DNA binding protein (Girling et al., 1993). Two mRNAs are transcribed from the *e2f3* gene, E2F3a and E2F3b (Adams et al., 2000; Leone et al., 2000). E2F3b is the result of an intronic promoter and differs from E2F3a by a single coding exon. DP2 is alternatively spliced and both splice variants are functional

(Ormondroyd et al., 1995). The second splice variant of DP2 is sometimes referred to as DP3 (Lavia and Jansen-Durr, 1999).

Both E2Fs and DPs can bind separately to DNA through their respective DNA binding domains, but the DNA binding affinity of the heterodimer is greater. E2F and DP bind synergistically to the E2F sites (Bandara et al., 1993; Girling et al., 1993; Helin et al., 1993b; Huber et al., 1993). There does not seem to be any specificity in the formation of heterodimer since both DP proteins seem to be able to associate with all six E2Fs (Dyson, 1998; Sidle et al., 1996).

The levels of certain members the E2F-DP heterodimer family has been found to vary with cell cycle progression. The expression of E2F1-3a is coupled with growth due to the presence of E2F binding sites in their promoters (Hsiao et al., 1994; Johnson et al., 1994; Neuman et al., 1994; Sears et al., 1997). On the other hand, DP1,2 and E2F3b,4,5 levels are constant during the cell cycle (Figure 1.2 Panel B). Because they do not share the same promoter even if they are situated at the same locus, transcription of E2F3a and E2F3b are differently regulated (Adams et al., 2000; Leone et al., 2000).

1.8.1 Structure of E2F/DP

Structurally, all E2Fs, except for E2F6, are very similar (Figure 1.3) (Helin, 1998; Lavia and Jansen-Durr, 1999; Sidle et al., 1996). The heterodimerization domain is conserved between DP and E2F and it allows tight association between the heterodimer partners (Bandara et al., 1993). The Marked box is a motif of unknown function that is conserved in all E2Fs (Lees et al., 1993). It has been shown to bind the human adenovirus E4 ORF6/7 protein (Cress and Nevins, 1996b). E2F1-5 encode a C-terminal transcriptional activation domain in which is embeded a non-LxCxE pocket protein binding site (Flemington et al., 1993; Harbour and Dean, 2000). E2F1-3 also contain in their N-terminus a cyclin A binding domain (CB) and unlike E2F4-5 they also have a

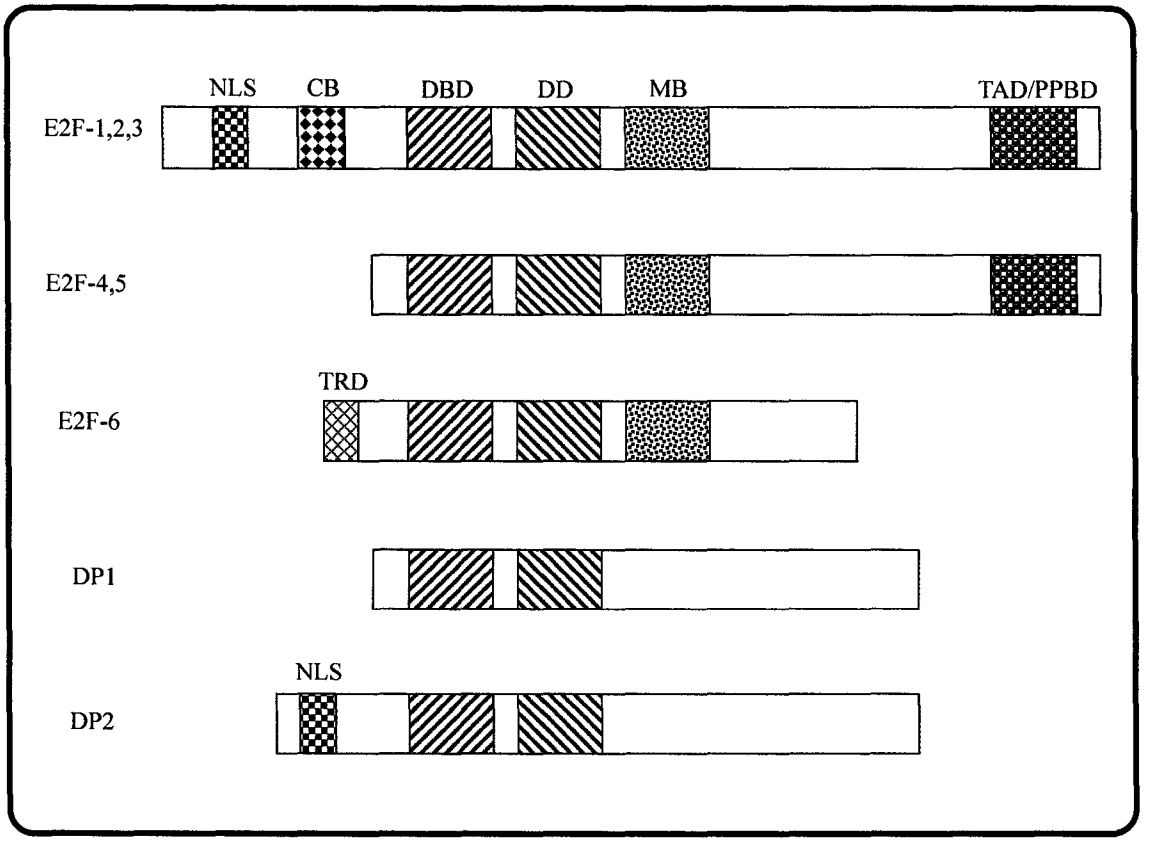


Figure 1.3: Structure of E2F and DP proteins. This figure is not made to scale. All E2F share homology in their DNA binding domain (DBD), their dimerization domain (DD) and their marked box (MB). E2F1-5 have a transcription activation domain (TAD) with its embeded pocket protein binding domain (PPBD). E2F1-3 have both a nuclear localization signal (NLS) and a cyclin A binding domain (CB) in the N-terminal region. E2F-6 differs from the other E2F in that it does not contain a transcription activation domain and in that it contains a transcription repression domain (TRD) in its extreme N-terminus. DP1-2 share homology in the dimerization domain (DD) with E2F and also have their own DNA binding domain (DBD). Unlike DP1, DP2 has a nuclear localization signal (NLS). Adapted from (Lavia and Jansen-Durr, 1999).

nuclear localization domain (NLS) (Sidle et al., 1996). DP2, and not DP1, contains a NLS in its N-terminus. Since it does not possess a transactivation domain, E2F6 is thought to act in a dominant negative fashion and to repress E2F dependent transcription (Morkel et al., 1997; Trimarchi et al., 1998). E2F6 also possesses an N-terminal transcription repression domain that can inhibit transcription when tethered to a promoter (Morkel et al., 1997). E2F6 can interact with DPs and has been shown to slow exit from S-phase when overexpressed (Cartwright et al., 1998; Gaubatz et al., 1998; Trimarchi et al., 1998). To facilitate reading from this point on, E2F will be used to refer to the E2F/DP heterodimer complex.

1.8.2 pRb binds E2F

As previously mentioned, E2F1 was cloned as a pRb pocket binding protein (Helin et al., 1992; Kaelin et al., 1992; Shan et al., 1992). It preferentially binds hypophosphorylated pRb (Adams, 2001; Chellappan et al., 1991; Harbour and Dean, 2000; Sellers and Kaelin, 1997). While p107 and p130 bind only E2F4 and E2F5, pRb seems to be able to bind E2F1-4 but shows preference to E2F1-3 (Beijersbergen et al., 1994; Dyson, 1998; Ikeda et al., 1996; Moberg et al., 1996; Nevins, 1998; Vairo et al., 1995) (Figure 1.4). Since E2F6 lacks the C-terminal transcription activation domain and its embeded pRb family binding domain, it does not interact with any pocket protein (Morkel et al., 1997).

Composition of the main pocket protein / E2F complexes varies with progression of the cell cycle (Figure 1.2 Panel C). In growth arrested cells, p130/E2F4-5 and pRb/E2F3b are the main complexes (Chittenden et al., 1993; Vairo et al., 1995) (Adams et al., 2000; Cobrinik et al., 1993; Corbeil and Branton, 1997; Dyson, 1998; Leone et al., 2000; Shirodkar et al., 1992). As cells enter G_1, p130 becomes phosphorylated and degraded, which results in E2F1-3a and p107 derepression (Smith et al., 1998). There is therefore a shift toward pRb/E2F1-3a and p107/E2F4-5 complexes (Beijersbergen and Bernards, 1996; Cobrinik et al., 1993; Leone et al., 2000; Moberg et al., 1996; Nevins, 1998; Schwarz et al., 1993). In differentiated cells, pRb/E2F and p130/E2F are the most

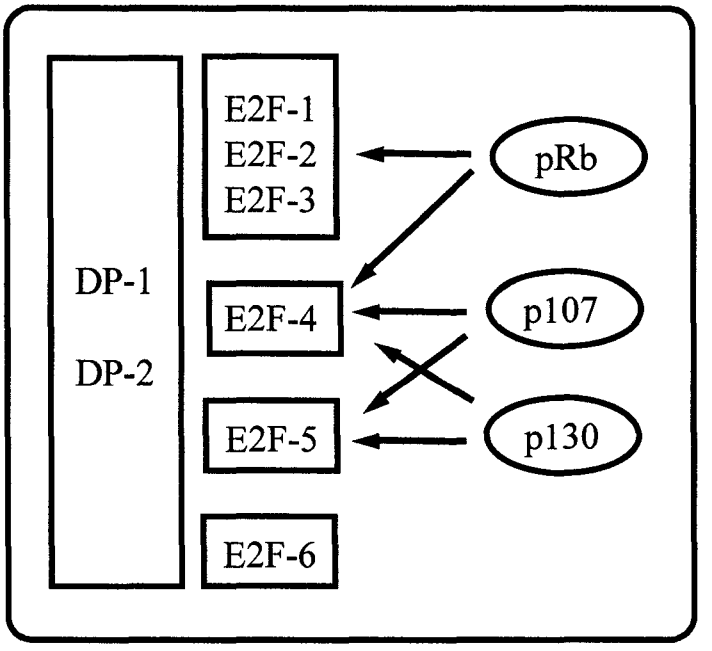


Figure 1.4: E2F/DP/pocket protein complexes. DP1 and DP2 are associated with all E2F. pRb associates preferentially with E2F1-3, but can also interact with E2F4. p107 and p130 interact with E2F4-5 complexes exclusively. E2F6 does not interact with pocket proteins since it lacks the pRb-binding domain situated inside the C-terminal transactivation domain of other E2F. Adapted from (Beijersbergen and Bernards, 1996).

important complexes (Shin et al., 1995; Yee et al., 1998) (Corbeil et al., 1995; Lipinski and Jacks, 1999).

1.8.3 Genes controlled by E2F

Many different genes have E2F binding sites in their promoters. These genes can be separated into two categories: 1) regulatory genes and 2) structural genes (Lavia and Jansen-Durr, 1999). Regulatory genes like *p107* control cell cycle progression while structural genes like DNA polymerase α are involved in biosynthetic pathways required for DNA replication. Table 1.1 lists other genes whose promoter have E2F sites.

Depending on the promoter, E2F sites have been found to be either positive or negative elements. The E2F binding sites in promoters of DNA synthetic enzymes, like DHFR, activate transcription in late G_1 (Means et al., 1992). On the other hand, E2F sites in promoters of *cdc2, cyclin A, cyclin E* and *p107* are negative elements whose elimination increases transcription (Johnson et al., 1994).

Table 1.1: Partial list of E2F responsive genes.

Regulatory genes		Structural genes	
pRb, p107	*e2f1, 2*	*DNA polα*	*ORC1*
cyclin E, A, D1	*cdc2*	*DHFR*	*Tk*
c-myc	*B-myb*	*TS*	*PCNA*
RanBP1	*cdc6*	*RRM2*	*Srp20*
cdc25A	*cdc25C*	*Histone H2A*	*topo.1*
dp1		*RPA2*	

Table 1.1: Partial list of E2F responsive genes. Source: (Helin, 1998; Hurford et al., 1997; Kalma et al., 2001; Lavia and Jansen-Durr, 1999; Sladek, 1997; Vigo et al., 1999)

1.8.4 E2F1 and cancer

Since they regulate genes important for cell cycle progression, one would intuitively think that *e2f* genes would be amplified or overexpressed in at least some cancers. In fact, E2F has some characteristics similar to oncogenes. E2F1-3 have been shown to function as an oncogene in transformation assays and E2F1 has been shown to cooperate with p53 inactivation in tumourigenesis (Pierce et al., 1998; Xu et al., 1995). However, overall E2Fs are very rarely overexpressed in tumours. The few examples include amplification of *e2f1* in HEL cells, an erythroleukemia cell line, in some cases of gastric and colorectal carcinomas and upregulation in small cell lung carcinoma (Eymin et al., 2001a; Saito et al., 1995; Suzuki et al., 1999). In some systems E2F can also function as a tumour suppressor. *e2f1* knock out mice display a high incidence of tumour development (Field et al., 1996; Yamasaki et al., 1996). Even though E2F inhibition is the endpoint of the pRb pathway, overexpression of E2F is clearly not as common in cancer as inactivation of *Rb*. This oncogene/tumour suppressor paradox is probably due to the ability of E2F1 to cause p53-dependent and -independent apoptosis when its activity escapes control by pocket proteins (Qin et al., 1994; Wu and Levine, 1994).

1.8.5 Modes of transactivation by E2F

E2F has been shown to activate transcription when tethered to a promoter (Helin et al., 1992; Kaelin et al., 1992; Shan et al., 1992). This activity maps to its C-terminal domain. The C-terminal transactivation domain of E2F was found to interact both in vitro and in vivo with the TATA Binding Protein (TBP) (Emili and Ingles, 1995; Hagemeier et al., 1993; Pearson and Greenblatt, 1997). E2F can therefore favour the formation of the RNA polymerase II initiation complex by recruiting TBP. The C-terminal region of E2F also recruits the p300/CBP histone acetyltransferase to activate transcription (Fry et al., 1999; Trouche and Kouzarides, 1996).

1.8.6 Regulation of E2F

Because E2F controls genes very important for cell cycle progression, its activity must be tightly regulated. The activity of E2F transcription is controlled both by regulating the levels of E2F protein and E2F transcription activity. As already explained, the expression of the E2F1-3a genes is controlled during the cell cycle due to the presence of E2F sites in the promoters (Hsiao et al., 1994; Johnson et al., 1994; Neuman et al., 1994). E2F1 and E2F4 cellular levels can also be controlled by the rate of degradation through the ubiquitin proteasome pathway (Campanero and Flemington, 1997; Hateboer et al., 1996; Hofmann et al., 1996).

The activity of free E2F can also be controlled through different post-translational modifications. E2F DNA binding affinity is controlled by phosphorylation. During S-phase, cyclin A-cdk2 binds to the E2F1 N-terminus and phosphorylates DP1, which leads to a decrease in DNA binding affinity (Krek et al., 1995; Xu et al., 1994). E2F2 and E2F3, unlike E2F4 and E2F5, have a similar cyclin A-cdk2 binding domain and thus their DNA binding affinity could also be regulated in a similar fashion (Nevins, 1998). The cyclin A-cdk2 binding site is very important for E2F1 regulation. Expression of mutant E2F1 lacking the cyclin A-cdk2 binding site causes S-phase arrest and apoptosis (Krek et al., 1995). Phosphorylation of E2Fs C-terminus by either TFIIH has also been suggested to favour degradation (Vandel and Kouzarides, 1999). E2F1-3 were also found to be regulated by acetylation (Martinez-Balbas et al., 2000; Marzio et al., 2000). Acetylation of the N-terminus of E2F1-3 results in increased DNA binding activity, increased half-life and increased activation potential.

The change in subcellular localization of E2F4 and E2F5 is another mechanism that regulates E2F activity. E2F4 and E2F5 lack a nuclear localization signal (NLS) and association with either pocket proteins or DP2 is required for their localisation to the nucleus (de la Luna et al., 1996; Lindeman et al., 1997; Magae et al., 1996; Muller et al., 1997; Verona et al., 1997). Furthermore, in differentiated cells, E2F1,3,5 have been

shown to be exported to the cytoplasm to prevent S-phase re-entry (Gill and Hamel, 2000).

The activity of E2F can also be controlled indirectly by methylation of E2F sites in promoters. CpG methylation of E2F sites in promoters prevents their recognition by E2Fs (Campanero et al., 2000).

Finally, the activity of E2F is controlled by binding to pocket proteins. This results in inhibition of the transactivation domain and the increase of their stability (Hateboer et al., 1996; Hofmann et al., 1996) (See below). Many different processes therefore tightly regulate the activity of E2F

1.9 pRb and E2F in apoptosis

As mentioned before, E2F is tightly regulated as loss of control of E2F activity can have dire consequences. Although overexpressing E2F1-3 is able to cause S-phase entry, only E2F1 has been demonstrated to induce apoptosis when overexpressed (DeGregori et al., 1997; Du et al., 1996; Guy et al., 1996; Shan and Lee, 1994b; Wu and Levine, 1994). Interestingly, disruption of E2F3 in $Rb^{-/-}$ embryos decreases the level of apoptosis, suggesting that E2F3 could play a role in promoting apoptosis in the absence of pRb (Ziebold et al., 2001). E2F1 mediated apoptosis has been found to be plurimechanistic and to be both p53-dependent and -independent (Holmberg et al., 1998; Macleod et al., 1996; Pan et al., 1998b). One of the main targets of p53-dependent apoptosis seems to be $p14^{ARF}$ (Bates et al., 1998). Free E2F1 activates transcription of ARF. ARF binds to MDM2 and inactivates MDM2-mediated p53 turnover and transcriptional silencing resulting in a build up of p53 levels, thus causing apoptosis (Pomerantz et al., 1998; Zhang et al., 1998b). Interestingly, ARF has also been shown to bind E2F1 and inhibit its transactivation activity (Eymin et al., 2001b). E2F on its own can also stabilize p53 by interacting with it via its marked box (Nip et al., 2001). Such interaction also blocks p53-mediated transactivation. The mechanism underlying p53

independent apoptosis caused by E2F1 is less clear. E2F1 can induce p53 independent apoptosis by downregulation of TRAF2 and inhibiting activation of anti-apoptotic proteins (Phillips et al., 1999). E2F1 has also been shown to activate transcription of p73, the p53 homologue (Irwin et al., 2000). The impact of p73 activation is twofold: it can induce apoptosis on its own; and it can also bind to and sequester MDM2 away from p53, resulting in increased p53 levels (Balint et al., 1999; Dobbelstein et al., 1999; Ongkeko et al., 1999; Zeng et al., 1999). Due to its importance in the control of apoptosis, it has been suggested that the role of E2F1 might be mainly in the apoptotic pathways protecting cells from pRb deregulation while other E2Fs might have more of a role in control of cell cycle (DeGregori et al., 1997).

It has been known for a few years that pRb protects cells from apoptosis. Inactivation of *Rb* in mice causes massive apoptosis in the central nervous system and inhibition of pRb by viral oncoproteins also leads to apoptosis (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Apoptosis caused by loss or inactivation of pRb can be p53-dependent or -independent (Macleod et al., 1996). It seems that disruption of the pRb pathway co-operates with p53 inhibition of tumorigenesis, which could explain the high incidence of p53 mutations in cancer (Symonds et al., 1994). The main target for pRb protection from apoptosis seems to be E2F1. Concomitant disruption of *Rb* and *e2f1* decreases the level of apoptosis compared to disruption of *Rb* alone (Liu and Zacksenhaus, 2000; Pan et al., 1998b). Furthermore, while overexpression of E2F1 can lead to apoptosis, overexpression of pRb along with E2F1 prevents apoptosis (Fan et al., 1996). By binding and inhibiting E2F1, pRb protects cells from apoptosis. Conversely, E2F1 protects cells from tumorigenesis by causing apoptosis in the event of pRb pathway disruption.

Interestingly, pRb has been found to be cleaved by caspases during apoptosis. The cleavage site is situated in the C-terminus and results in the loss of the last 42 amino acids of pRb (Janicke et al., 1996). This region is responsible for pRb/MDM2 interaction and is required for pRb inhibition of MDM2-mediated p53 degradation and MDM2 anti-apoptotic functions (Hsieh et al., 1999; Janicke et al., 1996).

No direct connection between p107 and p130 and apoptosis has been established. It is possible that due to their high homology with pRb they have a similar role to play. Disruption of *p130* in mice has recently been shown to cause massive apoptosis in the neural tube, in the brain, and in dermomyotomes, suggesting a role for p130 in protection from apoptosis (LeCouter et al., 1998).

Many viruses require free E2F activity to replicate efficiently (Shenk, 1996). Unfortunately, free E2F causes build up of p53 levels and ultimately apoptosis (DeGregori et al., 1997; Kowalik et al., 1998). Viruses had to evolve ways to deal with p53 to prevent apoptosis, which could kill the host cell prior to viral DNA replication. Adenovirus type 5 has evolved at least two such mechanisms. E1B-55K binds to p53 and inhibits its transcription activation function (Teodoro and Branton, 1997; Yew et al., 1994). Also, E4-ORF6 and E1B-55K together target p53 to the proteasome for ubiquitin dependent degradation (Querido et al., 2001).

1.10 pRb in differentiation

Differentiation and cell cycle progression are two opposing pathways. Depending on the signal received, cells will either proliferate or differentiate. Overexpression of E2F1 in differentiating cells blocks differentiation and cells enter S-phase and die by apoptosis (Guy et al., 1996). Differentiation is characterised by expression of tissue specific genes that are under the control of differentiation promoting transcription factors. The MyoD family is responsible for muscle gene expression and the C/EBP family is responsible for both adipocyte specific gene expression (C/EBPα, β, γ, NF-IL6) and hematopoietic gene expression (NF-IL6) (Darlington et al., 1998; Kitzmann and Fernandez, 2001). Differentiation in myoblasts has been extensively studied and will be used as an example to demonstrate the role of pRb in differentiation. The MyoD family of basic helix-loop-helix (bHLH) transcription factors is composed of four members that are expressed sequentially during differentiation (Myf-5, MyoD, myogenin and MRF4)

(Kitzmann and Fernandez, 2001). To bind to promoters, MyoD family members need to heterodimerize with E box proteins (Lassar et al., 1991).

The differentiation program is divided in two steps: 1) irreversible cell cycle exit and 2) expression of tissue specific genes. pRb seems to be an important factor for both steps. $Rb^{-/-}$ fibroblasts can differentiate, but upon serum stimulation, they can re-enter the cell cycle (Schneider et al., 1994). Furthermore, viral oncoproteins can reactivate the cell cycle machinery in terminally differentiated tissues by targeting pRb (Iujvidin et al., 1990; Pan and Griep, 1994; Tiainen et al., 1996). Both of these examples demonstrate importance of pRb in terminal cell cycle exit. pRb is also required for proper muscle gene expression (Novitch et al., 1996).

In the initial step of differentiation, the cellular level of pRb is increased (Coppola et al., 1990; Endo and Goto, 1992). This increase is in part caused by MyoD induction of pRb expression (Martelli et al., 1994). The increased level of pRb creates a surplus of pocket protein compared to E2F, thus ensuring that all E2F-DP complexes will be associated with members of the pRb family and that promoters of cell cycle genes will all be repressed (Ikeda et al., 1996). To ensure that pocket proteins remain in their active unphosphorylated form, the cdk inhibitor p21 is also induced in differentiating cells. The induction of p21 inhibits cdk complexes and prevents phosphorylation of pRb and p130 even upon serum stimulation (Halevy et al., 1995; Jiang et al., 1994; Steinman et al., 1994). This induction of p21 levels is mediated, at least in part, by MyoD (Guo et al., 1995; Halevy et al., 1995)

The excess pRb not bound to E2F has been shown to interact with another transcription factor during cell cycle exit prior to differentiation, HBP1 (Lavender et al., 1997; Tevosian et al., 1997). HBP1 is a member of the HMG box transcription factor family that was cloned in a yeast-two-hybrid screen using p130 (Lavender et al., 1997; Tevosian et al., 1997). It contains two LxCxE like motifs and it binds to the pocket of pRb (Lavender et al., 1997; Tevosian et al., 1997). Just like E2F, HBP1 has a potent transactivation domain that is blocked by pocket proteins (Lavender et al., 1997). It has

been shown to repress some cell cycle genes including N-myc and cyclin D1 (Gartel et al., 1998; Tevosian et al., 1997; Yee et al., 1998). Although both N-myc and cyclin D1 contain E2F sites, the presence of HBP1 sites allows further repression (Tevosian et al., 1997). Overexpression of HBP1 has been shown to cause cell cycle arrest (Tevosian et al., 1997). In fact, just like pRb, HBP1 levels have been shown to increase during differentiation (Tevosian et al., 1997). Surprisingly, HBP1 overexpression also inhibits differentiation (Shih et al., 1998). This differentiation block can be relieved by concomitant overexpression of pRb (Shih et al., 1998). This suggests that the decision between simple growth arrest and differentiation could be controlled by the pRb/HBP1 ratio (Shih et al., 1998). A low pRb/HBP1 ratio would cause cell cycle exit and inhibit differentiation while a high ratio would allow differentiation to proceed. This transient blockage of the differentiation program allows cells to build up sufficient levels of active pRb to ensure irreversible cell cycle exit prior to the onset of tissue specific gene expression (Shih et al., 1998).

The role of pRb in differentiation is not limited to irreversible cell cycle exit. pRb and MyoD have also been shown to cooperate in the activation of the transcription factor MEF2 (Novitch et al., 1999). It is not clear if this function of pRb involves direct interaction with MyoD as binding studies have yielded conflicting results (Gu et al., 1993b; Li et al., 2000; Zhang et al., 1999).

pRb has also been shown to have a similar function in differentiation in other cell lineages. In differentiating adipocytes and hematopoietic cells, pRb interacts directly with both C/EBP and NF-IL6 and this interaction increases the DNA binding activity of both transcription factors (Chen et al., 1996b; Chen et al., 1996a). pRb has also been shown to play an important role in osteoblasts differentiation as it interacts with and activates the osteoblast-specific transcription factor CBFA1 (Thomas et al., 2001).

1.11 RBP1

The first cellular protein to be cloned due to its ability to bind the small pocket of pRb was RBP1. It was cloned by two different groups using expression cloning (Defeo-Jones et al., 1991; Kaelin et al., 1992). It encodes a 1257aa ubiquitously expressed nuclear phosphoprotein (Fattaey et al., 1993). RBP1 exists in four splice variants (Otterson et al., 1993). This study focuses on isoform I. The first three isoforms share the same C-terminal region while the fourth encodes a different C-terminal domain. The differentially spliced exon encodes both putative cdc2 and casein kinase II sites. The level of phosphorylation could therefore be controlled in part by splicing as some isoforms lack some phosphorylation sites in the N-terminus (Fattaey et al., 1993; Otterson et al., 1993). Although its mRNA level is low, RBP1 is an abundant protein, suggesting that it is fairly stable (Defeo-Jones et al., 1991; Otterson et al., 1993). Even if its calculated size is 143kDa, it migrates between 180 and 200kDa (Fattaey et al., 1993; Otterson et al., 1993; Zhang et al., 1998a). This discrepancy between the theoretical and observed migration of RBP1 is probably due to its highly charged nature as 39% of its residues are charged, more than half of which are acidic (Fattaey et al., 1993).

1.11.1 RBP1 binds pRb

RBP1 was found to interact in vivo with pRb, showing preference for hypophosphorylated pRb (Fattaey et al., 1993; Otterson et al., 1993). Binding is through the small pocket since interaction with pRb can be competed with HPV E7 protein and peptide (Defeo-Jones et al., 1991). Deletion of the LxCxE domain of RBP1 or mutation of the pocket inhibits the interaction between RBP1 and pRb (Defeo-Jones et al., 1991; Lai et al., 2001). RBP1 was also found to interact with both p107 and p130 (Lai et al., 1999b; Lai et al., 1999a). The interaction with p107 is also pocket dependent since mutation of the p107 pocket disrupts RBP1 binding (Lai et al., 2001). The protein level of RBP1 is lower than that of pRb and thus RBP1 is only part of a subset of pRb complexes (Fattaey et al., 1993).

RBP1 was found by electrophoretic mobility shift assays (EMSA) to be part of a p130/E2F complex called C7 that was discovered both in differentiated myotubes and in growth arrested cells (Corbeil and Branton, 1997; Corbeil et al., 1995; Lai et al., 1999a).

1.11.2 Structural domains of RBP1

Since RBP1 has been cloned, many different structural domains have been recognized in its structure (Figure 1.5). RBP1 has previously been reported to contain both an ARID domain in the N-terminal region (aa 313-409) and a chromo domain (aa 592-634) (Chan et al., 1998; Koonin et al., 1995). Using the DART search tool from the NCBI web site, RBP1 was also found to contain a Tudor domain N-terminal to the ARID domain (aa 57-114). The RBP1 LxCxE motif is situated at the C-terminus (aa 957-961), a region that also contains a putative NLS (aa 1072-1093).

1.11.2.1 ARID domains

ARID domains or AT-Rich Interaction Domain are highly conserved evolutionarily. For example, *Drosophila* and human homologues of yeast SWI1 (Osa and BAF250/p270 respectively) both contain ARID domains just like their yeast homologue (Collins et al., 1999; Dallas et al., 1998). Examples of proteins containing an ARID are presented in Table 1.2. The core ARID domain is about 80 aa in length and contains 4 invariant residues (Kortschak et al., 2000). There exist two types of ARID proteins, those that contain only the core ARID domain and those that contain the extended ARID region (eARID) (Kortschak et al., 1998). eARID differs from ARID by the presence of a region of homology, the REKLES motif, just C-terminus to the core ARID sequence (Kortschak et al., 2000). Members of the eARID family include DRI, Bright, BDP and BDP/DRIL2 (Kortschak et al., 2000). The exact function of the REKLES domain is still not clearly understood, but in the case of Bright it was found to be necessary for tetramerization,

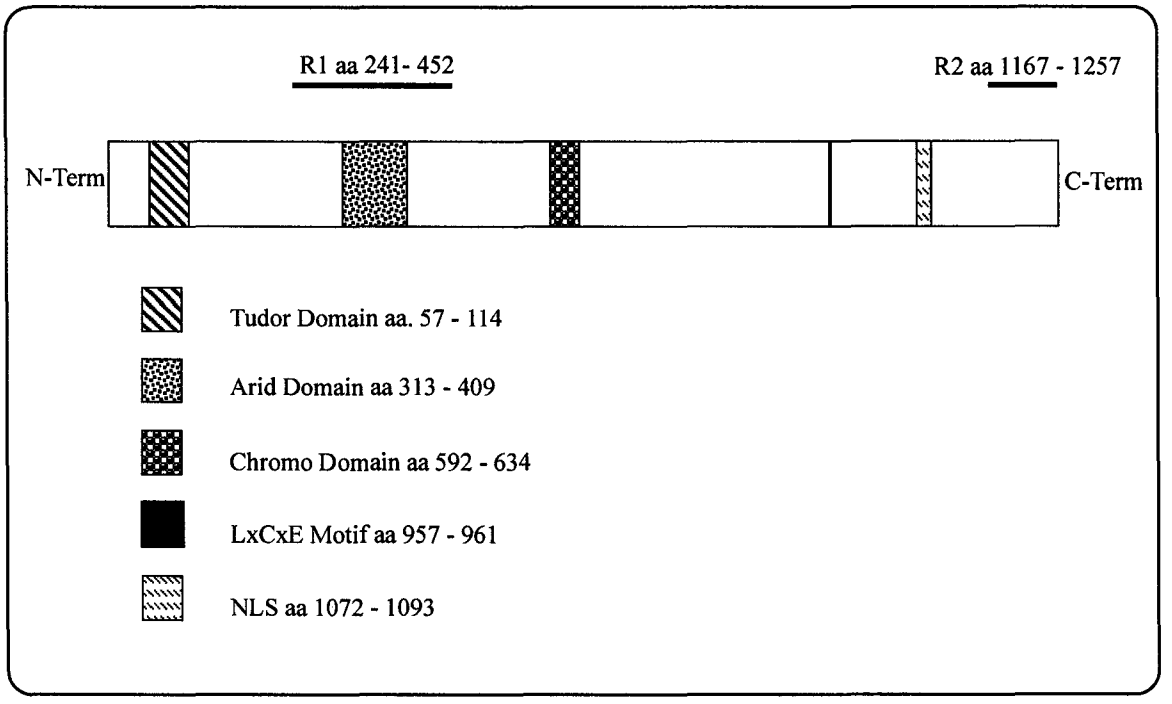


Figure 1.5: Structure of the RBP1 protein. This figure is made to scale. Three functional domains have been recognized in RBP1. It contains a Tudor domain at its N-terminus. The repression domain 1 (R1) contains an ARID domain. A chromo domain has been found in the middle of the protein. The repression domain 2 (R2) is situated at the extreme C-terminus and is preceded by the LxCxE pRb pocket binding motif and a putative NLS. Adapted from (Lai et al., 1999b).

Table 1.2: Partial list of proteins containing an ARID domain.	
Organism	Name
S. cerevisiae	SWI1
S. cerevisiae	YM8010.06
C. elegans	zk593.4
C. elegans	c09b11.3
C. elegans	T23D8.8
D. melanogaster	Dead Ringer (dri)
D. melanogaster	osa
D. rerio	Dri1 and Dri2
M. musculus	SmcX and SmcY
M. musculus	Jumonji
M. musculus	Bright
M. musculus	Desrt
M. musculus	BDP
S. scrofa domesticus	SMCp
H. sapiens	RBP1
H. sapiens	RBP1L1/BCAA
H. sapiens	RBP2
H. sapiens	MRF-1 and MRF-2
H. sapiens	p270
H. sapiens	PLU-1
H. sapiens	DRL1
H. sapiens	BDP/DRIL2

Table 1.2: Partial list of proteins containing an ARID domain. ARID containing proteins have been recognised in budding yeast (*S. cerevisiae*), nematode worm (*C. elegans*), fruit fly (*D. melanogaster*), zebra fish (*D. rerio*), mouse (*M. musculus*), swine (*S. scrofa domesticus*) and human (*H. sapiens*). Source: (Bulimo et al., 2000; Cao et al., 2001; Kortschak et al., 2000; Lahoud et al., 2001).

which is required for efficient DNA binding (Herrscher et al., 1995). As its name implies, the main function of the ARID domain is to bind DNA. This binding can be sequence specific as in the case of Bright, DRI and MRF2 or it can be non-specific as in the case of RBP2 and Osa (Collins et al., 1999; Fattaey et al., 1993; Gregory et al., 1996; Herrscher et al., 1995; Whitson et al., 1999). The structures of the ARID domain of MRF-2 and of the eARID domain of DRI have recently been resolved and it seems that both are very dissimilar. The ARID domain of MRF-2 resembles DNA polymerase and DNA repair enzymes structure while the eARID of DRI is more closely related to helix-turn-helix DNA binding domains (Iwahara and Clubb, 1999; Yuan et al., 1998).

ARID proteins are involved in many different cellular processes, including embryonic development, cell lineage gene regulation, cell cycle control and transcription regulation (Kortschak et al., 2000). Accordingly, mutations in ARID proteins can have dire consequences. Mutations in DRI in *Drosophila* or of Jumonji in mice lead to embryonic lethality while mutations in the ARID domain of Desrt in mice causes growth retardation and abnormalities of male and female reproductive organs (Lahoud et al., 2001; Shandala et al., 1999; Takeuchi et al., 1995).

Consistent with the presence of an ARID domain, RBP1 has been found to interact strongly with immobilized calf thymus DNA (Fattaey et al., 1993). This activity has not been mapped to a particular region of RBP1, nor has any specificity study been conducted, but the ARID domain is likely to be responsible. This could potentially allow RBP1 to be targeted to DNA in the absence of pRb.

1.11.2.2 Chromo domains

Chromatin organisation modifier domains also called chromo domains are 30-50aa long domains present in many proteins that bind to or are closely associated with chromatin (Koonin et al., 1995). This domain was first recognized in *Drosophila*

Polycomb (Pc) and HP1 proteins (Paro and Hogness, 1991). It has now been recognized in their mammalian homologues as well as in *Drosophila* SU(var)3-9, its mammalian homologue Suv39H1, *Drosophila* MES3, mammalian CHD-1 and yeast Swi6p (Delmas et al., 1993; Firestein et al., 2000; Koonin et al., 1995; Lorentz et al., 1994; Tschiersch et al., 1994). Proteins containing chromo domains play a role in transcription repression associated with heterochromatin and chromatin packaging and have also been found to be involved in gene silencing (Bienz and Muller, 1995; Lorentz et al., 1994; Messmer et al., 1992). Although a definite function of chromo domains has yet to be defined, many have been proposed. Chromo domains have been proposed to interact with themselves to allow for dimerization, they have been proposed to bind chromatin, possibly by binding to methylated lysine 9 of histone H3 in the case of HP1, and they have been suggested to function as RNA binding motifs (Akhtar et al., 2000; Bannister et al., 2001; Cowell and Austin, 1997; Jacobs et al., 2001; Messmer et al., 1992; Platero et al., 1995). If the chromo domain of RBP1 binds methylated histones like that of HP1, it could provide another attachment point for pRb-E2F complexes since pRb has recently been shown to recruit a histone methyl transferase (Bannister et al., 2001; Jacobs et al., 2001; Nielsen et al., 2001; Vandel et al., 2001)

1.11.2.3 Tudor domains

Tudor domains are present in many RNA binding proteins (Ponting, 1997). They were first described in the *Drosophila* protein tudor (tud) which is required for mitochondrial ribosomal RNA (mtrRNA) shuttling from the mitochondria to the cytoplasm (Amikura et al., 2001). Tudor domains are also present in proteins such as *Drosophila* homeless (hls), that is responsible for RNA localization during oogenesis, in human p100 nuclear protein that co-activates transcription by Epstein-Barr Virus Nuclear Antigen 2 (EBNA2) and binds ssDNA, and in human SMN that is involved in mRNA splicing and is essential for snRNP assembly prior to their export to the nucleus (Fischer et al., 1997; Gillespie and Berg, 1995; Pellizzoni et al., 1998; Tong et al., 1995). Currently, not much is known about the function of this particular domain, but the close

association of Tudor domain-containing proteins with RNA suggests that they are responsible for binding to RNA (Ponting, 1997). However, the crystalization of SNM Tudor domain demonstrated that Tudor domains are more likely to be protein-protein interraction domains, their association with RNA is probably indirect (Selenko et al., 2001). The presence of a Tudor domain in RBP1 is surprising since it has never been implicated in any RNA processing pathway.

1.11.3 RBP1 represses transcription

When tethered to a promoter via fusion with the Gal4 DBD, RBP1 was found to repress transcription (Lai et al., 1999a). The deletion of the LxCxE motif did not inhibit repression, suggesting that the repression function of RBP1 is independent of binding to pRb. RBP1 was also discovered to repress E2F dependent promoters when overexpressed (Lai et al., 1999a). This repression requires the presence of an intact LxCxE motif, which strongly suggests that it occurs via recruitment by pRb to E2F sites. In order to map the repression domain of RBP1, a panel of C-terminal deletion mutants was made. Using those deletion mutants in reporter assays, the repression domain of RBP1 was mapped to amino acids 241-452. This domain, referred to as R1, contains the ARID domain (aa313-409) preceded by a region that is predicted to form an α-helix (Lai et al., 1999a). When fused to a Gal4 DBD, the R1 region is capable of repressing transcription on its own. Surprisingly, deletion of the R1 domain does not totally inhibit repression by RBP1. This suggested the presence of another repression domain. This second repression domain termed R2 was found to encompass the extreme C-terminal region of RBP1 (aa1167-1257) (Lai et al., 1999b).

Following the identification of the two repression domains of RBP1, studies were conducted to establish the mechanism of repression associated with each domain. The R2 repression domain was found to recruit class I HDAC1-3 (Lai et al., 1999b). It recruits the Sin3 class I HDAC complex via direct interaction with the SAP30 subunit (Lai et al., 2001). In fact, RBP1 was found to be part of the mammalian Sin3 HDAC complex (Lai

et al., 1999b; Zhang et al., 1998a). Interaction studies between the R2 domain and class II and class III HDAC all proved negative (Lai et al., 2001; Lai, 2000a). R2 only recruits class I HDAC and the Sin3 complex is responsible for all of the HDAC activity associated with RBP1.

Repression by R1 is less understood. R1 does not recruit any HDAC activity whether TSA sensitive (class I & II) or NAD^+ dependent (class III) (Lai et al., 2001; Lai, 2000a). Strangely, the ARID domain, which is required for R1 repression, activates transcription when tethered to a promoter via a Gal4 DBD (Lai, 2000a). Furthermore, RBP1 has been reported to recruit histone acetyl transferase (HAT) activity (Chan et al., 1998). Preliminary results suggested that the ARID domain alone was able to recruit HAT activity, but that ARID was unable to recruit any HAT activity in the context of R1 or of RBP1 (Lai, 2000a). These results seem contradictory since RBP1 has been shown to repress transcription while HATs generally activate transcription (Hassig and Schreiber, 1997; Struhl, 1998; Turner, 1991).

1.11.4 Cell biology of RBP1

The cellular biology of RBP1 has not been extensively studied. Early on it was found that overexpression of RBP1 causes growth arrest (Lai et al., 1999a). This property is shared by many tumour suppressors, but deletion of RBP1 has never been demonstrated in cancer or in tumour cell lines. The discovery of RBP1 in an important p130/E2F complex in differentiated myotubes prompted the study of its role in myogenesis (Corbeil et al., 1995; Lai et al., 1999a). Preliminary results demonstrated that during the differentiation process, RBP1 was exported to the cytoplasm (Theberge, 2001). Although RBP1 is exported to the cytoplasm, the Sin3 HDAC complex seems to remain nuclear as HDAC1 did not exit the nucleus with RBP1 (Theberge, 2001). This effect is in opposition to class II HDACs that are exported to the cytoplasm during differentiation (Dressel et al., 2001; Grozinger and Schreiber, 2000; McKinsey et al., 2000). The cellular level of RBP1 was also found to be significantly higher in embryonic stem cells

as compared to more differentially committed cells like myoblasts (Binda and Branton, 2001). This suggests that RBP1 could play an important role in development.

1.11.5 BCAA / RBP1L1

Recently, a group isolated an IgG antibody that is overexpressed in the serum of breast cancer patients and this antibody was used to clone a new gene found to share extensive homology with RBP1 (Cao et al., 2001). This gene, called Retinoblastoma-Binding Protein-1-Like-protein-1 (RBP1L1), is also known as Breast Cancer-Associated Antigen (BCAA) (Cao et al., 2001). The RBP1L1 gene encodes a 1225 aa protein that shares 32% amino acid identity and 42% amino acid similarity with RBP1. It is overexpressed in many cancers (breast, lung, colon, pancreatic and ovarian cancers) (Cao et al., 2001). Unlike RBP1, RBP1L1 was found to localize mainly in the cytoplasm (Cao et al., 2001), which is not surprising since the RBP1 putative NLS is not conserved in RBP1L1. RBP1L1 presents the strongest homology with RBP1 in the N-terminal region, having both a Tudor and ARID domain, as well as in the C-terminal region. RBP1L1 exhibits high homology with RBP1 in the R1 and R2 regions and weak homology in the central region, resulting in the absence of both the chromo domain and the LxCxE motif in RBP1L1. It remains to be tested whether RBP1L1 R1 and R2 regions are functional but, should they be, RBP1L1 could function as a dominant negative mutant of RBP1 by sequestering the HDAC complex away from RBP1 and pRb. Similarly, an RBP1 mutant lacking the LxCxE motif works in a dominant negative manner and slightly activates E2F dependent promoters by competing with endogenous RBP1 for access to repression complexes (Lai et al., 1999a). This could explain RBP1L1 overexpression in many cancers since partial inhibition of pRb active repression, by sequestering repression complexes away, would favour growth.

1.12 pRb in cell cycle

The principal role of pocket proteins during progression in the cell cycle is the regulation of E2F transcription factors (Dyson, 1998; Harbour and Dean, 2000; Nevins, 1998; Sellers and Kaelin, 1997). Binding of pocket proteins to E2F inhibits their ability to activate transcription of DNA biosynthetic enzymes and actively represses transcription of many other genes responsible for cell cycle progression (Adnane et al., 1995; Helin et al., 1993a; Lavia and Jansen-Durr, 1999; Sladek, 1997; Weintraub et al., 1995). This inhibitory action of pocket proteins on E2F activity can be abolished by phosphorylation. Phosphorylation of pocket proteins causes the release of E2Fs that are free to activate different genes and promote S-phase entry (Adams et al., 2000; Chellappan et al., 1991; Harbour et al., 1999). This function of pRb takes place before the restriction point since overexpression of pRb before the restriction point causes growth arrest and accumulation of cells in the G_1 phase, while overexpression after the restriction point does not cause any blockage (Goodrich et al., 1991). Because they bind different E2Fs at different times in the cell cycle, pocket proteins do not regulate the same set of genes (Hurford et al., 1997).

1.12.1 Phosphorylation of pRb

As mentioned above, the activity of pRb and other pocket proteins is regulated by phosphorylation. The activity of pRb has also been shown to be regulated by cell cycle dependent acetylation (Chan et al., 2001a). The phosphorylation level of pRb varies during cell cycle (Buchkovich et al., 1989; Chen et al., 1989; DeCaprio et al., 1989). In G_0 and early G_1 phase of the cell cycle, pRb is hypophosphorylated, and during progression through G_1 it becomes increasingly phosphorylated. This hyperphosphorylation is maintained throughout S, G_2 and most of M-phase. In late M-phase, pRb binds to and is dephosphorylated by protein phosphatase 1 (PP1) in preparation for the next G_1 (Ludlow et al., 1990; Sellers and Kaelin, 1997; Tamrakar et al., 1999).

Phosphorylation of pRb is an ordered process with different cyclin-dependent kinases phosphorylating pRb sequentially at different sites during progression of the cell cycle (Chen et al., 1989; Dyson, 1998; Hinds et al., 1992; Lundberg and Weinberg, 1998). These are serine/threonine kinases that require cyclin regulatory subunits for activity (Pines, 1993). The interaction between cyclins and cdks has been shown to regulate the activity of cdk in different ways. Binding of cyclins to cdk causes conformational changes that remove stearic hindrance within the cdk catalytic site, changes the consensus requirement of cdk and targets cdk complexes to specific substrates (Adams, 2001; Jeffrey et al., 1995; Peeper et al., 1993). Expression of cyclins is sequential during cell proliferation allowing activation of different cyclin-cdk complexes one after the other as cells progress through the cell cycle (Figure 1.6).

pRb has a large number of cdk phosphorylation sites, 16 phosphorylation sites have been recognised (Figure 1.7), and since it is phosphorylated by many different kinase complexes, it is not surprising that phosphorylation of different sites regulate different processes (Adams, 2001; Kaelin, 1999). For example, phosphorylation of T821 and T826 causes the release of LxCxE proteins while phosphorylation of S807 and S811 inhibits the interaction between pRb and c-abl (Knudsen and Wang, 1996).

1.12.1.1 Cyclin D-cdk4/6 phosphorylation of pRb

Upon serum stimulation, transcription of D-type cyclins (D1, D2 and D3) is activated (Connell-Crowley et al., 1998). The expression of D-type cyclins peaks in mid to late G_1 and is required for G_0 exit (Matsushime et al., 1992; Sellers and Kaelin, 1997; Won et al., 1992). These cyclins form complexes with cdk4/6 and are responsible for the initial phosphorylation of pRb (Adams, 2001; Bates et al., 1994; Kato et al., 1993; Lundberg and Weinberg, 1998; Matsushime et al., 1992; Zhang et al., 2000). Sites such

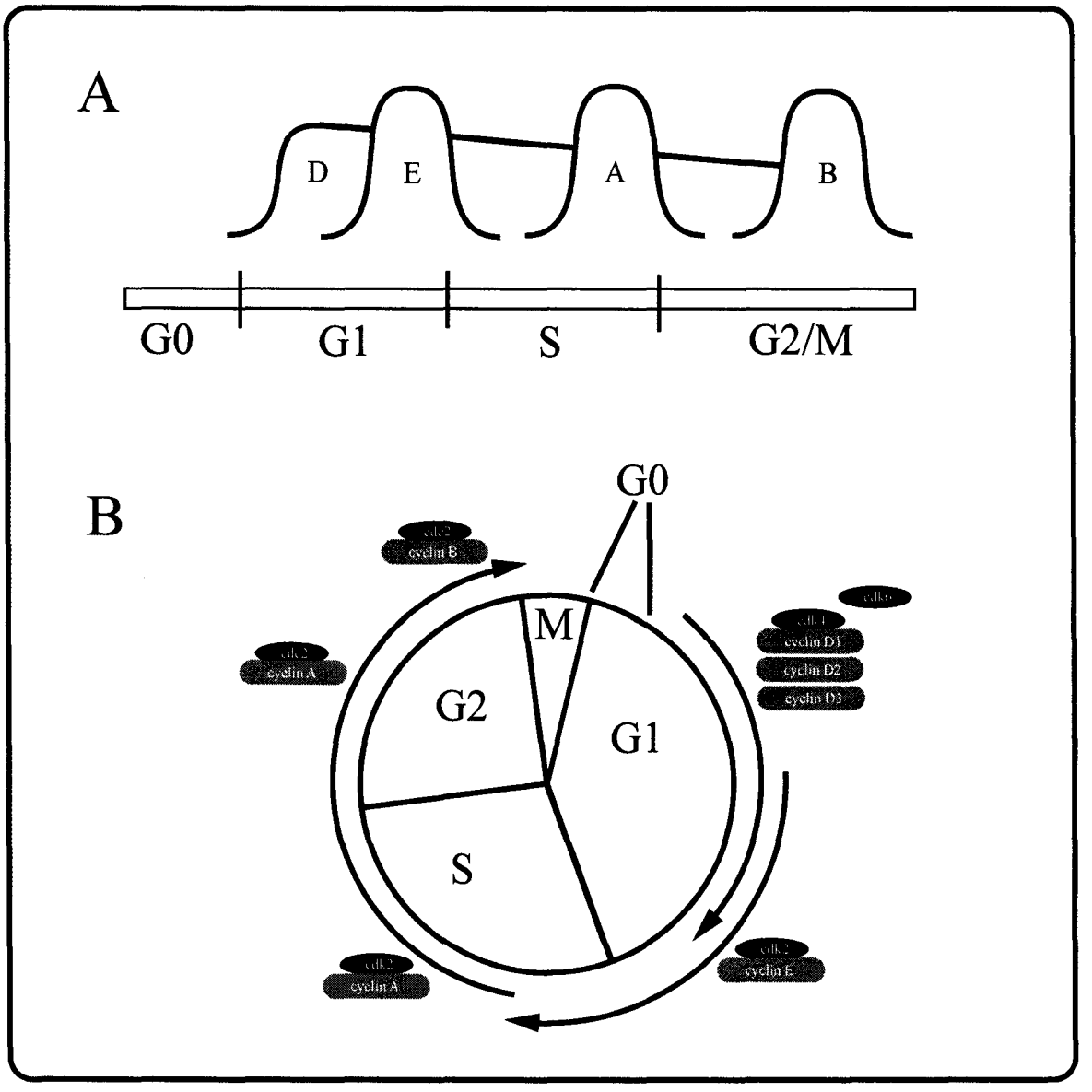


Figure 1.6: Cell cycle expression of cyclins and pattern of activation of cyclin/cdk complexes. Panel A: Pattern of sequential expression cyclins during the cell cycle. Panel B: Active cyclin-cdk complexes in the different phases of the cell cycle (Sellers and Kaelin, 1997; Sidle et al., 1996).

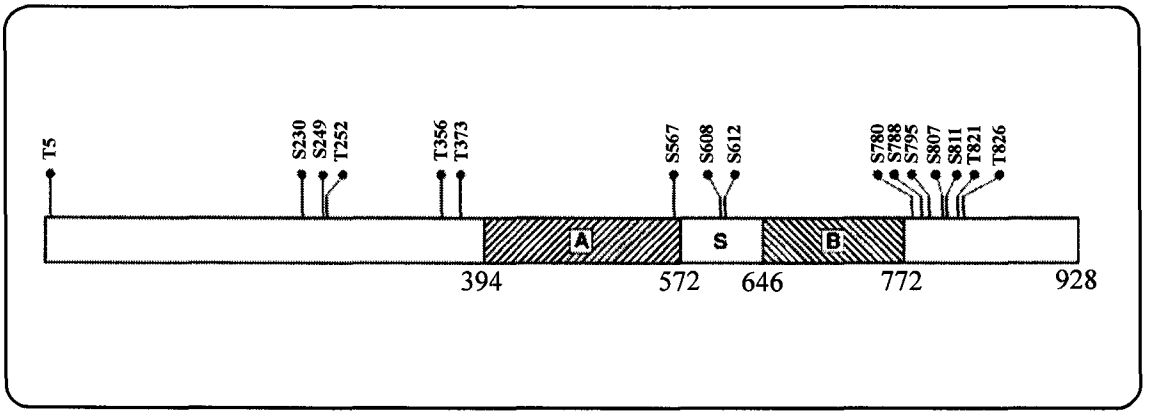


Figure 1.7: Cdk phosphorylation sites in pRb. There are 16 cyclin dependent kinase consensus sites in pRb. There is a large cluster of them in the C-terminus. Adapted from (Kaelin, 1999).

as S780, T826 and S795 have been shown to be preferred by cyclin D-cdk4/6 complexes (Connell-Crowley et al., 1997; Kitagawa et al., 1996; Pan et al., 1998a; Zarkowska and Mittnacht, 1997). The phosphorylation of these sites in the C-terminus causes intramolecular rearrangements allowing the C-terminus to bind to the B box, causing release of LxCxE proteins (Harbour et al., 1999; Lee et al., 1998). The LxCxE binding site in the B box is surrounded by basic residues and it is thought that the interaction between these basic residues and the acidic groups of the phosphates in the C-terminus is responsible for the intramolecular changes leading to the release of LxCxE proteins, like HDAC (Harbour et al., 1999; Knudsen and Wang, 1996; Lee et al., 1998). This intramolecular rearrangement also exposes S567, which is normally buried at the A/B interface, allowing its phosphorylation by cyclin E-cdk2 in late G_1 (Harbour et al., 1999; Lee et al., 1998).

1.12.1.2 Cyclin E-cdk2 phosphorylation of pRb

In late G_1, release of HDACs allows expression of the cyclin E gene (Zhang et al., 2000). Cyclin E levels peak at the G_1/S transition point (Adams, 2001; Dulic et al., 1992; Koff et al., 1992; Sellers and Kaelin, 1997). This cyclin complexes with cdk2, which phosphorylates pRb. The cyclin E-cdk2 complex can only phosphorylate pRb that has previously been phosphorylated by cyclin D-cdk4/6 (Dyson, 1998; Lundberg and Weinberg, 1998). Phosphorylation of S567 by cyclin E/cdk2 disrupts the interaction between the A and B boxes and causes the release of E2F from pRb (Harbour et al., 1999). Interestingly, S567 has been found to be mutated in cancer, which brings credence to this model (Lee et al., 1998). On the other hand, in vivo phosphorylation of S567 has never been shown (Harbour et al., 1999; Lee et al., 1998). Pocket proteins are not the only substrates of cyclin E-cdk2 complexes. This kinase complex has also been shown to phosphorylate E2F5 to increase its transactivation potential (Morris et al., 2000). Cyclin E-cdk2 activity is required for passage through the restriction point (Ekholm et al., 2001).

1.12.1.3 Phosphorylation of pRb after the G_1/S transition

In S-phase, cyclin A is activated by free E2F and replaces cyclin E in cdk2 complexes (Adams, 2001; Sellers and Kaelin, 1997; Tsai et al., 1991). This new kinase further phosphorylates pRb. It also binds to the N-terminus of free E2F1-3 and phosphorylates DP1 (Krek et al., 1995; Xu et al., 1994). This results in a decreased DNA binding activity and release of the transcription factor from the DNA. This phosphorylation step is required for S-phase exit. Prevention of this regulation step by mutation of the cyclin A binding site of E2F1 results in apoptosis (Krek et al., 1995). In G_2 and M phases, pRb is further phosphorylated by cyclin A-cdc2 and Cyclin B-cdc2 respectively (Sherr, 1993; Williams et al., 1992).

1.12.1.4 Phosphorylation of p107 and p130

Phosphorylation of p107 parallels that of pRb, but hypophosphosphorylated p107 appears at the beginning of S-phase (Beijersbergen et al., 1995; Grana et al., 1998; Sidle et al., 1996). This delay is probably due to the decreasing cyclin D-cdk4/6 activity at the time of p107 induction in late G_1. p130 is phosphorylated in G_0 and further phosphorylation in early G_1 results in both export to the cytoplasm and proteasome degradation (Canhoto et al., 2000; Smith et al., 1998).

1.12.2 Cyclins D and E in cancer

Due to the positive role played by D-type cyclins on cell cycle progression it is not surprising that their expression is often upregulated in cancer. Cyclin D1 is overexpressed in many cancers including, oesophageal carcinoma, lymphoma, breast carcinoma and squamous cell carcinoma (Jiang et al., 1992; Schuuring et al., 1992; Seto et al., 1992). Depending on the malignancy, the overexpression can be caused by translocation or gene amplification (Lammie et al., 1991; Rosenberg et al., 1991). In

mice, overexpression of cyclin D can also be induced by retrovirus insertion (Lammie et al., 1992). Although cyclin E also seems to play an important role in control of cell cycle progression, no amplification or gene translocation of the cyclin E gene has ever been reported in human tumours (Beijersbergen and Bernards, 1996). In fact, stable overexpression of cyclin E does not transform normal fibroblasts (Ohtsubo and Roberts, 1993).

1.12.3 Control of cdk activity by phosphorylation

The activity of cdk is also controlled by phosphorylation. Upon binding of cyclin A to cdk2, intramolecular changes expose a phosphorylation site in the catalytic cleft (Jeffrey et al., 1995). Phosphorylation of this site causes intramolecular changes that remove residues blocking the substrate-binding domain, resulting in complete activation of the kinase complex (Jeffrey et al., 1995). The enzyme responsible for this phosphorylation is cyclin H-cdk7 (Fisher and Morgan, 1994; Makela et al., 1994). This phosphorylation step in counteracted by cdc25A and cdc25C phosphatases.

1.12.4 Cdk inhibitors (CKI)

Finally, cdk inhibitors (CKI) represent another level of regulation for cdk. These fall into the $p21^{CIP1}$ and $p16^{INK4a}$ families (Sherr and Roberts, 1995). The $p21^{CIP1}$ family also comprises $p27^{KIP}$ and $p57^{KIP2}$. These are broad range cdk inhibitors that can regulate cyclin D-cdk4, cyclin E-cdk2 and cyclin A-cdk2 complexes (Gu et al., 1993a; Harper et al., 1993; Xiong et al., 1993). Strangely, $p21^{CIP1}$ can be found in active cdk complexes. It is possible that a low level of $p21^{CIP1}$ facilitates formation of active complexes while high expression would lead to inhibition (Harper et al., 1995; Zhang et al., 1994).

The $p16^{INK4a}$ family is composed of three other members, $p15^{INK4b}$, p18 and p19 (Chan et al., 1995; Guan et al., 1994; Serrano et al., 1993). In opposition to the $p21^{CIP1}$

family, these CKIs are more specific. Both $p16^{INK4a}$ and $p15^{INK4b}$, have been shown to compete with cyclin D for binding to cdk4 and cdk6 (Guan et al., 1994; Serrano et al., 1993). Their overexpression has been shown to cause G_1 arrest and leave pRb hypophosphorylated (Koh et al., 1995). Not surprisingly, $p16^{INK4a}$ overexpression does not have any effect in $Rb^{-/-}$ cells since pRb is the main target of cyclin D (Guan et al., 1994; Koh et al., 1995; Lukas et al., 1995; Medema et al., 1995). Being part of the pRb pathway, $p16^{INK4a}$ has also been found to be disrupted in cancer (Koh et al., 1995; Sheaff and Roberts, 1995).

1.13 Transcription repression by pRb

The binding site of pRb is situated in the transcription activation domain of E2Fs. It is therefore not surprising that binding of pRb to E2Fs results in inhibition of their transcriptional activation (Flemington et al., 1993; Harbour and Dean, 2000; Helin et al., 1993a; Ross et al., 1999). This passive repression mechanism is not sufficient to explain the observation that deletion of E2F sites in some promoters results in increased transcription, suggesting that these binding sites function as negative elements (Dalton, 1992). Furthermore, some pRb pocket mutants shown to be incapable of repressing transcription have been found to retain E2F binding, suggesting that E2F binding and transcription repression are separate functions (Sellers et al., 1998). The repression activity of pRb was mapped to the pocket and has been found to be independent of binding to E2F as targeting of the pocket to DNA by fusion with an heterologous DNA binding domain results in repression of transcription (Adnane et al., 1995; Bremner et al., 1995; Sellers et al., 1995; Weintraub et al., 1995). Five different mechanisms have been proposed to explain transcription repression by pRb: 1) interaction with and inhibition of basal transcription factors, 2) recruitment of histone deacetylases (HDAC), 3) recruitment of histone methyl transferases (HMT), 4) recruitment of DNA methylases and 5) recruitment of ATP dependent chromatin remodelling complexes. p107 and p130 are assumed to function similarly although implication of these five mechanism has yet to be proven.

1.13.1 Interaction with basal transcription factors

The homology between pRb and the TATA binding protein (TBP), which is a component of the TFIID transcription factor, led to the idea that pRb could repress transcription by interfering with basal transcription factors (Weintraub et al., 1995). For example, pRb inhibits the kinase activity of $TAF_{II}250$ that is required for initiation of complex formation (Dikstein et al., 1996; Ruppert and Tjian, 1995; Shao et al., 1995; Shao et al., 1997; Siegert and Robbins, 1999; Siegert et al., 2000; Solow et al., 2001).

1.13.2 Histone deacetylases (HDAC)

Histone acetylation has long been known to correlate with transcriptionnally active chromatin (Hassig and Schreiber, 1997; Struhl, 1998; Turner, 1991). The acetylation of lysine residues of N-terminal tails of core histones decreases the amount of positive charge in the core histone octamer. This results in a decreased association of these histone tails with DNA and with neighbouring nucleosomes, causing a loosening of the chromatin structure. The DNA is then more accessible to transcription factors. Conversely, histone deacetylation will compact the nucleosomes and be inhibitory to transcription factor recruitment. It is possible that histones are not the sole target of histone acetyltransferases (HAT) and histone deacetylases (HDAC). Transcription factors such as p53, E2F and pRb itself have all been shown to be regulated by acetylation and could be targeted by HATs and HDACs (Barlev et al., 2001; Chan et al., 2001a; Martinez-Balbas et al., 2000; Marzio et al., 2000).

So far, 17 human histone deacetylases (HDAC) have been cloned (Table 1.2) (Gray and Ekstrom, 2001; Khochbin et al., 2001). These enzymes are separated into three families depending on their homology to yeast HDACs. Class I HDACs are homologous to the co-repressor RPD3, class II HDACs are homologous to Hda1 and class III HDACs are homologous to SIR2 (Frye, 1999; Taunton et al., 1996; Wang et al., 1999).

Table 1.3 Human histone deacetylases (HDAC).		
Class I: RPD3-like	Class II: Hda1-like	Class III: SIR2-like
HDAC1	HDAC4	SIRT1
HDAC2	HDAC5	SIRT2
HDAC3	HDAC6	SIRT3
HDAC8	HDAC7	SIRT4
	HDAC9	SIRT5
	HDAC10	SIRT6
		SIRT7

Table 1.3 Human histone deacetylases (HDAC). Source: (Gray and Ekstrom, 2001; Kao et al., 2001; Zhou et al., 2001)

Class I and class II HDACs are both sensitive to trichostatin A (TSA), a specific HDAC inhibitor (Gray and Ekstrom, 2001; Yoshida et al., 1990). They both form high molecular weight complexes, but they are not part of the same set of complexes (Gray and Ekstrom, 2001; Khochbin et al., 2001). The main difference between class I and class II HDACs is that the activity of the latter is controlled by cellular localisation (Fischle et al., 2001; Grozinger and Schreiber, 2000; Verdel et al., 2000; Wang et al., 2000). Class II HDACs are actively maintained in the cytoplasm by binding to 14-3-3 proteins, which may mask their NLS or prevent them from interacting with importin α. Class III HDACs have a different activity. They possess TSA insensitive NAD^+-dependent HDAC activity (Imai et al., 2000; Landry et al., 2000). They also have been shown to possess ADP-ribosylation activity (Frye, 1999).

1.13.2.1 pRb recruits HDACs

It has recently been shown that pRb recruits HDAC1 to repress E2F dependent promoters (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998). Studies using TSA demonstrated that, in the case of pRb, the requirement for HDAC activity for repression was promoter dependent (Luo et al., 1998). Upon closer examination, it was shown that pRb and the other members of the pocket protein family, p107 and p130, recruit the class I HDAC (HDAC1-3, 8), but not class II (HDAC4-7, 9-10) or class III HDAC (SIRT1-7) (Ferreira et al., 1998; Lai et al., 2001; Lai et al., 1999b; Lai, 2000a). Strangely, p130 has been found to interact with HDAC1 via its C-terminal domain (Stiegler et al., 1998). Recruitment of HDAC could also play a role in the reversible acetylation of pRb (Chan et al., 2001a).

Both HDAC1 and HDAC2 posses an IxCxE motif which was thought to mediate direct interaction with the pocket in a similar fashion as the LxCxE motif (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998). On the other hand, HDAC3 does not possess such a motif and the mechanism of binding of HDAC3 to pRb family proteins remains unclear (Lai et al., 1999b). To study how HDAC interacts with pRb, mutations of the residues of the B box responsible for the interaction with the LxCxE motif have been made. Mutation of the LxCxE binding site resulted in inhibition of HDAC binding to pRb (Chan et al., 2001b; Chen and Wang, 2000; Dahiya et al., 2000). However, this result is controversial as another group reported that mutations that abolish E1A binding to pRb did not abolish HDAC1 binding (Dick et al., 2000). Strangely, no interaction between HDAC1 or HDAC2 and pRb has ever been detected by yeast two hybrid (Brehm et al., 1998; Lai et al., 1999b). It remains unclear whether the interaction between pRb and HDAC is direct.

1.13.2.2 RBP1 participates in HDAC recruitment by pRb

RBP1 has been found to be part of the Sin3 HDAC complex and has been shown to bind to the pocket of pRb via its LxCxE motif (Defeo-Jones et al., 1991; Lai et al., 2001; Lai et al., 1999b; Zhang et al., 1998a). Following a study that showed that neither pRb nor p107 were able to interact directly with HDAC, it was argued that RBP1 may, at least in part, be responsible for recruitment of HDAC to pRb (Lai et al., 2001; Lai et al., 1999b). Furthermore, immunodepletion assays showed that RBP1 is responsible for over half of the HDAC activity associated with pRb (Lai et al., 2001). Depletion of SAP30, which links the Sin3 HDAC complex to RBP1, abolished HDAC activity associated with RBP1 and reduced by half HDAC activity recruited by pRb (Lai et al., 2001). This strongly suggests that half of the HDAC activity recruited by pRb is provided by recruitment of the Sin3 HDAC complex via RBP1. The remaining HDAC activity could be provided by other pRb binding proteins, like c-ski and RBAP48, that also interact with HDAC (Kennedy et al., 2001; Tokitou et al., 1999). This led our group to propose the model depicted in Figure 1.8. The model suggests that RBP1 serves as a linker between the Sin3 HDAC complex and pRb. Hypophosphorylated pocket proteins recruit RBP1 to E2F dependent promoters in G_0 and early G_1. Active repression is brought to the promoter via interaction of the RBP1 R2 domain with the Sin3 HDAC complex and, through its R1 domain, RBP1 also recruits another unknown HDAC independent repression function. Upon phosphorylation by cyclin D-cdk4/6 in mid G_1, the small pocket is disturbed and RBP1 HDAC-dependent and HDAC-independent repression are released from pRb. Recruitment of HDAC to pocket proteins by RBP1 could also play a role in myogenesis due to the presence of RBP1 in the C7 p130/E2F complex in differentiated myotubes (Corbeil et al., 1995; Lai et al., 1999a).

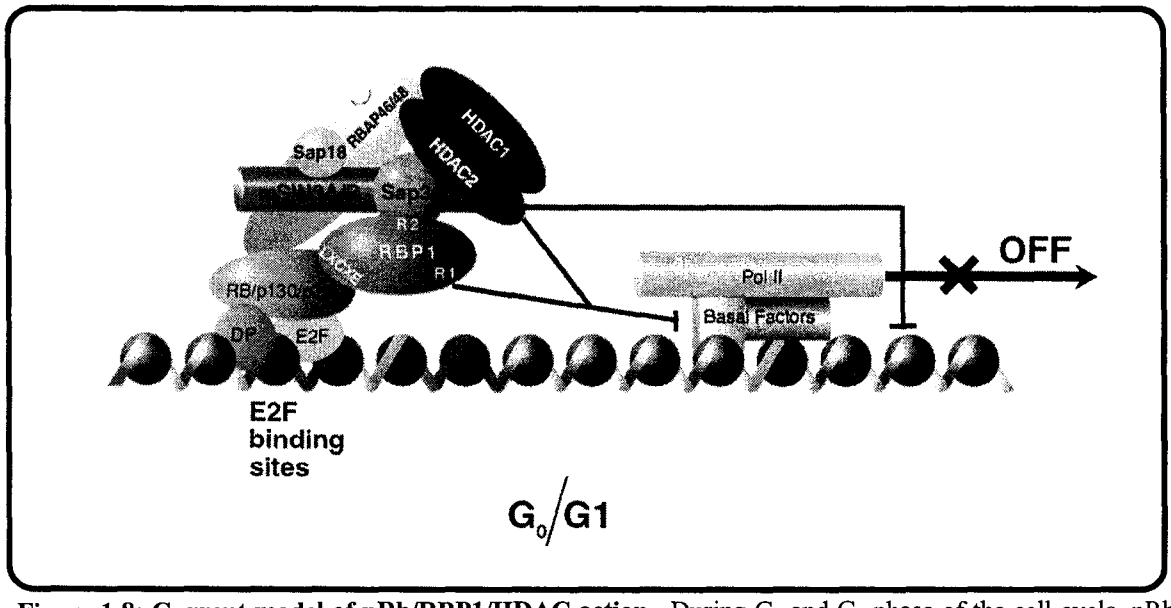


Figure 1.8: Current model of pRb/RBP1/HDAC action. During G_0 and G_1 phase of the cell cycle, pRb binds to E2F/DP heterodimers and is tethered to E2F binding sites. RBP1 binds to pRb pocket via its LxCxE motif and recruits the mSin3A/B HDAC complex via R2 through interaction with SAP30. Another unknown repression function is recruited via R1 to further repress transcription of E2F responsive promoters. This complex is destroyed in mid to late G_1 by phosphorylation of pRb by cyclin D-cdk4/6. Adapted from (Lai et al., 2001).

1.13.3 Suv39H1, a histone metyl transferase (HMT)

HDAC activity is not the only active repression mechanism used by pRb to repress E2F dependent transcription. Recently, the histone H3 lysine 9 specific methylase Suv39H1 has been found to associate and co-operate with hypophosphorylated pRb for repression of E2F transcription (Firestein et al., 2000; Nielsen et al., 2001; Rea et al., 2000; Vandel et al., 2001). In fission yeast, histone H3 methylation has been shown to correlate with heterochromatic silent regions (Nakayama et al., 2001). The absence of an LxCxE motif in Suv39H1 raises the possibility that this interaction could be indirect (Vandel et al., 2001). Methylation of histones can only occur on non-acetylated histones and is predicted to occur downstream of HDACs (Rea et al., 2000). HP1, a protein involved in heterochromatin silencing, has been found to bind methylated histones and could be involved in total shut off of E2F promoters (Bannister et al., 2001; Firestein et al., 2000; Lachner et al., 2001).

1.13.4 DNMT1, a DNA methyl transferase

DNA methylation has also been implicated in pRb active repression (Robertson et al., 2000). DNMT1, a DNA methylase, has been found to interact with pRb to repress E2F dependent promoters (Bestor et al., 1988; Robertson et al., 2000; Robertson et al., 1999). DNA methylation occurs at the 5-position of cytosine in the context of CpG dinucleotide and is thought to silence promoters by preventing consensus sequence recognition by transcription factors (Campanero et al., 2000; Tate and Bird, 1993). Repression of methylated promoters is also increased by binding of a methyl binding protein, MeCP2, that recruits HDAC to further decrease access of transcription factors to DNA (Jones et al., 1998; Nan et al., 1998; Tate and Bird, 1993).

1.13.5 BRG1, hBrm1 and human SWI/SNF complexes

BRG1 and hBrm1, the human homologues of yeast SWI2/SNF2, have also been shown to contain an LxCxE motif and to interact with pRb, p107 and p130 (Dunaief et al., 1994; Khavari et al., 1993; Muchardt and Yaniv, 1993; Singh et al., 1995; Strober et al., 1996). The association of hBRM1 and BRG1 with pRb has been shown to potentiate both repression of E2F promoters and pRb mediated growth arrest (Dunaief et al., 1994; Strober et al., 1996; Trouche et al., 1997). The human SWI/SNF complex remodels chromatin in an ATP dependent fashion by changing the location or the conformation of the nucleosomes without covalently modifying histones (Kingston and Narlikar, 1999). These changes can increase or decrease the accessibility of DNA to transcription factors. Studies in yeast demonstrated that the SWI/SNF complex can both activate or repress transcription in a promoter dependent fashion (Holstege et al., 1998; Sudarsanam et al., 2000). Although both hBrm1 and BRG1 contain an LxCxE motif, they may not interact with the pocket of pRb (Dahiya et al., 2000; Zhang et al., 2000). Complexes containing HDAC and BRG1 simultaneously bound to pRb have been found and have been postulated to be responsible for ordered expression of cyclin E and cyclin A during the progression of the cell cycle (Zhang et al., 2000).

1.14 Repression of non-mRNA genes by pRb

Transcriptional regulation of cell cycle genes and and genes encoding DNA synthetic enzymes by actively repressing E2F dependent transcription is not the only growth suppressive function of pRb. pRb has also been shown to regulate both RNA polymerase I and RNA polymerase III dependent transcription (Cavanaugh et al., 1995; Voit et al., 1997; White et al., 1996).

1.14.1 Regulation of RNA polymerase I

RNA polymerase I is responsible for transcribing rRNA genes that are required for de novo synthesis of ribosomes (Paule and White, 2000). The mechanism of pRb mediated inhibition of RNA polymerase has been studied by two different groups, but, although both groups agree that the main target of pRb is UBF, different conclusions have been reached concerning the mechanism involved (Hannan et al., 2000; Voit et al., 1997). UBF is an accessory transcription factor that enhances initiation complex formation for rRNA genes (Schnapp and Grummt, 1991). pRb has been shown to bind UBF directly, although it is controversial whether the interaction is through the C-terminus or through the pocket (Hannan et al., 2000; Voit et al., 1997). One group has shown that binding of pRb to UBF inhibits its intrinsic DNA binding activity (Voit et al., 1997). The other group showed that while binding of pRb did not affect DNA binding by UBF, it inhibited the interaction with SL-1, which is an essential transcription factor for rRNA genes (Hannan et al., 2000; Paule and White, 2000). Both mechanisms of inhibition of UBF prevent the efficient formation of the initiation complex at rRNA genes. Recently, UBF was also shown to be regulated by acetylation (Pelletier et al., 2000). The acetylation of UBF is suggested to be controlled by recruitment of the pRb-HDAC complex. The role of other pocket proteins was also examined and while p130 has been shown to both bind and inhibit UBF, no interaction between p107 and UBF has been detected (Ciarmatori et al., 2001; Hannan et al., 2000). Inhibition of rRNA synthesis prevents de novo synthesis of ribosomes and prevents cell growth. Ribosome biosynthesis is therefore yet another growth pathway controlled by the pRb pocket protein family.

1.14.2 Regulation of RNA polymerase III

RNA polymerase III is responsible for transcription of the 5S rRNA, tRNA and a few other small RNAs (Paule and White, 2000). Its activity has been found to vary with cell cycle progression (White et al., 1995). It is low in quiescent cells and early in G_1. Toward late G_1 it increases and remains high through S and G_2. Since the activity of

RNA polymerase III is negatively correlated with the activity of pRb, the effect of pRb on RNA polymerase III was examined and it was found that pRb inhibits transcription by RNA polymerase III (White et al., 1996). Because pRb mediated repression affects all promoters, pRb needs to target a factor that is required for transcription of all RNA polymerase III genes. TFIIIB is such a factor and has been found to be the prime target of pRb (Chu et al., 1997; Larminie et al., 1997). Other promoter specific factors including TFIIIC2 and $SNAP_c$ have also been shown to be inhibited by pRb (Chu et al., 1997; Hirsch et al., 2000). pRb binds to TFIIIB and inhibits its function. This interaction is dependent on the pocket of pRb and is specific for the hypophosphorylated form of pRb (Scott et al., 2001). Two mechanisms have been proposed to explain this inhibition. pRb shares homology with two subunits of TFIIIB, TBP and BRF, and it has been proposed that pRb interacts with TBP and BRF binding proteins thereby disrupting TFIIIB (Larminie et al., 1997). Binding of pRb to TFIIIB has also been shown to inhibit both interactions with TFIIIC2 and recruitment of RNA polymerase III by TFIIIB (Sutcliffe et al., 2000). Other members of the pocket protein family, p130 and p107 have been shown to similarly inhibit RNA polymerase III-dependent transcription and to bind to TFIIIB (Sutcliffe et al., 1999). In the absence of RNA polymerase III-dependent transcription, cell growth is inhibited since it requires a constant synthesis of tRNA. This constitutes another mechanism through which pRb controls cell growth. It is interesting to note that pRb is not the only tumour suppressor targeting RNA polymerase III since p53 also inhibits that biosynthetic pathway but in opposition to pRb, p53 targets only certain promoters (Chesnokov et al., 1996).

1.15 Role of pRb in S-phase?

Most of the research conducted so far on pRb focussed on its role in the G_1/S transition. Recently, pRb has also been proposed to play a role in control of S-phase. pRb was shown to inhibit DNA synthesis and to regulate S-phase completion (Bosco et al., 2001; Chew et al., 1998; Knudsen et al., 1998). Furthermore, an E2F-pRb-RBP1-HDAC complex was found to co-immunolocalize with BrdU foci in early S-phase of

primary cells and was suggested to prevent repetitive firing of replication origins (Bosco et al., 2001; Kennedy et al., 2000; Lai et al., 2001). Thinking of pRb only as the master controller of the G_1/S transition may therefore be an oversimplification of its actual role.

Project proposal

The Retinoblastoma (RB) family members, pRb, p107 and p130, are transcriptional regulators that function by masking the transactivation activity of E2F family transcription factors and by providing repression functions (Adnane et al., 1995; Helin et al., 1993a; Weintraub et al., 1995). Complex formation between RB and E2F modulates the expression of genes required for the progression of the cell cycle and DNA synthesis (Sladek, 1997). Several mechanisms by which RB represses transcription have been proposed. Histone deacetylases (HDAC) were cloned recently and were shown to account for part of the RB repression activity (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998). Such associations were mapped to the "small pocket" of RB. However, a direct interaction between the pocket and HDACs may not exist (Lai et al., 2001; Lai et al., 1999b). The crystal structure of the pocket suggests that proteins containing a LxCxE motif could physically interact with certain residues of the pocket (Lee et al., 1998).

RBP1 is a LxCxE containing protein and was cloned as an RB pocket binding protein (Defeo-Jones et al., 1991). Our group has shown recently that RBP1 accounts for both HDAC-dependent and -independent repression activities of the RB pocket (Lai et al., 2001; Lai et al., 1999b; Lai et al., 1999a). The repression activities provided by RBP1 were mapped to two domains (Lai et al., 1999b; Lai et al., 1999a). One of these domains, R2, functions by recruiting the SIN3/HDAC complex while the mechanism of the other domain, R1, remains elusive (Lai et al., 1999b). Preliminary results have demonstrated transcriptional activation and HAT activity associated with the ARID domain that were both abolished in the context of full length R1 (Lai, 2000a). Inhibition of the HAT activity of a basal transcription factor, like $TAF_{II}250$, was therefore hypothesized to be

responsible for repression by R1. The present studies were therefore conducted to characterize binding of HATs and other factors to R1 in an attempt to elucidate the mechanism of R1 repression activity.

Chapter 2: Experimental Materials and Methods

2.1 Plasmids

pGEX 2TK R1, pGEX 2TK ARID, pGEX 2TK dl747C are described elsewhere (Lai et al., 2001). pGEX 2T2C 4EBP3 was a gift from Nahum Sonnenberg (Poulin et al., 1998). pGEX E1A was made by Hugue Corbeil (unpublished). pGEX 2TK dl1016C was made by cloning the cDNA region coding for the first 240aa of RBP1 in frame with GST into the pGEX 2TK vector (Amersham Pharmacia Biotech). The structure of the RBP1 GST-fusion proteins is presented in Figure 2.1. pTind was made by replacing the CMV promoter of pcDNA3.1 Hygro+ (Invitrogen) with the Tetracycline inducible promoter (Tind) of pCA14 Tind. pTind RBP1-HA was made by cloning RBP1-HA from pCA14 Tind RBP1-HA into pTind. pTET-On is commercially available (Clontech).

2.2 Antibodies

The LY11 and LY32 antibodies are mouse monoclonal antibodies against the C-term of RBP1 that were raised by James Decaprio and William Kaelin (Lai et al., 1999b). The NM11 mouse monoclonal antibody against p300/CBP was a gift from Betty Moran (Dallas et al., 1997a; Dallas et al., 1997b). The rabbit polyclonal antibodies raised against SAP30 and actin were gifts from Danny Reinberg (Zhang et al., 1998a) and Gordon Shore respectively. The mouse monoclonal antibodies against HA, 12CA5 and HA11, are commercially available from Roche Diagnostics and Babco respectively. The M73 mouse monoclonal antibody against E1A is a gift from Ed Harlow (Harlow et al., 1985). The IF-8 mouse monoclonal antibody raised against pRb is commercially available from Santa Cruz Biotechnology.

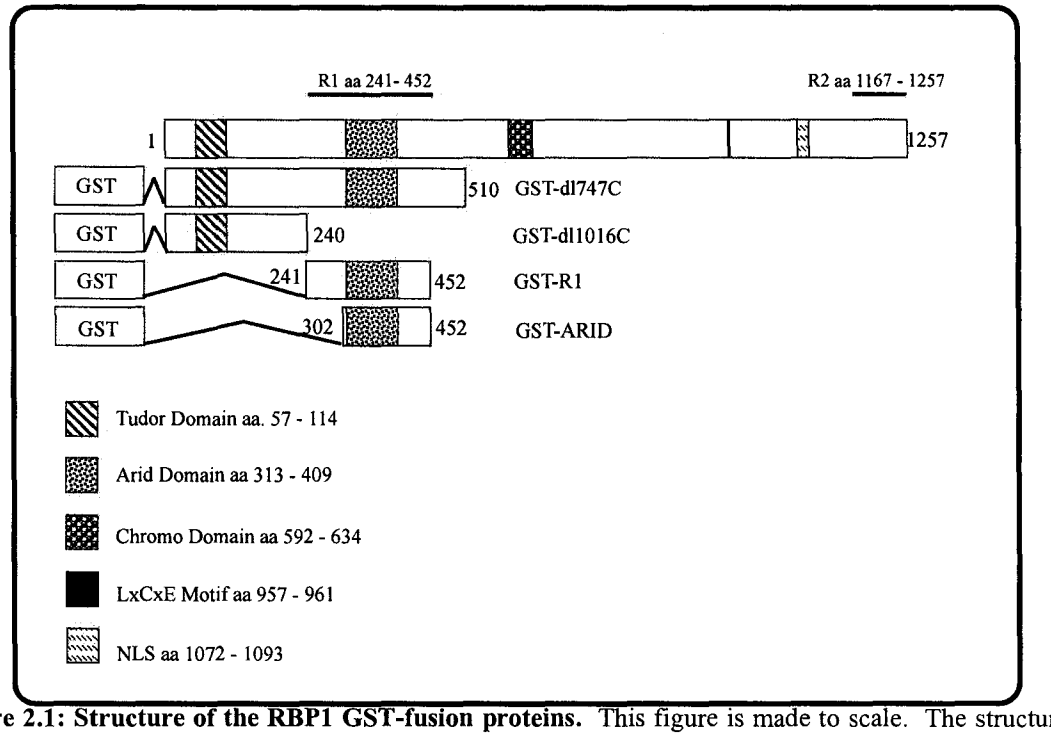


Figure 2.1: Structure of the RBP1 GST-fusion proteins. This figure is made to scale. The structure of the four GST-proteins used in HAT assays and GST-pulldowns is shown here. Adapted from (Lai et al., 1999b).

2.3 Cell Lines

H1299 cells, non small cell lung carcinoma (ATCC CRL-5893), were cultured in α-MEM with 10% Fetal Bovine Serum (FBS) and 1% PSG (100 units/ml penicillin, 100 units/ml streptomycin and 0.292 mg/ml glutamine). 293T cells, Kidney cells transformed with Adenovirus type 5 and SV40 DNA, were cultured in D-MEM with 10% FBS and 1% PSG. MCF7 cells, mammary gland adenocarcinoma (ATCC HTB-22), were cultured in α-MEM with 10% FBS, 20μg/ml bovine insulin and 1% PSG. Hela S3 cells, cervix adenocarcinoma (ATCC CCL-2.2), were cultured in D-MEM with 10% FBS and 1% PSG. C2C12 cells, mouse myoblasts (ATCC CRL-1772), were cultured in D-MEM with 20% FBS and 1% PSG. All cells were cultured in incubators at 37°C in the presence of 5% CO_2 and high humidity.

2.4 Crosslinking of antibodies to beads

12CA5 (α-HA) and LY11 (α-RBP1) antibodies were crosslinked to protein A sepharose 4 fast flow beads (Pharmacia) at 2μg/ml of wet beads according to the protocol provided by Harlow and Lane (Harlow and Lane, 1999). The effectiveness of the crosslinking was tested by SDS-PAGE followed by coomassie blue staining. Samples were taken before and after crosslinking. Good coupling was characterized by the disappearance of heavy chain after crosslinking. For IP, 10μl of wet beads (20μg of crosslinked antibody) were used for each sample.

2.5 GST-Miniprep

Competent BL21 DE3 bacteria (Invitrogen) were transformed with pGEX, pGEX 2TK and pGEX 2T-2C constructs and grown for 16 hours at 37°C on 2YT plates containing 100μg/ml ampicillin (Bioshop). The following morning the bacteria were

transferred to room temperature (RT) for continued growth during the day. Single colonies were used to inoculate 5ml of 2YT liquid media containing 100μg/ml of ampicillin. Liquid cultures were grown for 16 hours in a shaker maintained at 30°C. The volume was then doubled by adding 2YT liquid media containing 100μg/ml of ampicillin and 0.2mM IPTG (Bioshop) for a final concentration of 0.1mM IPTG. Induction was continued for 90min at 30°C. Bacteria were harvested by centrifugation at 3 200rpm in a Sorval RT7 centrifuge for 20min at 4°C. The pellets were resuspended in 800μl of ice cold GST Lysis Buffer (12mM Phosphate Buffer pH 7.4, 250mM NaCl, 2.7mM KCl, 1% (v/v) Triton and 5mM β-Mercapto-Ethanol (βME)) containing protease inhibitor (Aprotinin 2μg/ml, Leupeptine 5μg/ml and Pepstatin 0.4μg/ml) and lysed by two 10sec bursts of sonication. The extracts were cleared by centrifugation at 15 000rpm in a microfuge for 15min at 4°C. The lysates were then incubated with 60μl of ice cold Glutathione sepharose 4 fast flow beads (Amersham Pharmacia Biotech) in GST Lysis Buffer (50% slurry) for 90min at 4°C with gentle agitation. The beads were then pelleted by centrifugation at 2 000rpm in a Sorval RT7 centrifuge for 2min. The supernatant was discarded and the beads were washed 5 times with 1ml of ice cold GST Lysis Buffer and incubated at 4°C with gentle agitation for 10min between washes. The last wash was made using IP Lysis Buffer (20mM Tris-HCl pH 7.5, 150mM NaCl, 2mM EDTA, 1% (v/v) Triton, 2mM NaF, 2mM NaPPi, 500μM Na_3VO_4, 10% (v/v) glycerol and 2mM DTT). Before the last spin, the beads were separated into two new tubes, one half was used for the experiment and the other half was boiled with 60μl of 2X SDS sample buffer for 5min to analyze the input level on SDS-PAGE followed by coomassie blue staining.

2.6 S35 Labelling

Hela S3 cells were cultured as described above in 150mm plates. Upon reaching 80% confluency, the media from the plate was removed. The plates were rinsed once with 10ml of D-MEM media lacking methionine and cystine (D-MEM, Met-, Cys-). 7ml

of fresh D-MEM, Met-, Cys- media was added to the plates and they were incubated at 37°C in the cell culture incubator for 30min. EasyTag™ EXPRESS^{35}S^{35}S Protein Labeling Mix (NEN Life Science Products) was then added to the plate to obtain a final concentration of 100μCi/ml. Cells were labelled for 7 hours in the cell culture incubator at 37°C. Cells were harvested by scraping in 2ml of ice cold 1X PBS (12mM Phosphate Buffer pH 7.4, 137mM NaCl and 2.7mM KCl) and were then pelleted at 4°C by centrifugation at 2 000rpm in a Sorval RT6000B centrifuge.

2.7 Immuno-precipitation (IP) and GST-Pulldowns

Cells were harvested by scraping plates with 1X PBS and pelleted by centrifugation at 2 000rpm in a Sorval RT7 centrifuge for 5min at 4°C. The cell pellets were resuspended in 2.5 cell volumes of ice cold IP Lysis Buffer containing protease inhibitors by vortexing 30sec at the maximum setting and then lysed on ice for 30min. The extracts were cleared by centrifugation at 15 000rpm for 15min in a microfuge at 4°C. The cleared extract was diluted with IP Lysis Buffer containing protease inhibitors to a final volume of 800μl. The diluted lysates were precleared with 60μl of a 1:1 slurry of either protein A or protein G sepharose 4 fast flow beads (Amersham Pharmacia Biotech) in 1X PBS for IP or 60μl of a 1:1 slurry of glutathione sepharose 4 fast flow beads in 1X PBS for GST-pulldowns for 2 hours or overnight (O/N) at 4°C with gentle agitation. Precleared extracts were then incubated with antibodies and 30μl of a 1:1 slurry of either protein A or protein G sepharose 4 fast flow beads in 1X PBS for IPs or with purified GST-proteins still bound to glutathione sepharose 4 fast flow beads for 4 hours or O/N at 4°C with gentle agitation. The beads were then washed five times with 1ml of ice cold IP Lysis Buffer with 10min gentle agitation at 4°C between washes. Beads were then boiled in 60μl of 2X SDS Sample Buffer for 5min.

2.8 Silverstain and protein sequencing

Immunoprecipitations using 200µg of crosslinked LY11 antibody and 20mg of cell extract were performed as described earlier. Proteins present in the immunoprecipitates were separated by SDS-PAGE on a 5%-15% gradient gel. The gel was stained using the Silver Staining Plus kit from Biorad according the manufacturer indications. Bands were excised and destained by repeated washing with a 2% hydrogen peroxide solution. Destained bands were then given to the McGill Proteomic Facility of the Anatomy and Cell Biology Department for peptide mapping and sequencing.

2.9 Histone Acetyltransferase (HAT) assays

Prior to HAT assays, GST-proteins and their binding partners were eluted twice from the glutathione sepharose 4 fast flow beads by two steps of gentle agitation at 4°C in the presence of 15µl of HAT Buffer (50mM Tris-HCl pH 8.0, 10% (v/v) glycerol, 1mM DTT, 1mM phenylmethylsulfonyl fluoride (PMSF) and 100µM EDTA) containing 20mM reduced glutathione. The two eluate fractions were combined. To each eluate, 10µl of HAT Buffer containing 200µg/ml histone mix (Sigma) and 85nCi of ^{3}H acetyl coenzyme A (^{3}H-AcetylCoA) (Amersham Pharmacia Biotech) was added and, after mixing, the samples were incubated at 30°C for 20min. In the presence of HAT activity the ^{3}H-AcetylCoA was transferred to the histones. The reaction mixtures were then spotted on p81 paper disks of 2.5cm diameter (Whatman) and allowed to dry for 1 hour. The dried disks were then washed twice with gentle shaking in 250ml of 50mM sodium carbonate buffer (pH 9.2) at 37°C for 15min. The acetylated histones remained bound to p81 papers while the free ^{3}H-AcetylCoA was washed away. The activity was quantified by scintillation counting of the tritium content of the disks. 200ng of recombinant HIS-P/CAF (gift of Dr. Xiang-Jiao Yang) was used as a positive control for all experiments. For IP, the protocol was similar with the following modifications. The antibodies and their bound proteins were not eluted from the protein A sepharose 4 fast flow beads. In the initial step, the beads were resuspended in 20µl of HAT Buffer containing 100µg/ml

of histone mix and 85nCi of ^{3}H-AcetylCoA. Also, prior to spotting on p81 paper disks, the samples were centrifuged for 10sec at 15 000 rpm in a microfuge to separate the beads from the liquid phase and only the latter was spotted on p81 paper.

2.10 Western blotting

20μg of whole cell extract, quantified using bradford reagent (Biorad), or 20μl of gel filtration fractions were separated according to size on 6% or 10% polyacrylamide gels using SDS-PAGE. The proteins were then transferred, under a constant 1.2mA/cm^2 current, to methanol activated immobilon-P PVDF membranes (Millipore) using a semi-dry transfer apparatus. The membranes were then blocked in TBS-T (25mM Tris-HCl pH 7.2, 137mM NaCl, 2.7mM KCl, 1% (v/v) Calf Serum (CS), 1% (v/v) glycerol and 0.1% or 0.5% (v/v) Tween 20) with 5% powdered milk for 2 hours at RT or O/N at 4°C. See Table 2.1 for working dilutions of the different immunoblotting antibodies. The membranes were then rinsed three times with TBS-T to remove all traces of milk. They were then incubated with the different primary antibodies diluted in Antibody Dilution Buffer (25mM Tris-HCl pH 7.2, 137mM NaCl, 2.7mM KCl, 5% (v/v) CS, 5% (v/v) glycerol and 0.1% or 0.5% (v/v) Tween 20) for 2 hours at RT or O/N at 4°C. Following the incubation with the primary antibody, the membranes were then rinsed 3 times and washed 6 times with TBS-T with 5min of gentle agitation between washes. After the last wash, the membranes were incubated with the appropriate horse radish peroxidase (HRP) conjugated secondary antibodies (Jackson Immuno Research Laboratories) diluted in TBS-T with 5% powdered milk for 1 hour at RT. Again, the membranes were rinsed thrice and washed 6 times with TBS-T. Two more rinces were carried out with distilled water to remove serum wich inhibits HRP activity. The proteins recognized by the primary antibody were visualized by using enhanced luminol reagent according to the manufacturer's specification (NEN Life Science Products) followed by exposition on photographic films (Kodak).

Table 2.1: Working conditions for immunoblotting antibodies				
Antigen	Clone	Type	Dilution	% Tween20
RBP1	LY32	Mouse Monoclonal	1:10	0.1%
p300/CBP	NM11	Mouse Monoclonal	1:500	0.5%
HA Tag	HA11	Mouse Monoclonal	1:1000	0.1%
pRb	IF-8	Mouse Monoclonal	1:500	0.1%
E1A	M73	Mouse Monoclonal	1:1000	0.1%
SAP30	-----	Rabbit Polyclonal	1:1000	0.5%
Actin	-----	Rabbit Polyclonal	1:25000	0.5%

2.11 Infection with adenovirus vectors.

H1299 cells grown to 50% confluence in 150mm plates were infected with adenovirus vectors expressing RBP1-HA under the control of a tetracycline inducible promoter (Tind) and rTTA, a transcription factor activating the Tind promoter in the presence of doxycycline, at a total multiplicity of infection of 100 plaque-forming units per cell. Infections were performed for 1hr in 5ml of fresh media. Following infection, the volume of media was increased to 20ml and induction of the expression of RBP1-HA was continued for 48hrs in the presence of 1μg/ml of doxycycline, a tetracycline homologue.

2.12 Stable cell line establishment

H1299 cells from a 60mm dish were transiently transfected using lipofectamine plus reagent according to the manufacturer protocol (Gibco BRL) with 2μg of pTET-ON and 0,2μg of pTind-RBP1-HA. Cells were then selected for 2 weeks with 500μg/ml hygromycin B (Roche Diagnostic) until separate colonies were visible. Single colonies were then picked and grown separately while lowering the selection pressure to 400μg/ml

hygromycin B. Expression level of each clone was tested by inducing expression with 1μg/ml doxycycline (Sigma) for 48hrs. After establishment of clones, cells were kept in culture with 100μg/ml of hygromycin B.

2.13 Nuclear extract and cytoplasmic extracts

The cell fractionation protocol used was similar to the one described by Dignam (Dignam et al., 1983). Hela S3 cells were grown to 80% confluence and were harvested by scraping. The cells were pelleted by centrifugation at 2 000rpm for 5min at 4°C in a Sorval RT7 centrifuge and were washed with 5 cell volumes of ice cold 1X PBS. The cell pellet was then resuspended in 5 cell volumes of ice cold Buffer A (10mM HEPES pH 7.9, 1.5mM $MgCl_2$, 10mM KCL and 0.5mM DTT) and allowed to stand at 4°C for 10min. The cells were then pelleted by centrifugation at 2 000rpm for 5min at 4°C in Sorval RT7 centrifuge. The size of the cell pellet was seen to increase as cells swell in the hypotonic Buffer A. The cell pellet was resuspended in 2 initial cell volumes of ice cold Buffer A and lysed with 10 strokes of Kontes all glass homogenizer B type pestle. The extent of lysis was checked qualitatively using light microscopy. If lysis was complete, the lysed cell suspension was centrifuged for 10min at 2 000rpm in Sorval RT7 centrifuge at 4°C. The supernatant was decanted into another tube, mixed with 0.11 volume of ice cold Buffer B (0.3M HEPES pH 7.9, 1.4M KCL and 30mM $MgCl_2$), centrifuged for 60min at 15 000rpm in a microfuge at 4°C and dialysed O/N against Buffer D (20mM HEPES pH 7.9, 20% (v/v) glycerol, 0.1M KCl, 0.2mM EDTA, 0.5mM PMSF and 0.5mM DTT). The resulting extract constituted the cytoplasmic fraction. The pellet obtained after lysis with the homogenizer contained the nuclei and was re-centrifuged for 30min in a microfuge at 4°C to remove all traces of cytoplasmic extract. The nuclei were resuspended in 3ml of ice cold Buffer C (20mM HEPES pH 7.9, 25% (v/v) glycerol, 0.42M NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM PMSF and 0.5mM DTT) per 10^9 cells using a Kontes homogeneizer and were then incubated at 4°C with gentle mixing for 30min. The extract was then centrifuged for 30min in a microfuge at

4°C and dialysed against Buffer D O/N. The dialysed extract was centrifuged for 30min in microfuge at 4°C. This cleared extract constituted the nuclear extract.

2.14 Gel filtration

Chromatography was conducted at 4°C using the AKTA purifier HPLC system (Amersham Pharmacia Biotech). 1 ml of nuclear extract, 2mg/ml in Buffer D, was loaded in a Superdex 200 gel filtration column (Amersham Pharmacia Biotech). The complexes were eluted by size using 1.5 column volumes of ice cold GF Buffer (25mM HEPES pH 7.5, 150mM NaCl and 1mM EGTA) at a flow rate of 400µl/min. The proteins were collected in 250µl fractions. The absorption at 280nm was measured throughout the procedure to determine which fractions contained proteins. To calculate the size of complexes, a standard curve obtained with the same column by separating proteins of known sizes was used (Dr. D. Boivin personal communication). Proteins or complexes of size greater than 100kDa are predicted to elute before fraction 50 and the first fraction found to contain proteins by spectrophotometry was fraction 25. Therefore, to ensure the detection of both free RBP1 (143kDa) and complexed RBP1 (> 143kDa), fractions 25 to 50 were kept and analyzed.

Chapter 3: Experimental Results

3.1 HAT activity associated with RBP1

The repression mechanism of the R2 domain has been found to involve recruitment of the Sin3 HDAC complex (Lai et al., 2001; Lai et al., 1999b). On the other hand, that of the R1 domain remains elusive (Lai et al., 1999b). RBP1 has been reported to recruit histone acetyl transferase activity (HAT) (Chan et al., 1998) and preliminary results from our group suggested that HAT activity may be associated with the ARID domain of RBP1 (Lai, 2000a). Strangely, no HAT activity was detected with full length RBP1 or with complete R1 domain.

In order to clarify whether or not RBP1 interacted with HATs, HAT assays were performed using the same GST-fusion proteins that had previously been used (Lai, 2000a). The results are presented in Figure 3.1. No HAT activity was found to be associated with either GST-R1 or GST-ARID (Panel A). Recombinant P/CAF, a known HAT (Yang et al., 1996), showed significant HAT activity. E1A protein is known to interact with p300, another HAT (Jones, 1995; Moran, 1993; Ogryzko et al., 1996). As expected, GST-E1A interacted with p300 and was associated with significant HAT activity (Panel A and C). GST-E1A was expressed at a much lower level than either GST-R1 or GST-ARID (Panel B). Therefore, if any HAT activity was associated with either GST-R1 or GST-ARID, it should have been detected.

The initial report of HAT activity with RBP1 was obtained using co-immunoprecipitation experiments in MCF7 a human mammary gland adenocarcinoma cell line (ATCC HTB-22) (Chan et al., 1998). It is therefore possible that interaction between RBP1 and HATs is cell line specific. To verify this possibility, co-immunoprecipitation experiments were performed in MCF7, Hela-S3 and 293T cells. The averages of three such experiments are presented in Figure 3.2. Again, no HAT activity was found to be associated with RBP1 either in MCF7 or Hela-S3 cells (Panel A) even if significant amounts of RBP1 were precipitated with the LY11 antibody (Panel B). As expected, recombinant P/CAF showed high HAT activity. Immunoprecipitations using p300 antibody (NM11) in either Hela-S3 or MCF7 cells yielded significant HAT

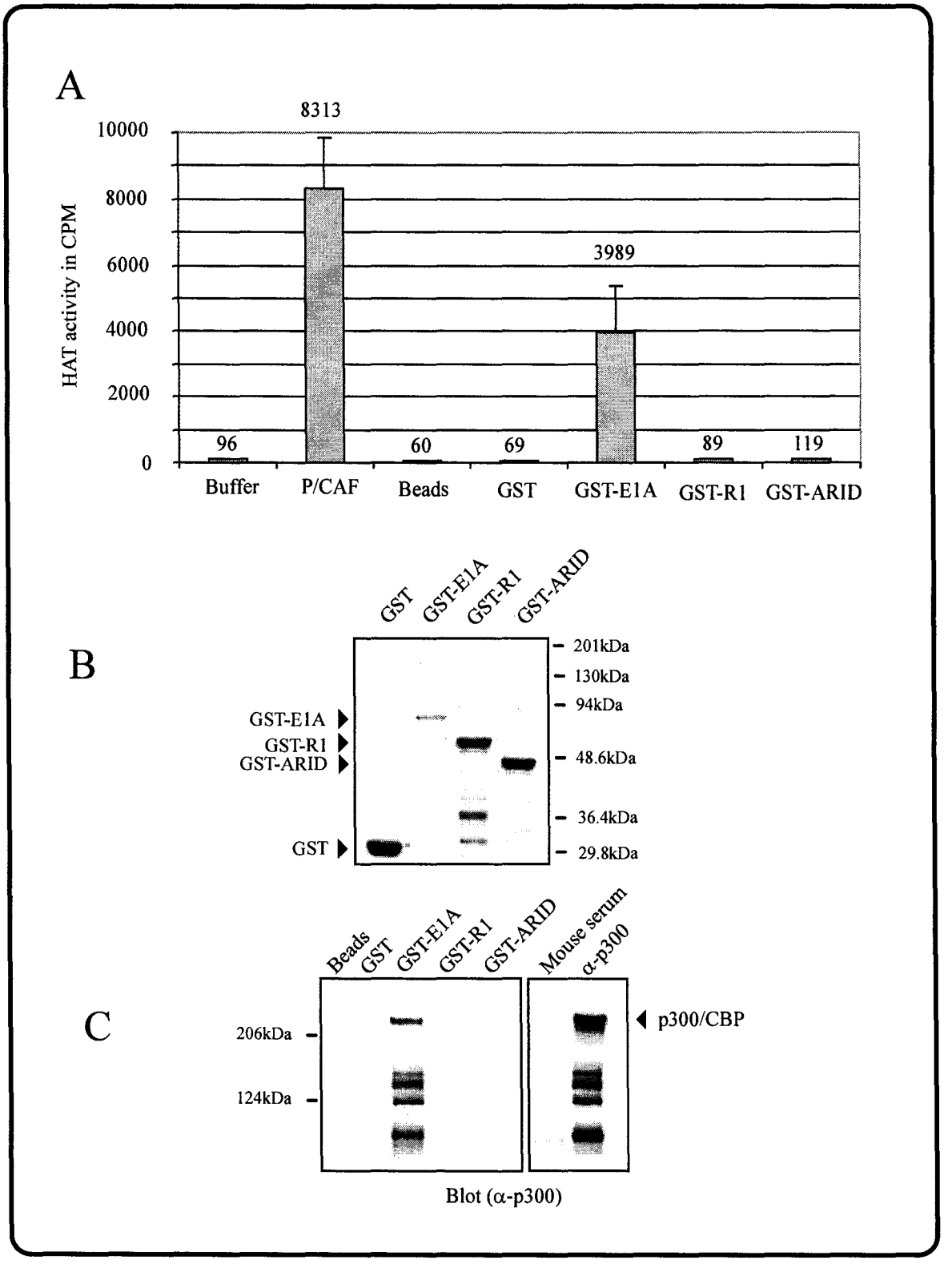
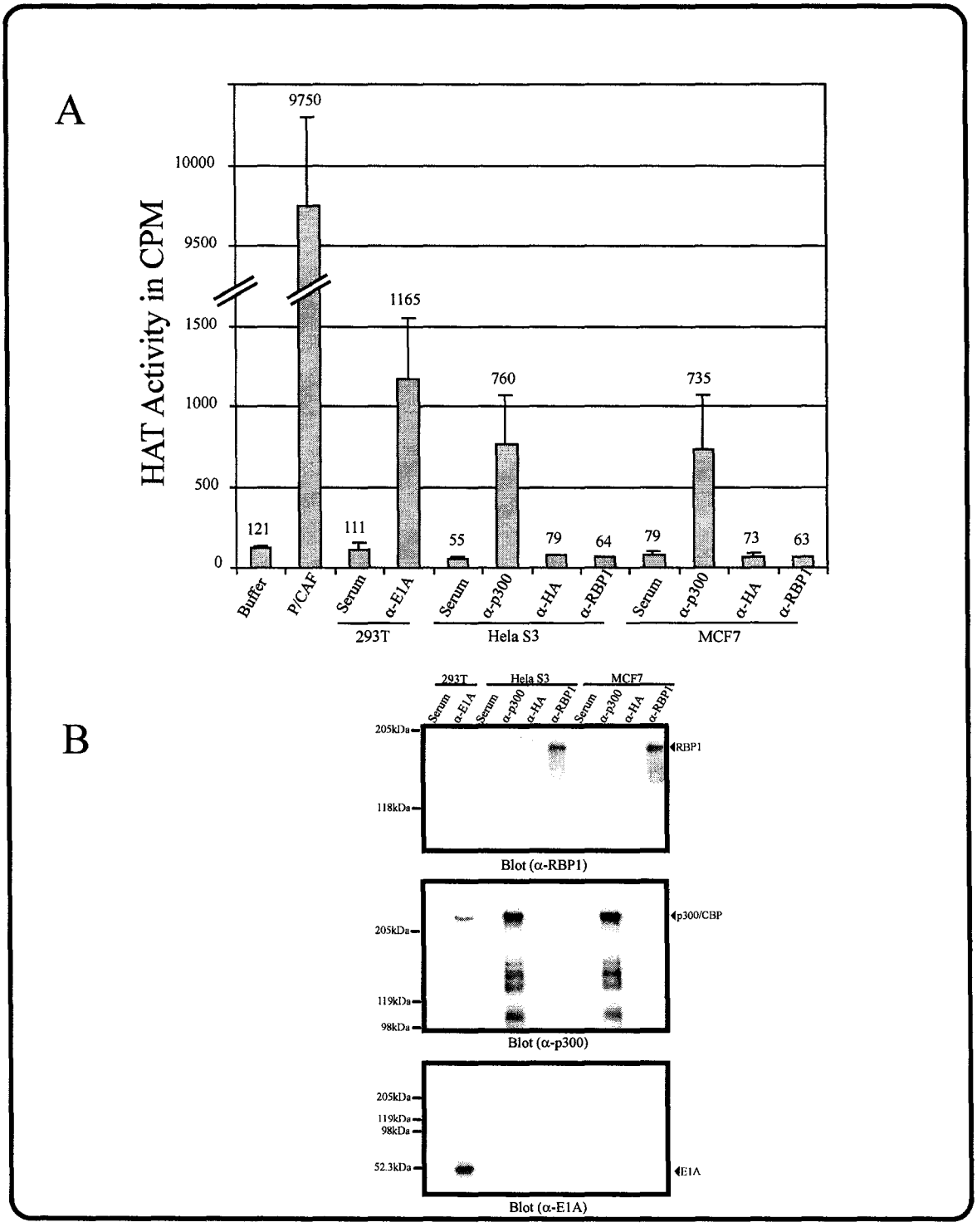

A
8313
3989
10000
8000
6000
4000
2000
0
HAT activity in CPM
96
60
69
89
119
Buffer
P/CAF
Beads
GST
GST-E1A
GST-R1
GST-ARID
B
GST
GST-E1A
GST-R1
GST-ARID
201kDa
130kDa
94kDa
48.6kDa
36.4kDa
29.8kDa
GST-E1A
GST-R1
GST-ARID
GST
C
Beads
GST
GST-E1A
GST-R1
GST-ARID
Mouse serum
α-p300
206kDa
124kDa
p300/CBP
Blot (α-p300)

Figure 3.2: HAT activity associated with RBP1 in Hela-S3 and MCF7 cells. Cells were harvested and lysed in IP Lysis buffer. Immunoprecipitations using either mouse serum, α-E1A (M73), α-p300 (NM11), α-HA (12CA5) or α-RBP1 (LY11) antibodies were performed. The last wash of the immunoprecipitations was done using HAT buffer to remove all traces of detergent. Immunoprecipitations were then used to conduct HAT assays. 200ng of P/CAF was used as a positive control for HAT activity. Panel A: Averages and standard deviations of three independent experiments. Panel B: Western blots on immunoprecipitations against RBP1, p300 and E1A for one representative experiment.


A
HAT Activity in CPM
10000
9500
1500
1000
500
0
9750
1165
760
735
121
111
55
79
64
79
73
63
Buffer
P/CAF
Serum
α-E1A
Serum
α-p300
α-HA
α-RBP1
Serum
α-p300
α-HA
α-RBP1
293T
Hela S3
MCF7
B
293T
Hela S3
MCF7
Serum
α-E1A
Serum
α-p300
α-HA
α-RBP1
Serum
α-p300
α-HA
α-RBP1
205kDa
118kDa
RBP1
Blot (α-RBP1)
205kDa
119kDa
98kDa
p300/CBP
Blot (α-p300)
205kDa
119kDa
98kDa
52.3kDa
E1A
Blot (α-E1A)

activity (Panel A). E1A was again used as a positive control and found to precipitate with p300 and HAT activity (Panel A and B). Strangely, the α-p300 antibody immunoprecipitated more p300 but less HAT activity in MCF7 and Hela-S3 cells than did the α-E1A antibody (M73) in 293T cells. This discrepancy is possibly due to interference between the NM11 antibody and the activity of p300.

3.2 Detection of proteins binding to RBP1 by GST-pulldowns

HATs do not seem to be involved in transcriptional repression by R1. However, R1 could be recruiting other co-repressors. When tethered to promoters by fusion with the Gal4 DNA binding domain, the R1 domain has been shown to repress transcription (Lai et al., 1999a). Preliminary experiments using EMSA suggested that GST-R1 may interact with cellular proteins (Binda and Branton, 2001). To identify R1-interacting proteins, GST-pulldown experiments were performed with GST-R1 and GST-ARID using whole cell extracts from ^{35}S-Met-labelled Hela-S3 cells. The goal is to detect potential R1 binding proteins that will ultimately be isolated from large scale GST-pulldowns from non-labelled extracts and identified by peptide mapping and sequencing. As the N-terminus of RBP1 has not been well characterized functionaly, GST-dl1016C and GST-dl747C were also included. In addition, GST and GST-4EBP3, a protein binding to and inhibiting the translation factor eIF4E, were used as negative controls. The results are presented in Figure 3.3. Five proteins were found to interact specifically with the the different RBP1 GST-fusion proteins. Their sizes and the specific mutants with which they interact are presented in Table 3.1. The intensities of the specific species are much lower than those of the non-specific species that interact with all fusion proteins. Because they occured at a fraction of the background of non-specifically binding proteins, these specific interactors could prove very difficult to isolate for sequencing.

Figure 3.3: GST-pulldowns on ^{35}S-Met-labelled extracts. Hela-S3 cells were labelled as described in the materials and methods section. They were harvested and lysed in IP Lysis buffer. GST pulldowns were conducted with whole cell lysate using GST, GST-4EBP3, GST-dl1016C, GST-dl747C, GST-R1 and GST-ARID. The pulldowns were separated by SDS-PAGE on a 5%-15% gradient gel. Panel A: Representative autoradiography of GST-pulldowns. The migration of large amounts of GST-fusion proteins disrupt the migration pattern of interacting proteins. To simplify analysis of the autoradiography, the position of the different GST-proteins is indicated on the left side of the figure. Different symbols (▸ , ✓ , *, ✦, #) have been inserted in the figure to mark the position of interesting species. Panel B: Coomassie blue staining of 10% of the GST-protein input.

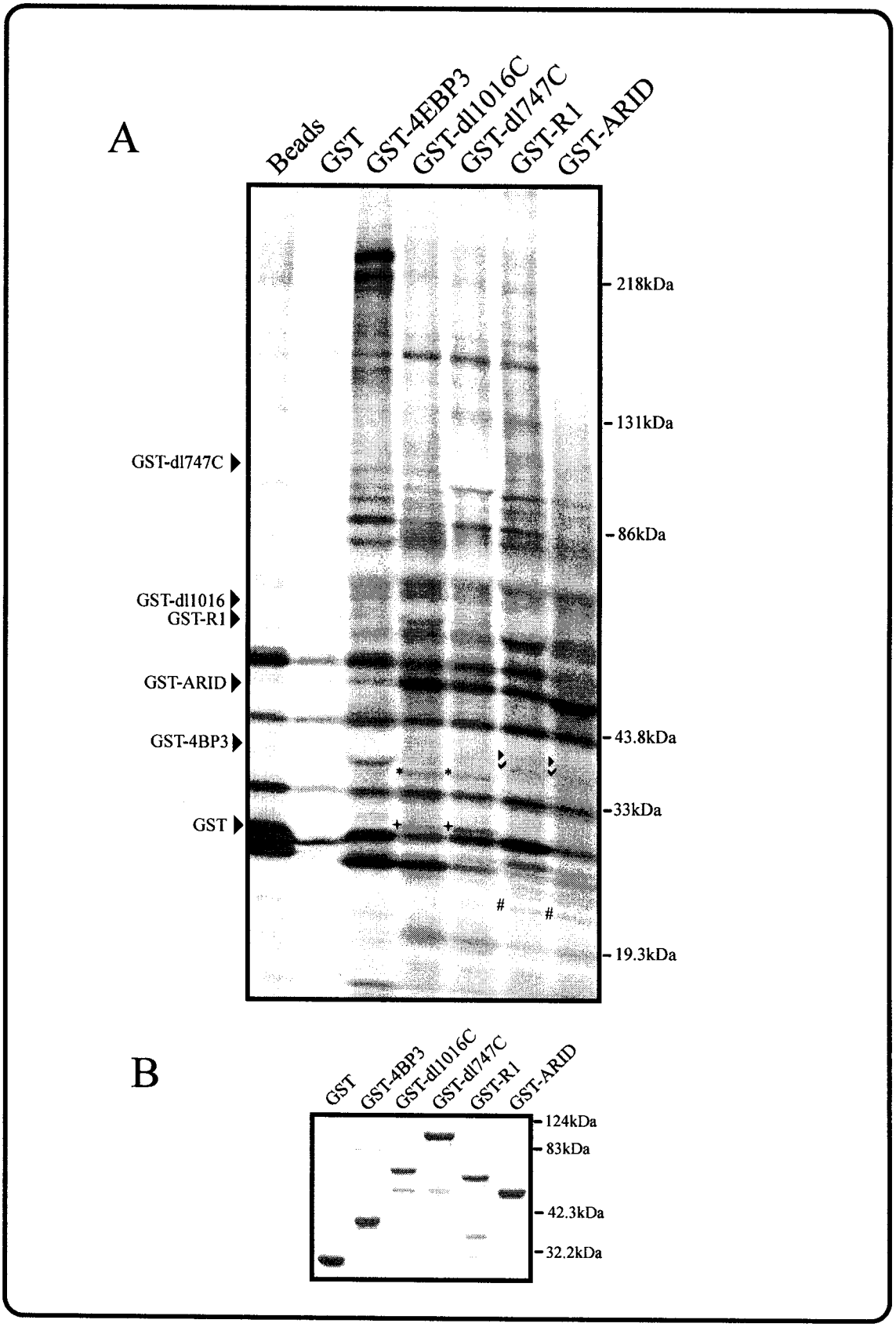

A
Beads
GST
GST-4EBP3
GST-dl1016C
GST-dl747C
GST-R1
GST-ARID
218kDa
131kDa
86kDa
43.8kDa
33kDa
19.3kDa
GST-dl747C
GST-dl1016
GST-R1
GST-ARID
GST-4BP3
GST
B
GST
GST-4BP3
GST-dl1016C
GST-dl747C
GST-R1
GST-ARID
124kDa
83kDa
42.3kDa
32.2kDa

Table 3.1 Putative binding proteins associated with GST fusion RBP1 mutants.					
Symbol	▶	✔	*	+	#
Size (approximate)	40kDa	38kDa	35kDa	29kDa	24kDa
GST	-	-	-	-	-
GST-4EBP3	-	-	-	-	-
GST-dl1016C	-	-	+	+	-
GST-dl747C	-	-	+	+	-
GST-R1	+	+	-	-	+
GST-ARID	+	+	-	-	+

Table 3.1 Putative binding proteins associated with GST fusion RBP1 mutants. "+" refers to a specific interaction and "-" refers to a lack of interaction.

3.3 Detection of proteins binding to RBP1 by immunoprecipitation

Isolation of proteins binding to R1 from GST-pulldowns does not seem to be practical. On the other hand, co-immunoprecipitation has successfully been used by our group to isolate proteins binding to the adenovirus type 5 E4-ORF6 protein (Querido et al., 2001). A similar approach was therefore used to isolate proteins binding to RBP1. Again, the goal is to isolate sufficient amounts of species present in α-RBP1 immunoprecipitates to allow identification by either peptide mapping or sequencing. A mouse monoclonal α-RBP1 immunoprecipitation antibody, LY11, has been shown to immunoprecipitate large amounts of RBP1 from extracts from asynchronously growing cells (Lai et al., 1999a). This antibody was used to immunoprecipitate RBP1 complexes from ^{35}S-Met-labelled Hela-S3 cells extracts (Figure 3.4). Although a very limited number of species are found to interact non-specifically with protein A sepharose (Beads), mouse pre-immune serum (Mouse Serum) or crosslinked 12CA5 α-HA

antibody, there is a great number of species interacting with crosslinked LY11 α-RBP1 antibody. The lower molecular mass species generally exhibited a much higher intensity than those having a higher molecular mass. RBP1 is known to migrate at an apparent molecular mass between 180kDa and 200kDa (Fattaey et al., 1993; Otterson et al., 1993; Zhang et al., 1998a). The major species in that size range was indicated as RBP1 on the right side of the figure. Surprisingly, this species is present at much lower levels than the RBP1 species reported in the initial characterization of the LY11 antibody (Lai et al., 1999a). The low levels of RBP1 in immunoprecipitations using the LY11 antibody is repeatable using several different cell lines (293T, H1299 and Hela S3). It has been suggested that RBP1 has a long half-life and a slow turnover rate (Defeo-Jones et al., 1991; Otterson et al., 1993). Since immunoprecipitations of ^{35}S-Met-labelled extracts only detect proteins synthesized during the labelling step, the low intensity of the RBP1 species could simply be due to a slow rate of de novo synthesis and this may not reflect the actual amount of RBP1 present in the immunoprecipitates. To confirm which species corresponds to RBP1 in α-RBP1 immunoprecipitates, the result of Western blotting against RBP1 on proteins immunoprecipitated with the LY11 antibody from non-labelled extracts was compared to the autoradiography from proteins immunoprecipitated with the LY11 antibody from ^{35}S-Met-labelled extracts. To permit comparison, proteins from both immunoprecipitates were separated alongside by SDS-PAGE. RBP1 was found to migrate below 200kDa, around 180kDa (Figure 3.5 Panel B). The intensity of the RBP1 species is much lower when compared to other species than what had previously been reported (Panel A adapted from (Lai et al., 1999a)).

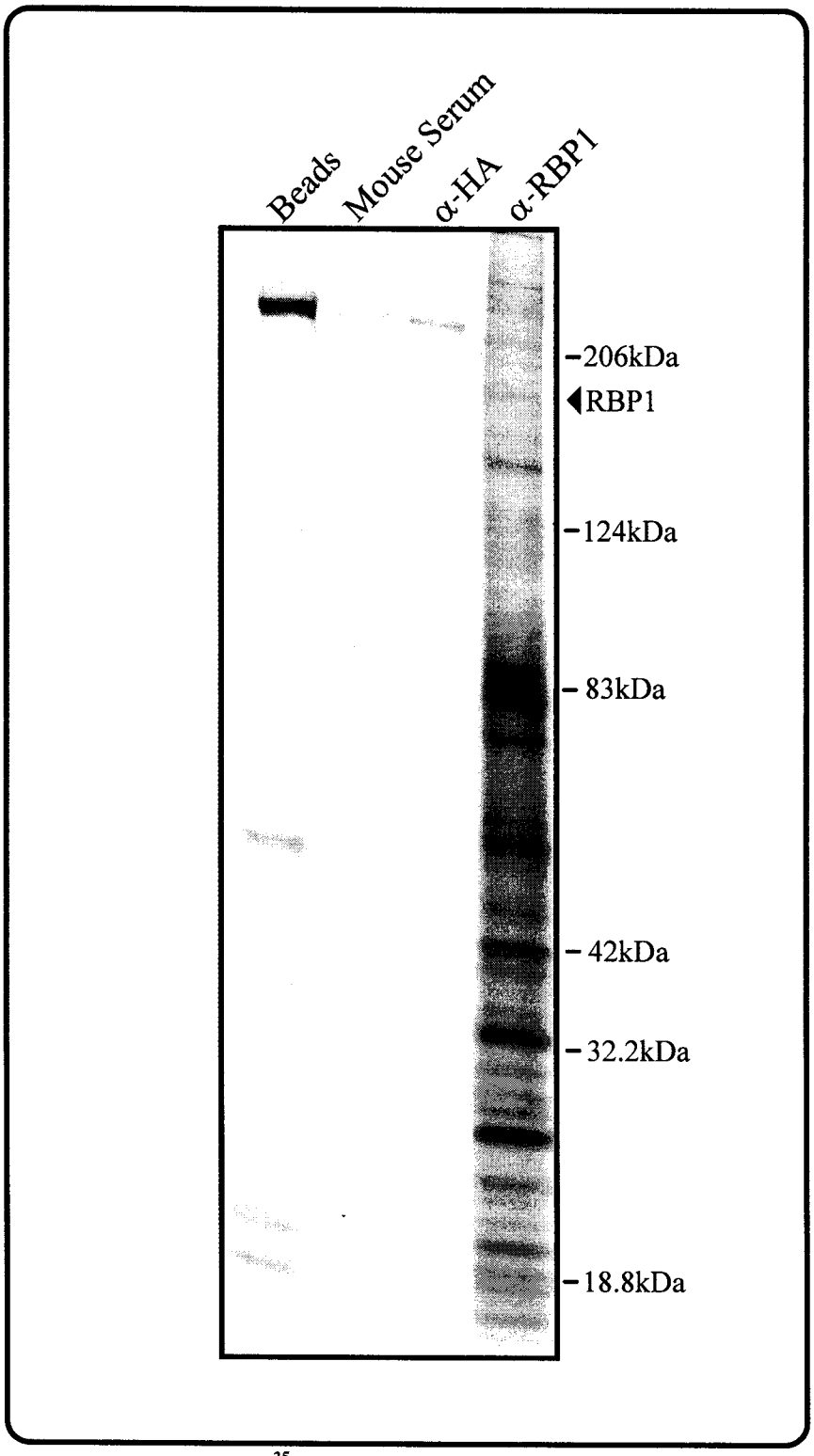
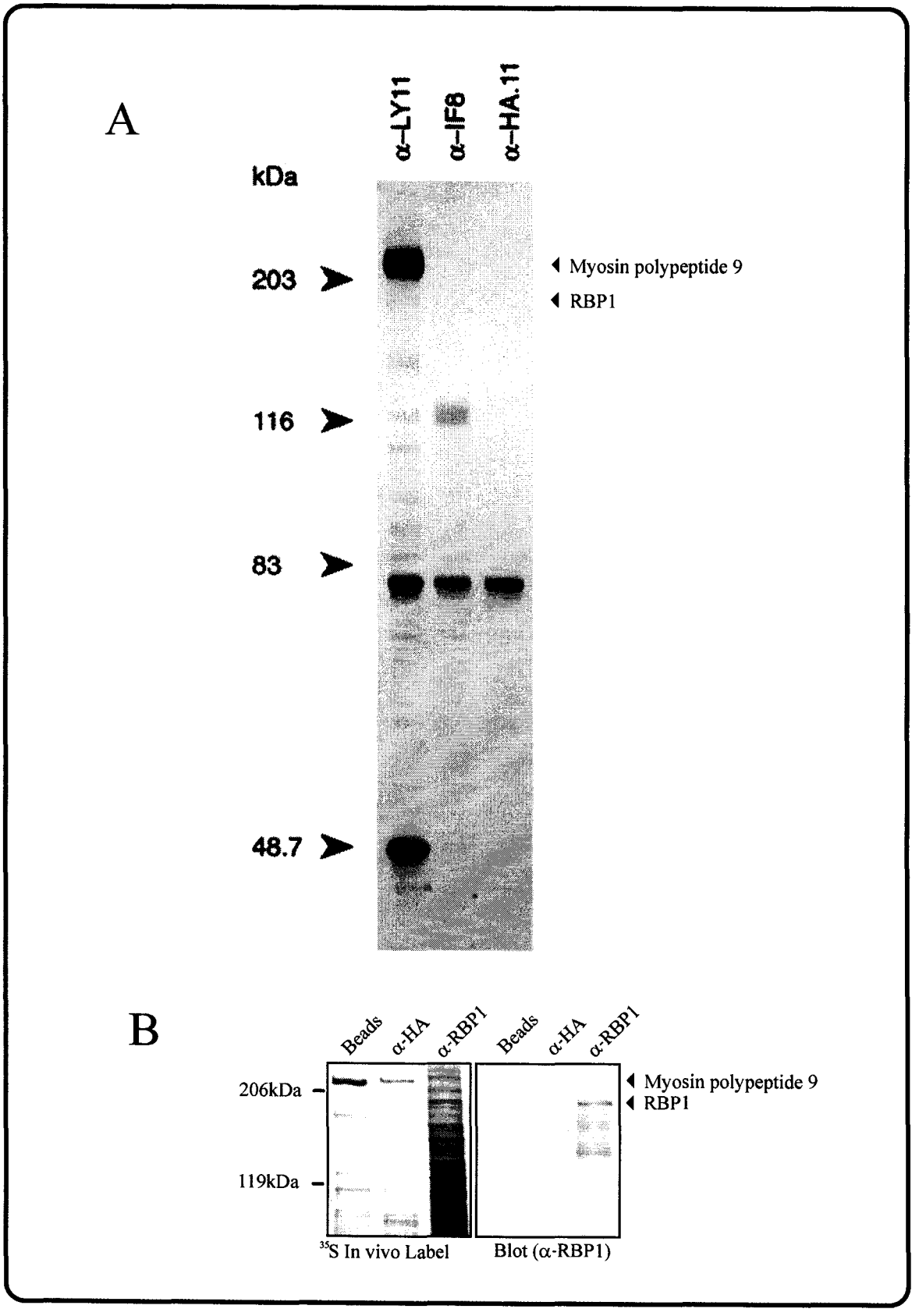


Figure 3.4: Immunoprecipitation on ^{35}S-Met-labelled extracts. Hela-S3 cells were labelled as described in the materials and methods section. They were harvested and lysed in IP Lysis buffer. The extracts were used for immunoprecipitation using α-HA (12CA5) and α-RBP1 (LY11) antibodies. Interacting proteins were eluted from protein A sepharose by boiling in 2X sample buffer and separated on a 5%-15% gradient gel by SDS-PAGE. This figure shows a representative autoradiography. The species assumed to be RBP1 is labelled on the right side of the figure.

Figure 3.5: Characterization of the amount of RBP1 immunoprecipitated with LY11. Hela-S3 cells were labelled as described. Cells were harvested and lysed in IP Lysis buffer. Immunoprecipitations were conducted simultaneously with ^{35}S-Met-labelled and non-labelled extracts. Interacting proteins were eluted from the protein A sepharose by boiling in 2X sample buffer and separated by SDS-PAGE on a 6% minigel. Proteins were transferred on PVDF membranes using a semi-dry transfer apparatus. Immunoprecipitates from ^{35}S-Met-labelled extracts were exposed for autoradiography and Western blotting against RBP1 was performed on immunoprecipitates from non-labelled extracts. Panel A: Labelled immunoprecipitations reported by Lai et al. (Lai et al., 1999a). Panel B: Immunoprecipitatates from ^{35}S-Met-labelled extracts and Western blot against RBP1 on immunoprecipitates from non-labelled extracts.


A
α-LY11
α-IF8
α-HA.11
kDa
203
116
83
48.7
Myosin polypeptide 9
RBP1
B
Beads
α-HA
α-RBP1
Beads
α-HA
α-RBP1
206kDa
119kDa
Myosin polypeptide 9
RBP1
35S In vivo Label
Blot (α-RBP1)

3.4 Establishment of an inducible stable cell line expressing RBP1-HA

Affinity purification of RBP1 complexes may not be feasible using the LY11 antibody. On the other hand, overexpression of tagged RBP1 could be used to isolate RBP1 complexes. Establishment of an inducible stable cell line overexpressing RBP1 was therefore undertaken to isolate RBP1 complexes. Outside of its ability to cause growth arrest, not much is known about RBP1 cell biology (Lai et al., 1999a). A cell line overexpressing tagged RBP1 could therefore be used to study cellular functions of RBP1 as well. To circumvent growth inhibition by RBP1, an inducible system was used. The TET-ON system was selected as it has been found to function well in inducible adenovirus vectors expressing RBP1-HA and adenovirus type 5 E4-ORF4 (Lai and Branton, 200b; Marcellus et al., In preparation). This system comprises two components, a plasmid with a tetracycline inducible promoter linked to RBP1-HA, pTind RBP1-HA and a plasmid expressing the rTTA transcription factor, pTET-ON. The pTind plasmid was constructed by replacing the CMV promoter of pcDNA3.1 *hygro+* with a tetracycline inducible promoter (Tind). The Tind promoter is activated by binding of the rTTA transcription factor. rTTA can only bind to the Tind promoter if it has previously been activated by binding to doxycycline, an analogue of tetracycline. However, this system is somewhat leaky and some transcription from the Tind promoter still occurs even in the absence of rTTA binding.

Before making the cell lines, the expression of the pTind RBP1-HA construct was tested in transient transfection experiments in three cell lines, 293T, C2C12 and H1299 cells (Figure 3.6 Panel A). In all three cell lines, the expression of RBP1-HA was induced upon doxycycline treatment. The level of expression varies between the cell lines. This is in part due to the varying efficiency of transfection. The efficiency was very high in 293T cells and very poor in C2C12. As the construct seemed to be expressing well upon induction, establishment of an inducible cell line in both H1299 and C2C12 was undertaken. To ensure minimal expression of RBP1 prior to induction, ten times less pTind RBP1-HA DNA related to pTET-ON DNA was transfected into cells prior to selection of stable transfectants. This ratio has two advantages. First, the low

number of pTind RBP1-HA plasmid per cell prevents high expression in the absence of doxycycline. Second, double selection for both plasmids would no longer be required as cells containing pTind RBP1-HA would be highly likely to also contain the pTET-ON plasmid. The expression of RBP1 in 20 colonies of H1299 and C2C12 cells was tested with and without induction with doxycycline. The expression pattern of the H1299 positive clones is presented in Figure 3.6 Panel B. No positive clones were obtained for the C2C12 cell line. Out of the 12 positive H1299 clones, HR12 and HR20 were kept for further use because of their high RBP1-HA expression levels and lack of background expression in uninduced cells, respectively. Clone HR10 was also considered, but upon passage of the cells the high level of expression of RBP1-HA was lost.

The kinetics of induction were studied in both cell lines to determine the optimal induction conditions. It was found that maximal expression is obtained 48hrs post induction with doxycycline (Figure 3.7 Panel A). The concentration of doxycycline required to optimise the expression of RBP1-HA was also investigated and it was discovered that the addition of 2.00μg/ml of doxycycline in the culture media ensured maximal expression (Figure 3.7 Panel B). Extracts from induced and uninduced cells were also separated by SDS-PAGE and immunoblotted against RBP1 to analyze the level of overexpression (Figure 3.7 Panel C). Endogenous RBP1 cannot be separated from RBP1-HA on minigels as these proteins co-migrate in a thick band that is recognized by α-RBP1 immunoblotting antibody. Quantification of the bands of RBP1 by densitometry demonstrated that, when induced, HR12 cells express twice the level of normal cells, meaning that the cellular concentration of RBP1-HA is equal to the endogenous RBP1 concentration. An inducible system to overexpress tagged RBP1 has therefore been established and could be used in the future to study both proteins interacting with RBP1 and the cell biology of RBP1.

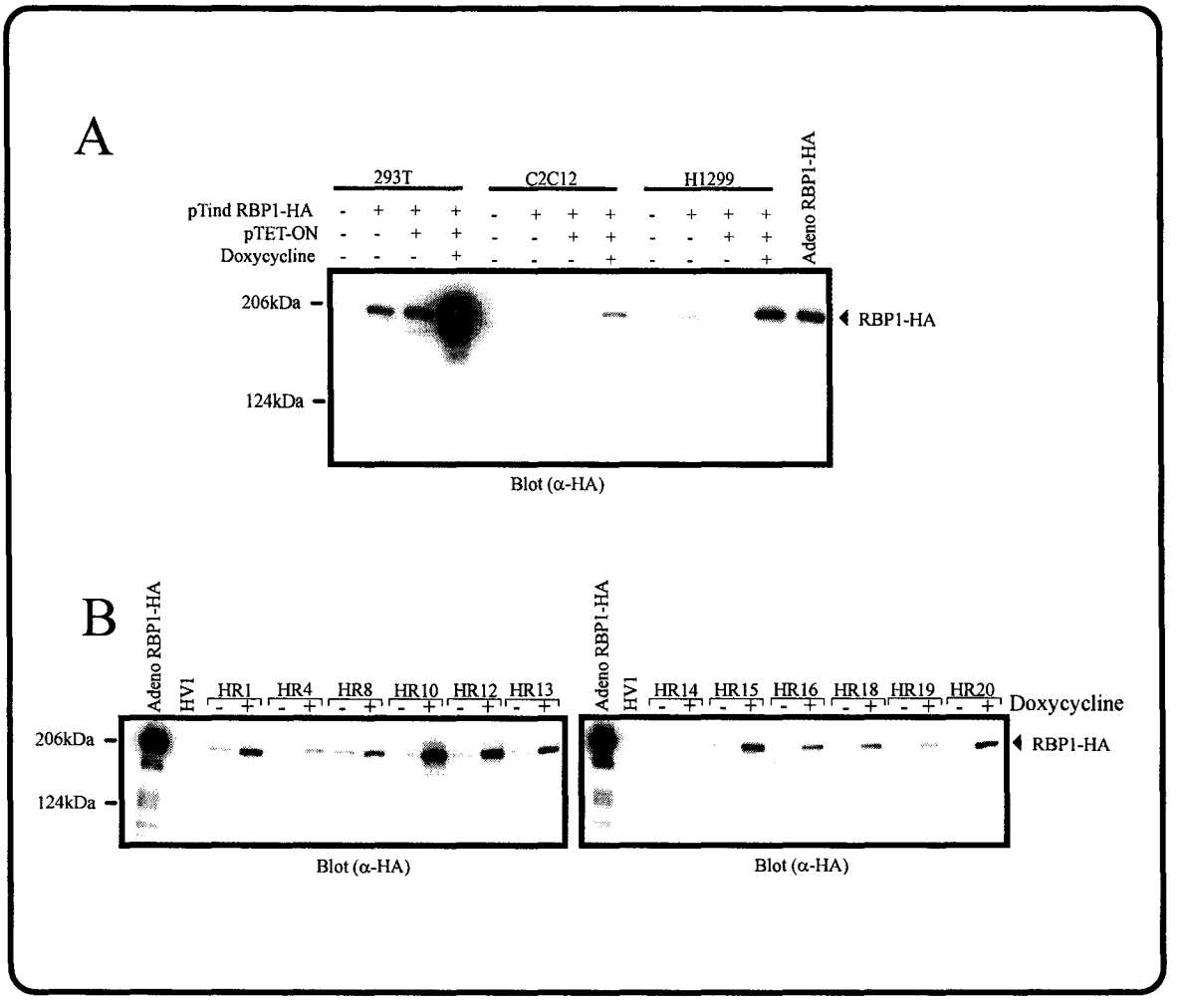


Figure 3.6: Transient expression of pTind RBP1-HA and expression of stable cell line positive clones. Panel A: Transient expression of pTind RBP1-HA in 293T, C2C12 and H1299 cells. 293T, C2C12 and H1299 cells were grown in 6 well plates to 50% confluence. They were transfected with 2μg of pTind RBP1-HA and 2μg of pTET-ON. Expression of RBP1-HA was induced with 1μg/ml of doxycycline for 48Hres. Cells were harvested and lysed in IP Lysis buffer. Protein concentration was quantified using Bradford reagent and 20μg of whole cell extract was separated by SDS-PAGE on a 6% minigel. Proteins were transferred on a PVDF membrane using the semi-dry transfer apparatus. Western blotting was performed using α-HA (HA11) antibody. 5μg of whole cell extract from H1299 cells infected with RBP1-HA adenovirus vector was used as a positive control. Panel B: Expression of all pTind RBP1-HA positive clones. H1299 cells were transfected and selected for resistance to hygromycin B as described in the materials and methods section. Expression of clones was assessed by inducing expression for 48hres with 1μg/ml doxycycline and was compared to expression in uninduced cells. Extracts were prepared and analyzed by Western blot similarly as in A. Again 5μg of whole cell extract from H1299 cells infected with RBP1-HA adenovirus vector was used as a positive control and 20μg of whole cell extract from pTind plasmid stable cell line (HV1) was used as a negative control.

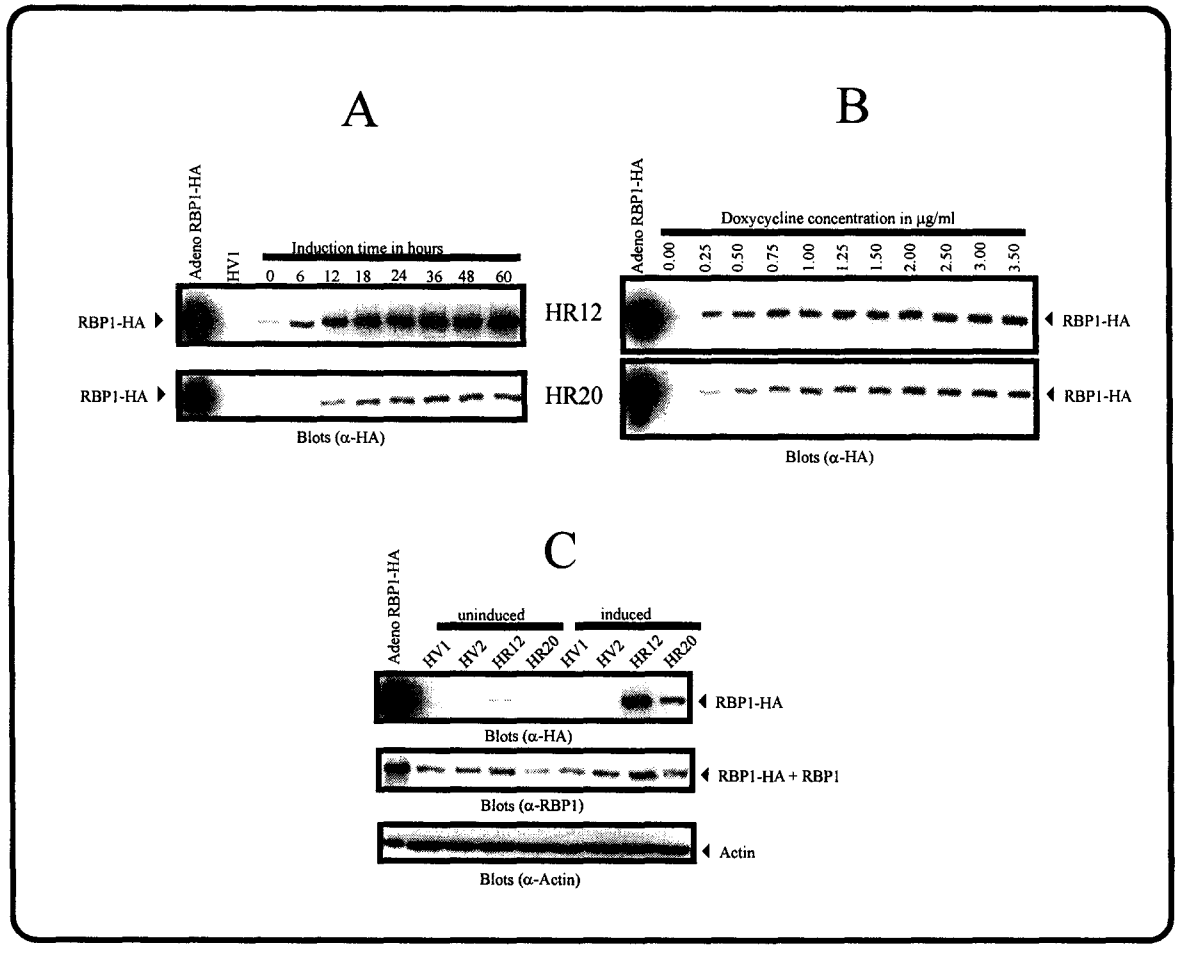
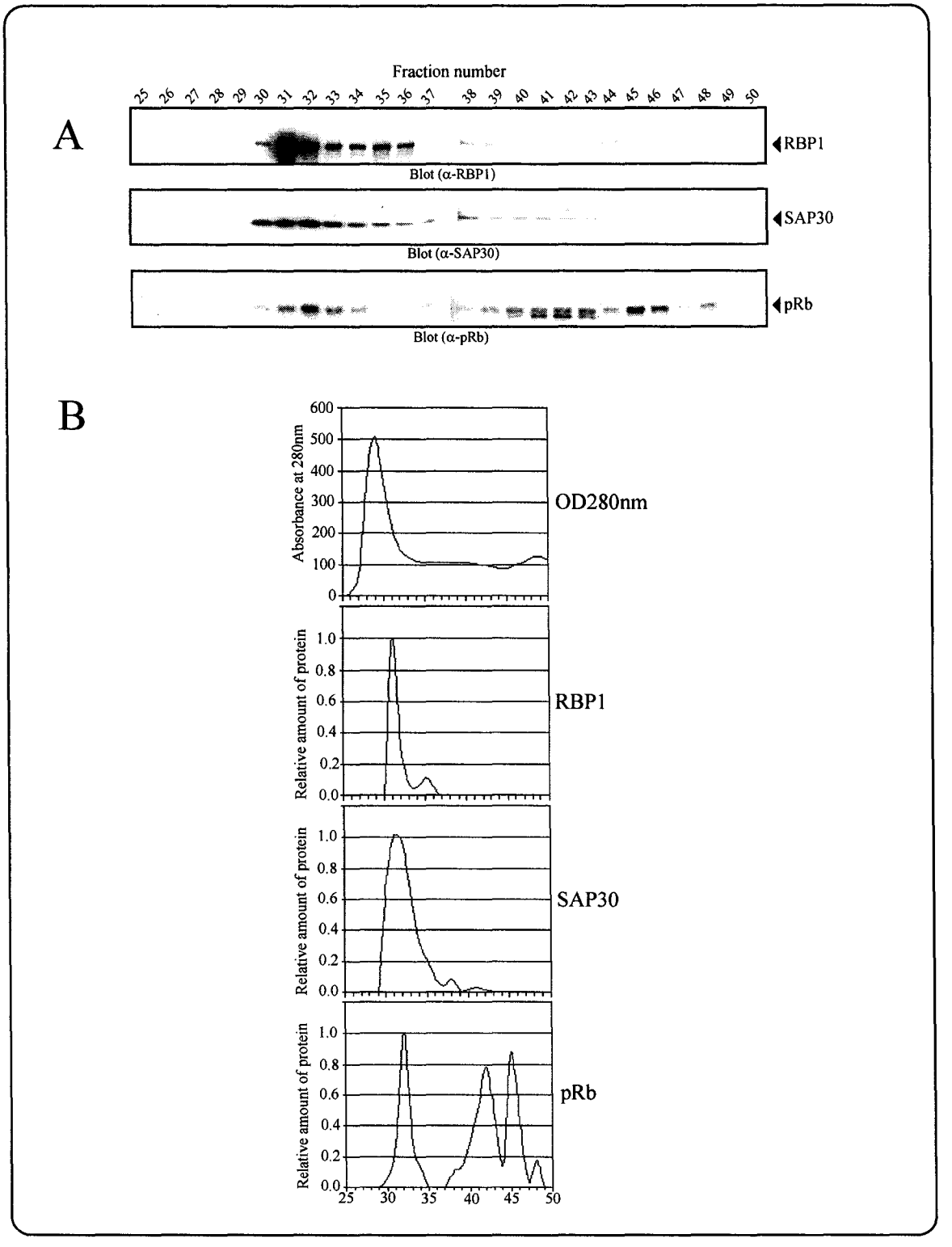


Figure 3.7: Pattern of expression of RBP1-HA in HR12 and HR20. Panel A: Expression at varying times post induction. Cells were induced for different lengths of time using 1μg/ml of doxycycline in 6 well dishes. Cells were harvested and lysed with IP Lysis buffer. 20μg of whole cell extract was separated by SDS-PAGE on 6% minigels by SDS-PAGE and transferred to PVDF membranes using semi-dry transfer apparatus. Protein expression was determined by Western blotting analysis using α-HA (HA11) antibody. 5μg of whole cell extract from H1299 cells infected with RBP1-HA adenovirus vector was used as a positive control and 20μg of whole cell extract from cells stably transfected with pTind plasmid (HV1) was used as a negative control. Panel B: Expression at different doxycycline concentrations. Induction was conducted for 48hres using varying concentrations of doxycycline. Whole cell extracts were prepared and analyzed as in A. C: RBP1-HA expression compared to endogenous RBP1. Cells were induced for 48hres using 2μg/ml of doxycycline. Extracts were prepared as in A. Protein expression was analyzed by Western blotting using α-HA (HA11), α-RBP1 (LY32) and α-actin antibodies.

3.5 Separation of RBP1 containing complexes by gel filtration

RBP1 is known to be part of the Sin3 HDAC complex (Lai et al., 2001; Zhang et al., 1998a), but it is unknown if it is part of other high molecular complexes. To study RBP1 complexes, protein and complexes from nuclear extract were separated according to size by gel filtration. The different fractions were immunoblotted with antibodies against RBP1, SAP30 and pRb (Figure 3.8 Panel A). RBP1 elutes in a narrow range of fractions (Panel B), suggesting that it mainly exists as a single complex. All of RBP1 was found to co-fractionate with SAP30. The size of complexes can be determined by the elution pattern, high molecular complexes eluting in earlier fractions than lower molecular weight complexes. The size of the main RBP1 complex was calculated to be 800kDa. It is also possible to know if a protein is in complexes or not, in which case it would elute at around its molecular weight. No RBP1 was found uncomplexed as it was not detected in fraction 47, which contains proteins of 143kDa in size. Two forms of pRb were detected by Western blotting analysis of column fractions. The faster migrating species corresponded to hypophosphorylated pRb and the slower migrating, to hyperphosphorylated pRb. Interestingly, hypophosphorylated pRb does not significantly co-fractionate with RBP1. On longer exposures, a small amount of hypophosphorylated pRb was detected in fractions containing RBP1. Most if not all of RBP1 therefore seems to be in complexes containing SAP30 but no hypophosphorylated pRb.

Figure 3.8: Gel filtration of RBP1 complexes. Nuclear extract was prepared as described in the materials and methods section. 20µl of fractions 25 to 50 were separated on 6% and 10% minigels by SDS-PAGE and were transferred to PVDF membranes using the semi-dry system. Proteins were analyzed by Western blotting using α-RBP1 (LY32), α-SAP30 and α-pRb (IF-8) antibodies. Levels of RBP1, SAP30 and pRb in the different fractions were quantified using densitometry. Panel A: Western blots against RBP1, SAP30 and pRb for fractions 25 to 50. Panel B: Chromatograms of absorbance at 280nm and of quantified level of RBP1, SAP30 and pRb for fraction 25-50.


A
Fraction number
25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50
RBP1
Blot (α-RBP1)
SAP30
Blot (α-SAP30)
pRb
Blot (α-pRb)
B
Absorbance at 280nm
600
500
400
300
200
100
0
OD280nm
Relative amount of protein
1.0
0.8
0.6
0.4
0.2
0.0
RBP1
SAP30
pRb
25 30 35 40 45 50

Chapter 4: Discussion and future work

Acetylation of histones is generally associated with active chromatin structure (Hassig and Schreiber, 1997; Struhl, 1998; Turner, 1991). Protein recruiting HATs therefore usually activate transcription when tethered to promoters. Association of HAT activity with RBP1 would have been surprising given that RBP1 was shown to repress transcription and to interact with class I HDACs (Chan et al., 1998; Lai et al., 1999b; Lai et al., 1999a; Lai, 2000a). On the other hand, RBP1 would not be the first protein reported to interact with both HATs and HDACs. The YY1 transcription factor interacts with both types of enzymes (Thomas and Seto, 1999; Yao et al., 2001). HATs and HDACs have also been shown to regulate the activity of proteins that recruit them. E2F1-3, YY1, pRb and p53 activities have all been shown to be regulated by reversible acetylation (Barlev et al., 2001; Chan et al., 2001a; Martinez-Balbas et al., 2000; Marzio et al., 2000; Yao et al., 2001). The activity of RBP1 could be similarly regulated by reversible acetylation. It would therefore be worthwhile to study the potential acetylation of RBP1 in vivo.

Preliminary results suggested that the HAT activity was recruited by the ARID domain, but not by the R1 domain (Lai, 2000a). Interestingly, when tethered to promoters, the ARID domain activates transcription while tethering of the R1 domain represses it (Lai et al., 1999a; Lai, 2000a). This could suggest that the ARID domain recruits HAT activity to promoter and that R1 prevents such an interaction. This HAT was suggested to be $TAF_{II}250$, which is part of the TFIID basal transcription factor (Lai, 2000a; Mizzen et al., 1996).

Results from HAT assays presented in Figure 3.1 suggested that there is no HAT activity associated with either the ARID domain or with complete R1 domain. The initial study reported HAT activity associated with RBP1 in MCF7 cells (Chan et al., 1998). Assays were therefore performed in these cells, but no HAT activity was detected. For their experiments, Chan et al. used a different antibody, a rabbit polyclonal serum that they had raised (Chan et al., 1998), while we used a mouse monoclonal antibody raised by DeCaprio and Kaelin (LY11) (Lai et al., 1999a). When the rabbit polyclonal antibody was tested in immunoprecipitation in our lab, it was not found to precipitate any

detectable amount of RBP1. Therefore, it was not possible to reproduce results from Chan et al. Although the possibility that our immunoprecipitating antibody prevents recruitment of HATs by RBP1 cannot be dismissed, it is unlikely since the epitope of the LY11 antibody maps to a region of the middle of the protein that has not been found to affect transcriptional repression (Lai et al., 1999a). The preliminary study in our laboratory demonstrating interaction of HATs with the ARID domain of RBP1 was only done once while the HAT assays described here have been performed three times. It is therefore possible that both preliminary studies represent artefacts and that there never was any HAT activity associated with the ARID domain of RBP1.

GST pulldowns using different domains of RBP1 precipitated too many non-specific species to allow proper isolation of proteins for sequencing or peptide mapping. It is unclear what causes the binding of non-specific species to RBP1 GST-fusion proteins as many of these are absent in the GST alone control. It is possible that the larger size of the RBP1 mutants allow more access for non-specific binders. Another possibility is that GST-RBP1 deletion mutants are not folded properly in bacteria. This could also explain the low level of specific interaction detected with these GST mutants. On the other hand, GST-R2, another RBP1 deletion mutant that has not been used in this study, has been shown to be functional (Lai et al., 2001).

It is not clear which species corresponds to RBP1 in immunoprecipitations. Three groups reported that RBP1 migrates between 180kDa and 200kDa (Fattaey et al., 1993; Otterson et al., 1993; Zhang et al., 1998a). The species corresponding to RBP1 in LY11 immunoprecipitations has been reported to migrate significantly over 200kDa (Figure 3.5 Panel A adapted from (Lai et al., 1999a). A very intense species migrating above 200kDa was also consistently seen in LY11 immunoprecipitates reported here (Figure 3.4 and 3.5 Panel B). Large scale immunoprecipitation permitted the isolation of sufficient amounts of this protein to permit protein sequencing. This protein was found to be Myosin polypeptide 9. It seems to bind non-specifically to protein A. The high molecular weight and the strong intensity of the species that has been reported by Lai et al. to be RBP1 strongly suggest that it is in fact Myosin polypeptide 9. The faint species just below the

203kDa marker is more likely to be RBP1 (Figure 3.5 Panel A adapted from (Lai et al., 1999a)).

Many proteins of low molecular mass were found to be immunoprecipitated by the LY11 antibody. The strong intensity of these species suggests that they are present in large quantities. These proteins could be non-specific interactors that bind to the LY11 antibody. On the other hand, the large number of interacting proteins could simply reflect interaction of RBP1 with polyribosomes on RNA or binding of RBP1 to chromatin since chromo domains, ARID domains and Tudor domains have all been suggested to interact with DNA and/or RNA (Akhtar et al., 2000; Bannister et al., 2001; Collins et al., 1999; Gregory et al., 1996; Herrscher et al., 1995; Jacobs et al., 2001; Messmer et al., 1992; Platero et al., 1995; Ponting, 1997; Whitson et al., 1999). This issue could be resolved by performing immunoprecipitations in the presence of RNAse and/or DNAse.

Contrary to what has been reported, the LY11 antibody does not seem to immunoprecipitate large amounts of RBP1. Use of the LY11 mouse monoclonal antibody in immune-affinity chromatography is also impractical due to its non-specificity in immunoprecipitations. Other methods should therefore be used to isolate binding proteins.

With the TET-On system, a RBP1-HA inducible cell line was established in H1299. Expression of RBP1-HA is comparable to levels of endogenous RBP1 expression. This low level of expression could allow studies of much more physiologic relevance than overexpression by transient transfection. The HA antibody has been shown by our group to be very specific and to permit much cleaner immunoprecipitations. As RBP1-HA is probably part of RBP1 complexes due to its physiological expression, this cell line could be an interesting tool to study proteins interacting with RBP1. Unfortunately, preliminary experiments suggested that the HA epitope is masked by complex formation at the C-terminal R2 repression domain as very limited amounts of RBP1-HA were successfully immunoprecipitated using the α-HA antibody. This problem could be circumvented by establishing another cell line expressing an N-

terminally tagged RBP1 using the same system. Since no protein interaction has been detected so far within the N-terminus of RBP1, the HA epitope would most likely not be masked and could be used to isolate complexes. The effect of RBP1 in cell cycle progression could also be studied. RBP1 is known to inhibit cell growth if overexpressed, but the exact mechanism involved it is still unclear (Lai et al., 1999a). By using flow cytometry, it would be possible to study the behaviour of cells overexpressing RBP1-HA.

Problems were encountered while attempting to establish an inducible cell line in C2C12 mouse myoblasts. Many colonies started to differentiate or exited the cell cycle before their expression could be tested and thus no positive clones could be obtained. It is possible that overexpression of RBP1-HA in C2C12 is too growth inhibitory to permit establishment of an inducible cell line. On the other hand, since C2C12 cells have a tendency to exit the cell cycle and to differentiate in response to contact with other cells, growth in colonies might therefore not be possible. It would be possible to circumvent this problem by using double selection with green fluorescent protein (GFP) and hygromycin B. To do so, the *hygro* selection marker of the pTind plasmid would need to be replaced by an *hygro*-IRES-*gfp* marker where IRES is an internal ribosomal entry site allowing translation of both proteins from one mRNA. Using a fluorescent cell sorter, transfected cells would be selected for expression of GFP. Stable transfectants would then be selected for hygromycin B resistance. Cells would be passaged regularly to prevent them from reaching confluence. This system would allow establishment of a RBP1-HA inducible C2C12 line without stressing cells by growing them in colonies.

The pattern observed in gel filtration shows that RBP1 perfectly co-fractionates with SAP30. This observation suggests that the main RBP1 complex is the Sin3 HDAC complex that is recruited via the R2 repression domain (Lai et al., 2001; Lai et al., 1999b; Zhang et al., 1998a). Repression by the R1 domain does not involve HDACs (Lai et al., 1999b) and no HDAC-independent mechanism is known at the present in the Sin3 HDAC complex (Zhang et al., 1998a). Therefore, a complex containing RBP1 and repression complexes associated with both R1 and R2 may not exist. It does not seem that the R1 repression domain forms a stable complex with repression functions. This would be

expected if repression by R1 involves interaction with transcription factors or DNA. Interestingly, RBP1 was not found to co-fractionate with hypophosphorylated pRb. This was somewhat expected because pRb was not found to be part of the Sin3 HDAC complex (Zhang et al., 1998a). Furthermore, pRb is known to form weakly associated complexes that are cell cycle specific (Morris and Dyson, 2001). Complexes involving pRb are therefore very hard to isolate.

Many questions remain unanswered concerning the function of RBP1. One such question of paramount importance is the mechanism of repression by R1. With roughly 6000 genes (Goffeau et al., 1996; Harrison et al., 2002), yeast offers a simpler system to study binding proteins. As R1 strongly represses transcription in yeast (Kennedy, B. personal communication), it would be possible to study the mechanism of repression of R1 in this species. The small number of proteins in yeast would permit an easier analysis of proteins that co-immunoprecipitate with RBP1. Furthermore, as libraries of yeast deletion strains exist, the impact of deleting certain genes on the repression by R1 could be studied. This functionality also has its drawbacks. Conventional yeast two hybrid studies cannot be performed using R1 because transcription would always be repressed. Yeast two hybrid systems that are not transcription based could therefore be useful in this context. Of course, as R1 contains an ARID domain, it is possible that repression is due to interaction of the ARID domain with DNA, thereby preventing transcription factors from binding to the promoter. However, the ARID domain alone activates transcription when tethered to a promoter by fusion with an heterologous DNA binding domain (Lai, 2000a). Studies in yeast will probably shed some light on the mechanism of repression by the R1 domain.

Two new repression activities have recently been found to be recruited by pRb. Both DNMT1 (a DNA methyl transferase) and Suv39H1 (a Histone H3 methyl transferase) have been found to interact with the small pocket of pRb to repress E2F transcription (Bestor et al., 1988; Firestein et al., 2000; Nielsen et al., 2001; Rea et al., 2000; Robertson et al., 2000; Robertson et al., 1999; Vandel et al., 2001). It is still unclear if these proteins interact with pRb directly or not. As both of these proteins target

HDAC-independent activity to the small pocket of pRb, they would be potential causes for R1 repression. Furthermore, DNMT1 has been shown to be part of a complex containing HDAC1 (Fuks et al., 2000; Robertson et al., 2000). RBP1 could serve as a linker between HDAC1 and DNMT1. It would therefore be worthwhile to investigate the possibility of interaction between RBP1 and both DNMT1 and Suv39H1. GST pulldown analysis of R1 and ARID binding proteins did not detect any interacting proteins of the molecular mass of Suv39H1 or DNMT1, 48kDa and 194kDa respectively (Aagaard et al., 1999; Bestor et al., 1988; Robertson et al., 2000). However, the presence of these proteins could have been masked by other non specific interactors. By conducting GST-pulldowns with non-^{35}S-Met labelled extracts followed by immunoblotting using antibodies against DNMT1 and Suv39H1, interaction between R1 and these two repressors could be detected. Also, classical histone methyl transferase assays could be performed on RBP1 immunoprecipitates or on GST-R1 pulldowns.

Another question that remains to be answered is the function of the different structural domains in RBP1. ARID domains from many different proteins have been demonstrated to bind DNA (Collins et al., 1999; Gregory et al., 1996; Herrscher et al., 1995; Whitson et al., 1999). RBP1 is known to bind specifically to DNA (Fattaey et al., 1993). Therefore, the DNA binding activity of RBP1 ARID domain should be studied both alone and in the context of R1 to allow for the possibility of a direct interaction of RBP1 with DNA when it is recruited to promoters. Since the ARID domain of RBP1 seems to activate transcription (Lai, 2000a), it would also be worthwhile to study other ARID domains to determine if this activity is unique to RBP1 or if it is generalized.

The chromo domain of RBP1 has not been extensively studied either. The function of chromo domains is less clear than the function of ARID domains. They have been proposed to allow for dimerization, to bind chromatin and to work as RNA binding motifs (Akhtar et al., 2000; Bannister et al., 2001; Cowell and Austin, 1997; Jacobs et al., 2001; Messmer et al., 1992; Platero et al., 1995). Dimerization of RBP1 has been suggested before (Theberge, 2001) and should be confirmed. Chromatin immunoprecipitation studies could also be performed to analyze potential binding of

RBP1 to chromatin. Finally, interaction between the chromo domain of RBP1 and RNA could be detected by supershift assays.

The recent discovery of a Tudor domain in RBP1 is perplexing since this domain is usually found in proteins involved in RNA metabolism. It seems that Tudor domains are protein-protein interaction domains (Selenko et al., 2001). However, they also have been suggested to bind RNA (Ponting, 1997). Binding studies should be carried out to isolate Tudor domain binding proteins. The Tudor domain of RBP1 has not been found to modulate transcription (Lai et al., 1999b; Lai et al., 1999a), but it could serve as an interaction domain to allow recruitment of RBP1 to other transcription factors.

The genomic sequence of the RBP1 gene has recently been isolated by our group (Binda and Branton, 2001). The disruption of this gene will soon be attempted in mice. Knock out cells will be very useful to study the function of RBP1. For example, it would be possible to study the impact of *RBP1* loss on pRb repression of E2F transcription by comparing repression in *RBP1*$^{-/-}$ and parent cells. Disruption of proteins encoding Tudor or ARID domains has often been found to be lethal or very deleterious (Buhler et al., 1999; Lahoud et al., 2001; Lefebvre et al., 1995; Shandala et al., 1999; Takeuchi et al., 1995). Therefore, the phenotype of knock out mice is expected to be very dramatic. Making a conditional knock out could be informative in this case.

Although RBP1 has never been reported to be mutated or deleted in cancer, the expression of a new protein highly homologous to RBP1, RBP1L1/BCAA, has been found to be increased in breast cancer patients as well as in other malignancies (Cao et al., 2001). This new protein is very interesting as it potentially encodes both functional R1 and R2 domains without having an LxCxE pRb-binding motif. It could therefore function as a dominant negative mutant and inhibit RBP1-mediated E2F transcription repression. An RBP1 mutant lacking the LxCxE has previously been found to function in a similar fashion and to inhibit function of endogenous RBP1 when overexpressed (Lai et al., 1999a). Theoretically, mutation of the LxCxE motif of RBP1 could favour malignancy by sequestering HDAC-dependant and -independent repression functions from pRb and

wild type RBP1. However, RBP1 does not seem to be the only protein linking pRb and HDACs (Kennedy et al., 2001; Tokitou et al., 1999). Therefore, disruption of RBP1 and mutation of the LxCxE may not completely inhibit active repression by pRb and may not favour oncogenesis.

In conclusion, the present study demonstrated that there is no HAT activity associated with RBP1. Furthermore, it was shown that neither GST-pulldowns nor immunoprecipitations using the LY11 antibody are useful in isolating RBP1 binding proteins. A system was worked out to establish inducible stable cell lines overexpressing RBP1-HA. The H1299 cell line that was established could be used in the future to study both proteins binding to RBP1 and the cellular roles of RBP1. Finally, RBP1 was found to exist almost exclusively in a complex or in complexes containing SAP30 but not hypophosphorylated pRb.

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