The role of RBP1 in myoblast differentiation

By

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Abstract

Previous studies demonstrated that growth arrest and terminal differentiation are associated with the formation of the C7 complex. This complex contains E2F, pRb, p130 and RBP1. Because of RBP1 ability to induce growth arrest and to repress E2F-dependent transcription, and because pRb participates in myogenesis, we studied whether or not RBP1 is implicated in the differentiation pathway.

We demonstrate here that RBP1 is being shuttled from the nucleus to the cytoplasm during differentiation. We recently demonstrated that RBP1 acts as a linker protein between pRb and HDACs complex, bringing a repressor complex to E2F promoters (Lai *et al.,* 1999a). RBP1 shuttling suggests that it would play a role in the initial stages of myogenesis, possibly in the induction of the growth arrest. As differentiation proceeds, RBP1 would be translocated to the cytoplasm. Without RBP1, pRb/RBP1/HDAC repression complex would fall apart, enabling pRb to activate myogenic proteins, leading to terminal differentiation.

Résumé

Des résultats préliminaires ont démontré que la senescence et la différentiation sont associés à la formation du complexe C7. Ce complexe contient E2F, pRb, p130 et RBP1. À cause de la capacité de RBP1 à induire l'arrêt de croissance et puisque pRb participe à la myogénèse, nous avons étudié si RBP1 est impliqué au cours de la différentiation.

Nous démontrons ici que RBP1 est transporté du noyau vers le cytoplasme au cours de la différentiation. RBP1 agit comme une protéine lien entre pRb et le complexe HDAC, amenant un complexe répresseur aux promoteurs d'E2F (Lai *et al.*, 1999a). Le transport de RBP1 suggère qu'il jouerait un rôle dans les stages initiaux de la myogénèse, probablement dans l'induction de l'arrêt de croissance. Au cours de la différentiation, RBP1 serait transporté vers le cytopasme. Sans RBP1, le complexe pRb/RBP1/HDAC ne pourrait exister, permettant à pRb d'activer les protéines myogéniques.

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List of Abbreviations

aa	amino acid
Ad5	Adenovirus type 5
ADN	acid deoxyribonucleique
APL	aprotinin-pepstatin-leupeptin
ARID	A/T-rich interacting domain
ATP	adenosine triphosphate
ATCC	Americamn Type Culture Collection
bHLH	basic helix-loop-helix
BSA	bovine serum albumin
bp	base pair
C	celcius
CDK	cyclin-dependent kinase
CDI	cyclin-dependent kinase inhibitor
CKII	casein kinase II
cDNA	complementary DNA
C-terminus	carboxy terminal
Da	Dalton
DNA	deoxyribonucleic acid
ds	double-stranded
DTT	dithiothreitol
E2F	Early region 2 factor
FBS	fetal bovine serum
GST	glutathione-S-transferase
HCL	hydrochloric acid
HDAC	histone deacetylase
HLH	helix-loop-helix
HMG	high mobility group
HRP	horse raddish peroxidase
HS	horse serum
kb	kilobase
kDa	kilodalton
g	gram
IPTG	Isopropyl β-D-thiogalactopyranoside

LXCXE	Leucine-X-Cysteine-X-Glutamic acid, where X represent any amino acid
M	molar
MEF	muscle enhancer factor
$MgCl_2$	magnesium chloride
mRNA	messenger RNA
mg	milligram
min	minute
ml	milliliter
mM	milliMolar
NaCL	sodium chloride
NaDoc	deoxycholic acid sodium salt
NaF	sodiun fluoride
$NaVO_4$	sodium vanadate
ng	nanogram
nM	nanomolar
N-terminus	amino terminus
NP-40	nonidet P-40
PBS	phosphate buffer saline
PMSF	phenylmethylsulfonyl fluoride
PP1	protein phosphatase 1
pRb	retinoblastoma susceptibility gene product
PSG	penicillin, steptamycin, glutamine
PVDF	polyvinylidene difluoride
RB1	retinoblatoma gene
RBP1	pRb-binding-protein
RNA	ribonucleic acid
RPM	revolution per minute
SDS	sodium dodecylsulfate
SDS-PAGE	SDS-polyacrylamide gel eletrophoresis
TAF	TBP-associated factor
TBP	TATA binding protein
TBS	Tris-buffered saline
Tris	Tris (hydroxymethyl) aminomethane
ul	microliter

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Chapter 1

General Introduction

The restriction point is one of the key control points late in the G_1 phase of the cell cycle. Prior to this point, the cell is responsive to extracellular signals, and depending on the nature of those, it may take alternative routes such as differentiation, senescence, or cell death. This period ends with the phosphorylation of pRb, enabling the cell to pass through that control point. pRb can thus be considered as a key regulator that holds proliferation in check (Goodrich and Lee, 1993; Hatakeyama *et al.,* 1994; Mittnacht *et al.,* 1994; Sherr *et al.,* 1994).

1.1 Discovery of Retinoblastoma Gene

Retinoblastoma is a rare pediatric tumour of the eye afflicting about 1 in every 20,000 children (Knudson, 1971). The disease presents itself in both heritable and sporadic forms, the latter being the most common. Both forms have as their root cause the loss of pRb function, the former through a germline mutation in one *RB1* allele and acquisition of somatic mutations in the second (Knudson, 1977). Most of the *RB1* mutations are not observable at the cytogenetic level, but rather involve point mutations, small deletions or insertions, or skipping of specific exons. In addition, point mutations and small deletions in the promoter region of *RB1* have also been found (Bookstein *et al.,* 1990).

Despite the dominant manifestation of the disease, retinoblastoma occurs as a consequence of a recessive mutation. Demonstration that *RB1* is a tumour suppressor gene first came from experiments in which the *RB1* gene was reintroduced into pRb-deficient cells. As a result, cellular growth and tumorigenecity were both suppressed *in vitro* and *in vivo*, respectively (Banerjee *et al.,* 1992; Bookstein *et al.,* 1990; Sumegi *et al.,* 1990; Xu *et al.,* 1991). The retinoblastoma susceptibility gene was the first human tumour suppressor gene having growth inhibitory activity to be identified (Weinberg *et al.,* 1995; Wang *et al.,* 1994; Hollingsworth *et al.,* 1993).

RB1-related genes have been found in a wide variety of organisms (see table 1.1). Its conservation throughout evolution suggests that pRb plays critical functions in eukaryotic systems.

Table 1.1 pRb is Found in a Variety of Organisms
Chickens
Rodents
Xenopus
Drosophila
C. elegans
Plants

Table 1.1 pRb is Found in a Variety of Organisms
pRb is found in a variety of organisms. Its conservation throughout evolution suggests that it plays important roles in eukaryotic systems. (Lu *et al.*, 1998; Boehmelt *et al.*, 1994; Du *et al.*, 1996; Huntley *et al.*, 1998; Bernards *et al.*, 1989; Destree *et al.*, 1992)

The *RB1* gene encompasses upwards of 200 kb of DNA on chromosome 13q14 (Bookstein *et al.,* 1988). A 4.8 kb mRNA is formed through the fusion of 27 exons clustered into three groups separated by large introns (Brookstein *et al.,* 1988; McGee *et al.,* 1989; Hong *et al.,* 1989). The major promoter activity for the *RB1* gene is contained within a 71-bp G/C-rich region between –264 and –194 relative to the translation initiation codon (Hong *et al.,* 1989). DNA sequence analysis of the promoter region has revealed consensus DNA-binding sites for the Sp1, ATF, and E2F transcription factors (Goodrich and Lee, 1993; Sakai *et al.,* 1999). The Sp1 and ATF sites are critical for the basal level transcriptional control of the promoter. The E2F1 recognition sequence located next to the Sp1 and ATF sites is involved in S phase boosting of *RB1* transcription (Shan *et al.,* 1994). Findings have shown that pRb is negatively autoregulated through E2F1 (Rhode *et al.,* 1996; Sandig *et al.,* 1996). In addition to retinoblastomas, mutations in the *RB1* gene have been detected in various other cancers (see table 1.2) (Friend *et al.,* 1986; Lee *et al.,* 1987a; Dunn *et al.,* 1988).

1.2 Autoregulation of the *RB1* gene

There seem to be two types of transcription factors regulating *RB1* gene expression; one for controlling its basal level of transcription, and the other for regulating its transcription in response to environmental changes (Hong *et al.,* 1989; Sakai *et al.,* 1991; Zacksenhaus *et al.,* 1993). The *RB1* promoter contains potential binding sites for transcription factors with which pRb can interact and either stimulates or represses its own expression depending on the physiological situation (Rhode *et al.,* 1996). Shan *et al.* provided evidence demonstrating that pRb expression is negatively autoregulated through E2F1 (Shan *et al.,* 1994). They showed that E2F1 specifically binds to pRb promoter and transactivates its expression. Moreover, their data indicate that overexpression of pRb suppresses its E2F1-mediated stimulation and that pRb expression is paralleled by the expression of the E2F1 during cell cycle progression (Shan *et al.,* 1994).

Table 1.2 pRb is Mutated in a Variety of Cancers
osteosarcomas
glioblastoma
small-cell lung carcinomas
prostate carcinomas
breast carcinomas
bladder carcinomas
some types of leukaemias
cervical carcinomas

Table 1.2 pRb is Mutated in a Variety of Cancers
This table gives a partial list of cancers in which *RB1* is mutated. pRb is a key regulator of the cell cycle and any mutation leading to its loss of function can result in uncontrolled growth. (Issig *et al.*, 1993; Chen *et al.*, 1990; Ichimura *et al.*, 1996; Yokota *et al.*, 1988; Murakarni *et al.*, 1991; Xu *et al.*, 1994; Helin *et al.*, 1997; Bookstein *et al.*, 1990; Theodorescu *et al.*, 1997; Tamura *et al.*, 1994; Takahashi *et al.*, 1991; Goodrich *et al.*, 1992).

1.3 Structural Features of the Retinoblastoma Protein

The *RB1* gene encodes a nuclear phosphoprotein spanning 928 amino acids (aa) (Lee *et al.,* 1987a). pRb has a computed molecular mass of 106 159 Daltons (Da). Despite the restricted number of tissues that are affected by *RB1* mutations, pRb mRNA and protein can be detected in almost all cell types (Bernards *et al.,* 1989; Friend *et al.,* 1987; Lee *et al.,* 1987a).

pRb has at least three protein binding regions (see figure 1.1). One of them is the well known 45 kilodalton (kDa) A/B pocket region (Hu *et al.,* 1990; Huang *et al.,* 1990; Kaelin *et al.,* 1990). This region consists of domain A (aa 394-572) and domain B (aa 646-772) separated by an insert region (aa 573-645) (Huang *et al.,* 1990; Hu *et al.,* 1990; Kaelin *et al.,* 1990). Viral oncoproteins as well as other cellular pRb-interacting proteins bind to a conserved groove on the B-box portion via a conserved leucine-X-cysteine-X-glutamic acid motif (LXCXE, where X represents any amino acid) (Yandell, 1989; Hu *et al.,* 1990; Huang *et al.,* 1990; Lee *et al.,* 1998). The A-box provides a scaffold for proper protein folding. Even less of a recognition site is required by MyoD and myogenin, which appear to bind only within the B domain (Gu *et al.,* 1993).

Unlike its relatives p107 and p130, no proteins have yet been found to interact with the spacer region of pRb. This region provides a physical space between domain A and B, enabling formation of the A/B pocket. Small deletions or amino acids changes within that domain do not affect pRb activity, but deletion of the entire insert region inactivates the protein-binding function of the A/B pocket (Hu *et al.,* 1990; Huang *et al.,* 1990; Qian *et al.,* 1992).

Another group of pRb-binding proteins requires not only the A/B pocket, but also a region within the C-terminus (referred to as pRb large pocket). These include the D-type cyclins, the E2F/DNA complex, and protein phosphatase type 1 and 2 (PP1, PP2). The large pocket of pRb is necessary for its growth-suppressive activities as demonstrated by the fact that pRb-dependent arrest of Saos-2 cells requires that whole region (Sellers *et al.,* 1998).

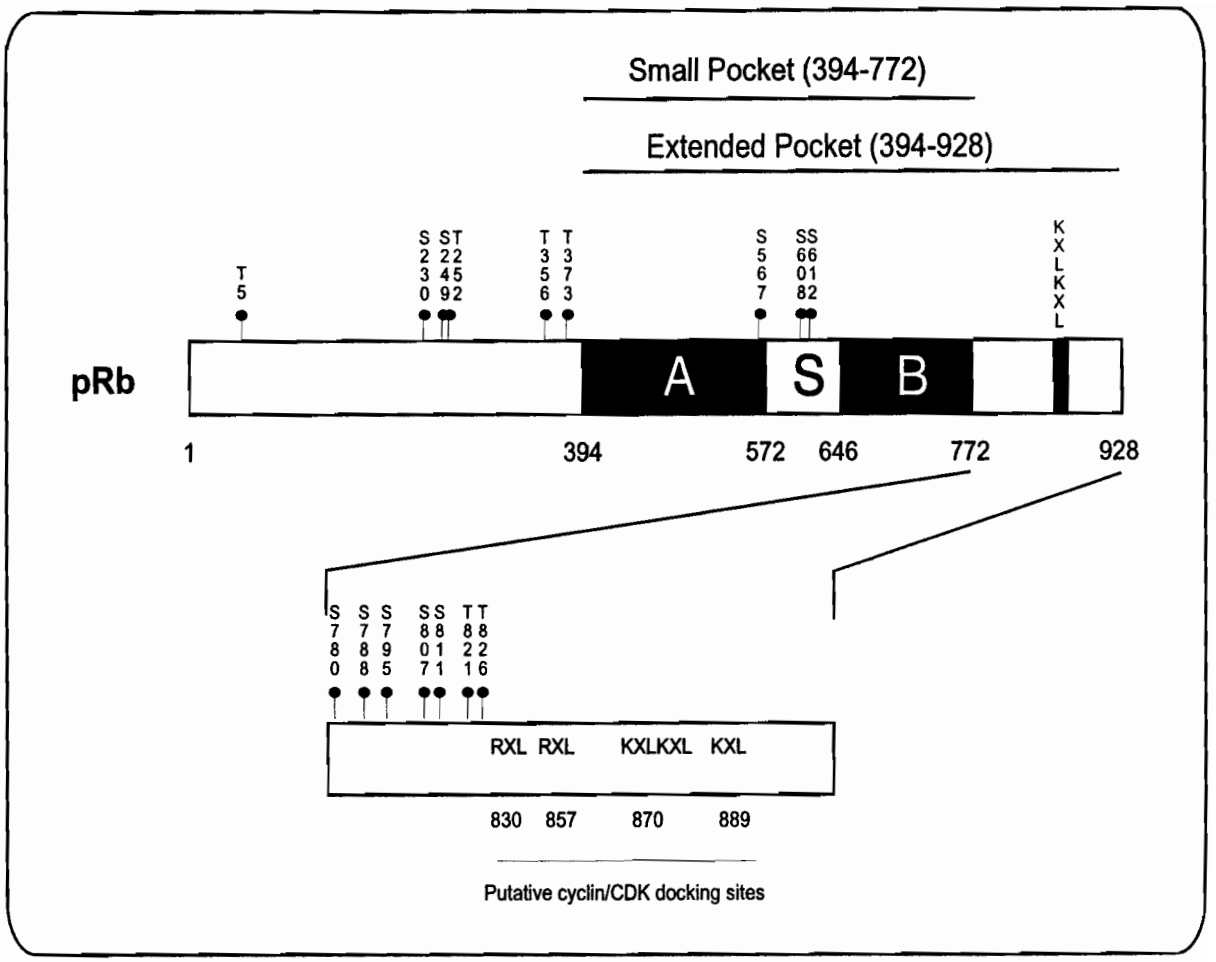


Figure 1.1 pRb Structure and Phosphorylation Sites
pRb is a 928 amino acid residues protein having many protein-binding domains. The A/B pocket is composed of two noncontiguous domains (A and B) as well as a spacer domain. This region forms the binding site for viral oncoproteins and cellular proteins containing the LXCXE motif. In contrast to its homologues p107 and p130, the insert domain of pRb do not possess any protein-binding activity. The C-terminal region of pRb is part of the large pocket which contributes to stable binding to E2F. This region also possesses protein-binding activity independently of the A/B pocket. As well, the C-terminal region contains a bipartite nuclear localization signal. 16 potential CDK phosphorylation sites are found throughout the structure of pRb. Circles above represent Ser or Thr residues recognized and phosphorylated by cyclin-dependent kinases. (Adapted from Kaelin, 1999) This figure is drawn to scale.

The interaction of the c-abl tyrosine kinase led to the definition of the C pocket, which functions independently of the A/B pocket. The C-terminal region of pRb also contains a bipartite nuclear localization signal (aa 860-876) as well as non-specific DNA-binding activity (Lee *et al.,* 1987b; Wang *et al.,* 1990). Many of the cell cycle-regulated phosphorylation sites are located in the C terminus (see figure 1.1) (Lin and Wang, 1992).

Finally, the N-terminal region of pRb, outside of the A/B pocket, has also been shown to have protein-binding activity (Sterner *et al.,* 1995, 1996; Chen *et al.,* 1994).

Interestingly, many of the naturally occurring pRb mutations found in tumour cells are ones that disrupt the integrity of the A/B pocket (Hamet *et al.,* 1993).

1.4 Role of pRb in Cell Cycle

pRb protein and transcript levels remain about the same during the cell cycle, although there is a slight increase in mid-to late G_1. This might be due to the fact that E2F1 binds specifically to an E2F recognition sequence in the pRb promoter and transactivates pRb expression during that same period (Shan *et al.,* 1994; Nevins *et al.,* 1997; Smith *et al.,* 1998). Cell cycle regulated changes in the phosphorylation status of pRb regulate its protein binding capacity as well as its cellular functions (Buchkovich *et al.,* 1989; Chen *et al.,* 1989; DeCaprio *et al.,* 1989; Kiess *et al.,* 1995a; Sidle *et al.,* 1996; Richon *et al.,* 1997; Dong *et al.,* 1998; Grana *et al.,* 1998; Cheng *et al.,* 2000). The present model of how pRb suppresses cell division is that hypophosphorylated pRb binds to and inhibits the activities of transcription factors such as E2F, whose activity is required for S phase entry (Nevins *et al.,* 1992a, b). Phosphorylation of pRb results in the release of E2F and subsequent activation of genes necessary for cell cycle progression.

Several CDK complexes target pRb for phosphorylation (see section 1.5.1) (Chen *et al.,* 1989; Mihara *et al.,* 1989; Ludlow *et al.,* 1990; Lin and Wang, 1992; Connell-Crowley *et al.,* 1997). Sixteen CDK phosphorylation sites are predicted from the primary sequence of pRb and at least seven of these have been shown to be phosphorylated *in vivo* (see figure 1.1). Phosphorylation of specific pRb

phosphoacceptor sites probably modulates distinct biochemical activities (Knudsen *et al.,* 1996, 1997; Zarkowska *et al.,* 1997). For instance, phosphorylation of Thr 821 and Thr 826 disrupts pRb binding to LXCXE proteins, whereas phosphorylation of Ser 608, Ser 621 or any sites in the C-terminal region of pRb disrupts its binding to E2F (Knudsen *et al.,* 1996, 1997; Zarkowska *et al.,* 1997).

1.5 Regulation of pRb by Phosphorylation

1.5.1 Cyclin/CDK as mediator of pRb phosphorylation

Cyclins are a family of proteins that were first identified by virtue of their cyclical appearance during the cell cycle in marine invertebrates and in yeast (see figure 1.2) (Lees *et al.,* 1993). They are the positive regulatory subunits of a class of serine-threonine protein kinases, named cyclin-dependent kinases (CDKs) (Pines *et al.,* 1993). Ten different cyclins and seven CDKs are encoded by the mammalian genome (Sher *et al.,* 1993). Homology among the cyclins is limited to a 100-residue cyclin box. This region is responsible for CDK binding and activation (Lees *et al.,* 1993). One way of classifying cyclins is according to their expression pattern throughout the cell cycle in G_1, S, G_2 and M phases (Pines, 1993).

Mitogens stimulation triggers expression of type D cyclins (D1, D2, and D3). Cyclin D1, D2, and D3 are synthesized in a cell type-specific manner (Sherr *et al.,* 1993). Although they are very unstable, there is a very little fluctuation of D-cyclin levels during the cell cycle. These proteins preferentially heterodimerize with CDK4 and CDK6 (Bates *et al.,* 1994; Matsushime *et al.,* 1992; Meeker, 1991; Meyerson *et al.,* 1994; Motokura *et al.,* 1991, 1992; Tsai *et al.,* 1991; Xiong *et al.,* 1991, 1992). Expression of cyclin D is necessary for G_o exit (Connell-Crowley *et al.,* 1998).

Cyclin E is expressed in mid G_1, peaking at the G_1/S boundary (Dulic *et al.,* 1992; Koff *et al.,* 1992; Ohtsubo *et al.,* 1995). This cyclin is necessary for the restriction point transition to occur and plays an important role in the onset of DNA synthesis (Harbour *et al.,* 1999). Cyclin E protein levels drop rapidly after the entry of cells into S phase due to phosphorylation-mediated proteolysis.

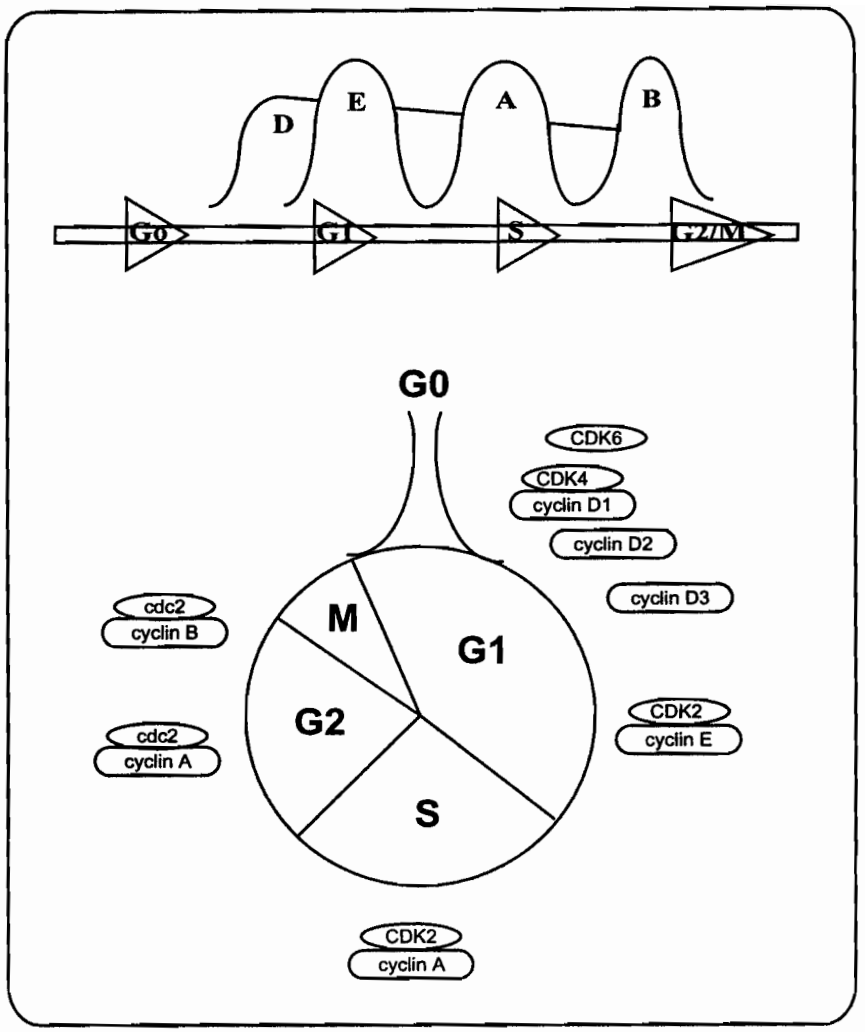


Figure 1.2 Cyclins Expression and Cyclin/CDK Complexes Formation during the Cell Cycle

Various cyclins are expressed at different time during the mammalian cell cycle. Although they are very unstable, type D cyclins are expressed at about the same level throughout the cell cycle. These cyclins associate with CDK4 and CDK6 in early G1. These complexes play an important role in Go exit. Cyclin E expression begins in mid G1 and peaks at the G1/S boundary. This cyclin associates with CDK2 and this complex plays a role in the onset of DNA synthesis. Cyclin A is expressed at the G1/S boundary and its expression level is maximum in late G1. Cyclin A initially associates with CDK2 in S phase but switches to cdc2 as cells approach the G2/M boundary. Cyclin B1/B2/cdc2 complexes regulate the G2/M transition. D, E, A, and B refer to cyclins. (Adapted from Sidle *et al.*, 1996)

Cyclin A is expressed at the G_1/S boundary and reaches maximal levels late in G_1 phase (Girard *et al.,* 1991; Pagano *et al.,* 1992; Zacksenhaus *et al.,* 1993). It forms an active complex with CDK2 in S phase and with cdc2 in G_2 and M phases. It enables the cell to exit S phase by phosphorylating both E2F and DP, causing their release from DNA (Krek *et al.,* 1995; Bernards, 1997; Dynlacht *et al.,* 1997; Dyson, 1998). Cyclin A is also targeted for degradation at the end of S phase (Yam *et al.,* 2000).

Cyclin B1 and B2 expression begins in S phase and peaks in mitosis (Lew *et al.,* 1991; Pines *et al.,* 1989). These cyclins, in association with cdc2, regulate G_2/M transition (Lapidot-Liffson *et al.,* 1992; Morgan, 1995; Pines *et al.,* 1989; Riabowol *et al.,* 1989; Sherr *et al.,* 1995). Ubiquitin-mediated degradation of these proteins is necessary for exiting mitosis (Pines and Hunter, 1989).

Cyclin F, the largest member of this family, is known to play a role in the G_2 phase of the cell cycle as its overexpression produces cell populations enriched in the G_2 phase (Bai *et al.,* 1994).

Cyclin G is a transcriptional target of p53. It may play a role in the growth inhibitory function of p53 (Okamoto *et al.,* 1994; Tamura *et al.,* 1993; Zauberman *et al.,* 1995).

Different sites of pRb are phosphorylated by different CDKs, at different periods throughout the cell cycle (Kitagawa *et al.,* 1996; Zarkowska *et al.,* 1997; Harbour *et al.,* 1999). Members of the cyclin D family as well as cyclins A and E have been implicated in this process (Kato *et al.,* 1993; Ewen *et al.,* 1993; Dowdy *et al.,* 1993). In G_o and early G_1, pRb is hypophosphorylated (see figure 1.3). Mitogenic signals trigger accumulation of D-type cyclins (cyclin D2 and D3), as well as their association with CDK4/6. This active complex binds to pRb via the cyclin N-terminal LXCXE motif, and phosphorylation is mediated on a number of serine and threonine residues on the pRb C-terminus (Dowdy *et al.,* 1993; Ewen *et al.,* 1993; Kato *et al.,* 1993; Connell-Crowlew *et al.,* 1998; Dyson *et al.,* 1998). This phosphorylation event causes the C-terminus and the pocket region to interact via basic lysine patches in the B box region of pRb. This inhibits LXCXE-protein binding and allows access to a normally buried residue; S 567. Phosphorylation of S 567 disrupts the pocket

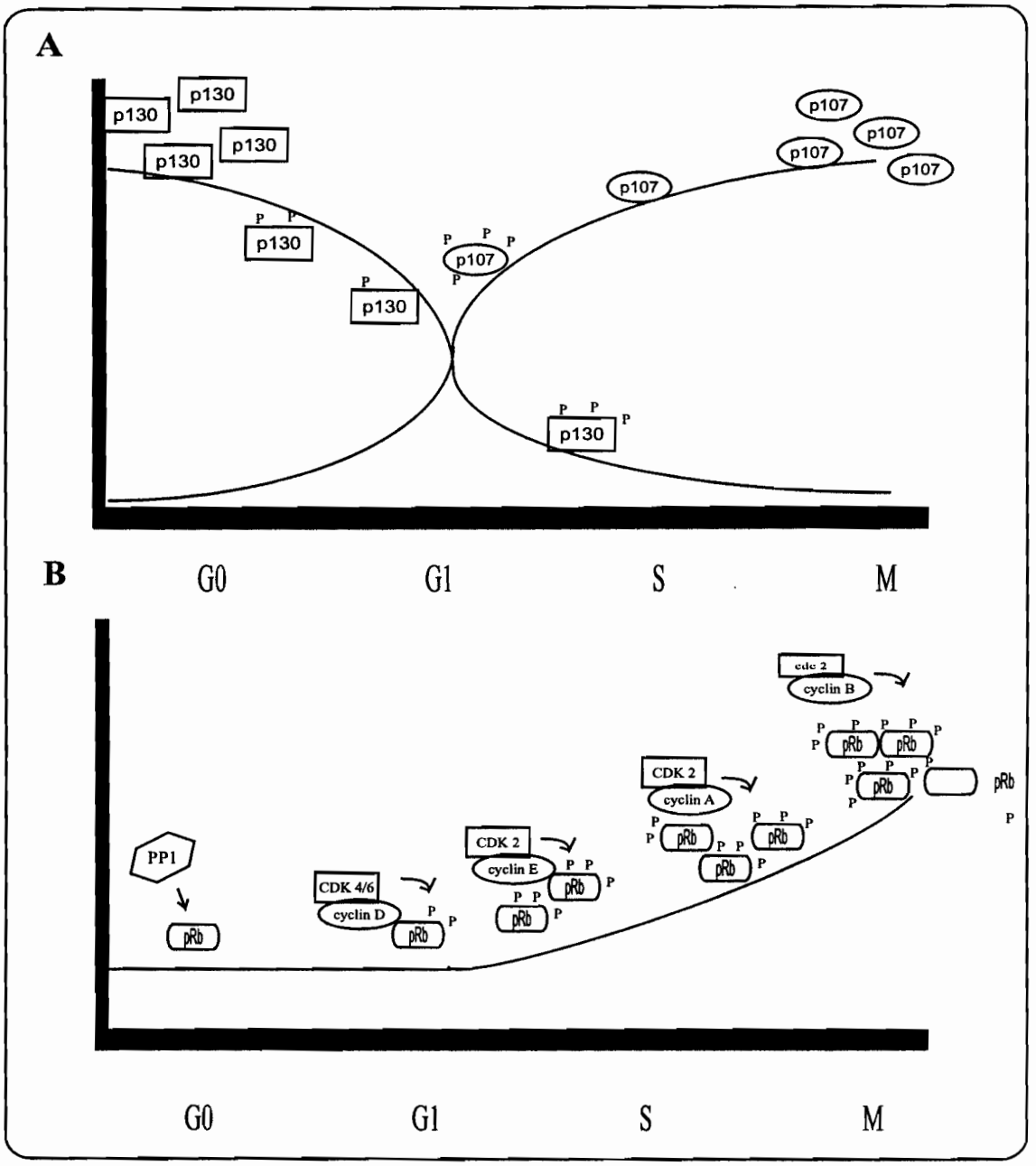


Figure 1.3 Pocket Proteins Levels and Phosphorylation Pattern during the Cell Cycle Panel A illustrates the phosphorylation pattern of p107 and p130 proteins. As shown above, p130 is phosphorylated whereas p107 loses its phosphate groups in actively proliferating cells. Panel B pictures the phophorylation pattern of pRb. pRb is found in its inactive hyperphosphorylated state in cycling cells. Ths figure is not to scale and the levels pictured above are not quantitative. (Adapted from Nevins, 1998).

structure, abolishing binding of E2F to pRb (Harbour *et al.,* 1999, Driscoll *et al.,* 1999). This provides a binding site for cyclin E/CDK2, which phosphorylates pRb at late G_1. This is followed by cyclin A/CDK2-mediated phosphorylation (Hatakeyama *et al.,* 1994; Ezhevsky *et al.,* 1997; Lundberg and Weinberg, 1998). pRb is further phosphorylated by cyclin B/cdc2 kinase (Hunter *et al.,* 1994; Sherr *et al.,* 1995). This state of phosphorylation is maintained until during or shortly after mitosis (Ludlow *et al.,* 1990; Mittnach *et al.,* 1994; Chew *et al.,* 1998). As cells progress from M to G_1 phase, pRb loses multiple phosphate groups and regains a hypophosphorylated form (Ludlow *et al.,* 1993). Evidence strongly suggests a major role for PP1 in the dephosphorylation and activation of pRb (Ludlow *et al.,* 1993; Durfee *et al.,* 1993; Nelson *et al.,* 1997; Puntoni and Villa-Moruaai, 1997; Rubin *et al.,* 1998; Edwards and Thomas, 2000).

1.5.2 CDK Regulation

As CDKs are present at constant levels throughout the cell cycle, their activity must be regulated. The regulation of the cyclin/CDK holoenzyme activity depends on parameters such as: (i) specific phosphorylation and dephosphorylation of cyclins and CDKs; (ii) assembly of CDKs with their regulatory subunits (cyclins); and (iii) association/dissociation of CDIs.

Phosphorylation of CDKs by CDK-Activating kinase (CAK) (cyclin H/CDK7) makes the catalytic pocket accessible for the protein substrate (Fisher *et al.,* 1994; Hunter *et al.,* 1994). In contrast, dephosphorylating enzymes such as cdc25A or cdc25C inhibit CDKs activities (Hengstschlager *et al.,* 1999).

Another way of regulating CDK activities is through cell cycle dependent expression of their specific cyclin partners (see section 1.5.1).

Cyclin-dependent-kinase inhibitors (CDIs) play a major role in the negative regulation of cyclin/CDK activity (Hunter and Pines, 1994; Sherr and Roberts, 1995). These proteins are involved in the G_1 arrest of cells in response to anti-proliferative signals. This arrest enables cells to enter processes such as terminal differentiation

and cellular senescence. There are two different families of CDIs: the proteins of the INK4 locus and the Cip/Kip family (Roussel, 1999; Sher *et al.,* 1999).

Members of the $p16^{INK4a}$ (mts1, cdkn2, cdk4i) family include $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$. Their expression results in a G_1 growth arrest, which prevents the assembly of the D cyclins with CDK4/6 and phosphorylation by CAK (Serrano *et al.,* 1993; Tam *et al.,* 1994; Aprelikova *et al.,* 1995). $p16^{INK4a}$ specifically inhibits the kinase activity of cyclin D/CDK4/6 complexes, blocking cell cycle progression in an pRb-dependent manner (Sherr *et al.,* 1995; Serrano *et al.,* 1993; Hirai *et al.,* 1995; Hannon *et al.,* 1994; Hall *et al.,* 1995; Carnero and Hannon, 1998).

Members of the $p21^{WAF1}$ family, including $p21^{WAF1}$, $p27^{Kip1}$ and $p57^{Kip2}$, are universal CDIs since they are able to associate with and inhibit the activity of a wide range of cyclin/CDK complexes (Xiong *et al.,* 1993). p21 family interacts with and inactivates cyclin/CDK complexes containing cyclins A, B, D, and E (Harper *et al.,* 1993; Xiong *et al.,* 1993; Zhang *et al.,* 1993, 1994). The discovery that p21 is under the transcriptional control of p53 has highlighted the possibility that p53 itself may indirectly regulate pRb function (El-Deiry *et al.,* 1993; Harper *et al.,* 1993).

1.6 pRb Family Members

Studies with E1A and T antigens led to the cloning of p107 and p130. These two proteins are classified as members of the *RB1* gene family as they are highly related in amino acid sequence (Ewen *et al.,* 1991; Hannon *et al.,* 1993; Li *et al.,* 1993a; Friend *et al.,* 1986). The A and B domains are the regions of highest homology between pRb family members. However, the sequences flanking them are relatively distinct in pRb compared with p107 and p130. p107 and p130 show about 50% amino acid identity and about 30% when compared with pRb (Ewen *et al.,* 1991; Hannon *et al.,* 1993; Li *et al.,* 1993a; Mayol *et al.,* 1993; Zhu *et al.,* 1993; Dyson, 1994; Wang, 1997; Mulligan *et al.,* 1998). Human p107 is a phosphoprotein of 1068 residues migrating as a 120 kDa species on SDS-PAGE, while human p130, also a phosphoprotein, is made up of 1082 residues and migrates at about 126 kDa (see figure 1.4) (Zhu *et al.,* 1993; Hannon *et al.,* 1993; Li *et al.,* 1993a; Mayol *et al.,* 1993).

In contrast to pRb, p107 and p130 possess a spacer region capable of interacting with cyclin E/CDK2 and cyclin A/CDK2 complexes (Zhu *et al.,* 1995b; Cao *et al.,* 1992a, b; Ewen *et al.,* 1992; Faha *et al.,* 1992; Huang *et al.,* 1992; Lees *et al.,* 1992; Shirodkar *et al.,* 1992; Smith and Nevins, 1994, 1995). When in free form, all three pocket proteins localize to the nuclear compartment of the cell (Lee *et al.,* 1987a; Ewen *et al.,* 1991; Baldi *et al.,* 1995).

1.6.1 Phosphorylation of Pocket Proteins

p107 exits in hyper- and hypophosphorylated forms. Its phosphorylation status throughout the cell cycle follows that of pRb (see figure 1.3) (Beijersbergen *et al.,* 1995; Sidle *et al.,* 1996; Grana *et al.,* 1998). However, in contrast to pRb, hypophosphorylated p107 reappears at the beginning of S phase. This is probably due to the fact that the newly synthesized p107 cannot be efficiently phosphorylated by the declining cyclin D1-associated kinase activity (Cobrinik *et al.,* 1993).

Four different phosphorylation species of p130 are detected throughout the cell cycle (Mayol *et al.,* 1995; Grana *et al.,* 1998). In quiescent cells, p130 is hypophosphorylated (see figure 1.3). As for p107, p130 gets phosphorylated in mid G_1 (Mayol *et al.,* 1995). However, in contrast to pRb and p107, two hyperphosphorylated forms (form 1 and 2) are detected in early G_1 and G_o (Baldi *et al.,* 1995; Mayol *et al.,* 1995). This suggests that p130 might play a role in driving cells to exit the cell cycle (Baldi *et al.,* 1995; Mayol *et al.,* 1995). Evidence from inhibitor studies suggest that p130 protein levels might be regulated by phosphorylation-mediated proteolysis (Ikeda *et al.,* 1996; Smith *et al.,* 1996).

1.6.2 Pocket Proteins Cell Cycle

p130 and p107 have a different pattern of accumulation during the cell cycle (see figure 1.3). p130 is mostly found in quiescent or differentiated cells. In contrast to p107, p130 mRNA levels are relatively constant in growing and G_o cells. p130 protein accumulates when cells exit the cell cycle (Mayol *et al.,* 1995). Its

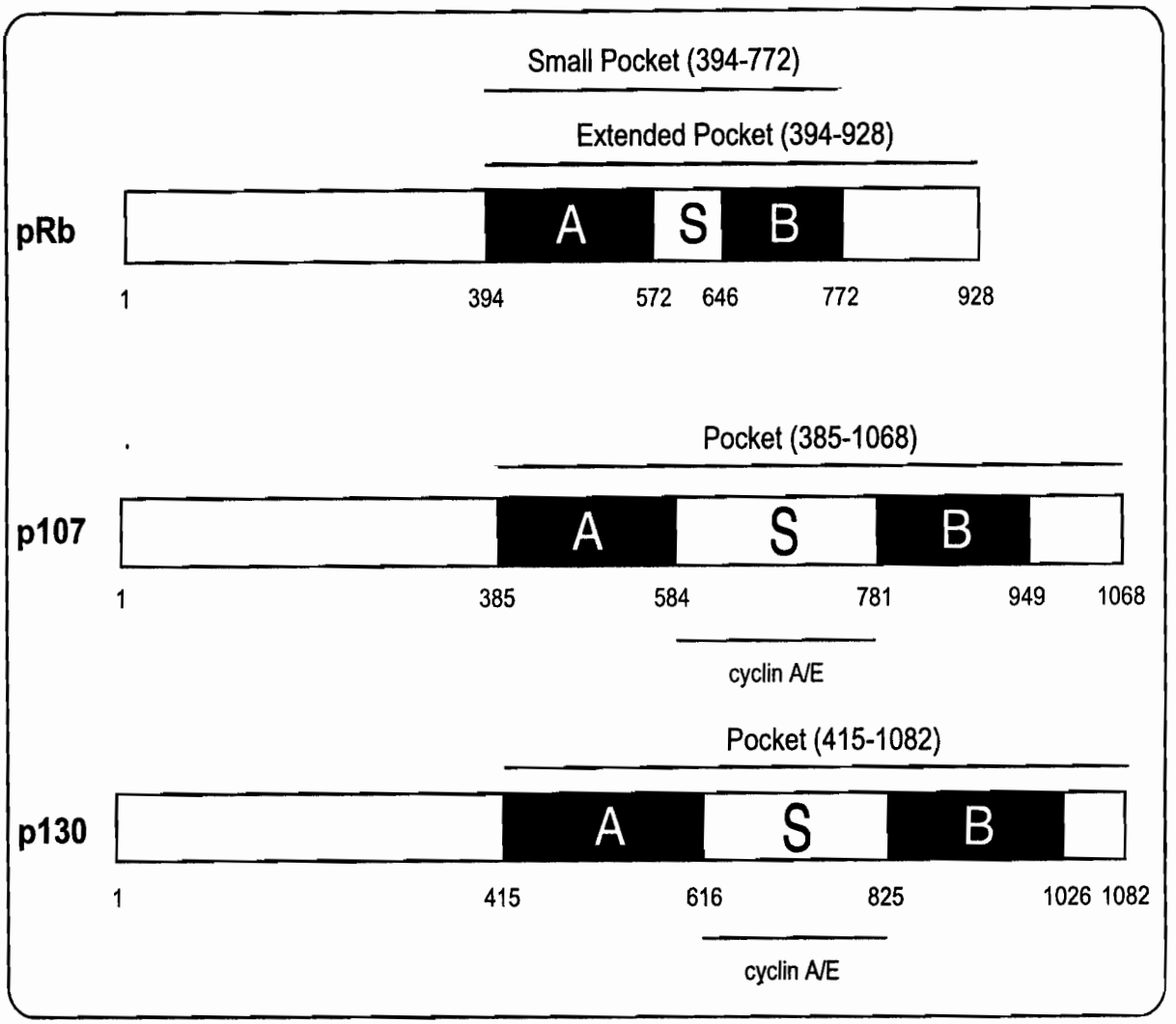


Figure 1.4 General Structure of the Pocket Proteins
The functional domains are illustrated as boxes. The pRb family members, pRb, p107 and p130, share two highly conserved domains: the A box and the B box. Together with the spacer region, the A and B domains form what is known as the small pocket. The A/B pocket serves as a binding site for LXCXE-containing proteins. E2F binding occurs in an extended region referred to as the large pocket. This pocket comprises the A/B pocket as well as the C-terminus. Regions flanking the pocket as well as the spacer region are highly conserved between p107 and p130. p107 and p130 spacer region mediates cyclin A and E binding. (Adapted from Sidle et al., 1996). This figure is drawn to scale.

accumulation reflects the entry into a quiescent state rather than the transient passage through G_1. As cells exit their quiescent state, p130 becomes phosphorylated, triggering its translocation to the cytoplasm followed by its degradation via the ubiquitin-proteasome pathway (Mayol *et al.,* 1995: Verona *et al.,* 1997; Smith *et al.,* 1998). It then reappears at late mitosis.

In contrast, p107 protein levels are controlled at the mRNA level via an E2F-responsive promoter. Quiescent cells do not express detectable p107. p107 first appears at mid G_1 and its level is maintained until the end of S phase (Suzuki *et al.,* 1995). Ginsberg *et al.* showed that p130/E2F down-regulates the p107 promoter in quiescent and early G_1 cells (Ginsberg *et al.,* 1994; Zhu *et al.,* 1995b). As p130 is degraded in late G_1/S phase, activation of p107 transcription occurs by relieving the p107 promoter from p130-mediated repression. High amounts of p107 protein are thus found in proliferating cells (from G_1 to G_2/M) (Stiegler *et al.,* 1998). p107 protein levels remain relatively constant in proliferating cells until the end of S phase (Suzukj *et al.,* 1995; Nevins, 1998).

1.7 Mouse knockout studies

Knockout mouse studies provided evidence that beside their overlapping biochemical properties, pRb family members also have very distinct functions. These studies revealed that the pocket proteins are implicated in processes such as cellular differentiation and inhibition of apoptosis during development (Cobrinik *et al.,* 1996). The phenotype of these mice varied depending on their genetic background. Different tissues appear to have different requirements for pRb family members. Only pRb-deficient mice developed tumours, supporting the fact that pRb plays a unique role as a tumour suppressor gene (Clarke *et al.,* 1992; Jacks *et al.,* 1992; Lees *et al.,* 1992).

pRb is absolutely required for normal mouse development as pRb-nullizygous embryos die by the 14^{th} embryonic day. This suggests that p130 and/or p107 are able to compensate for pRb in early embryonic development (Clarke *et al.,* 1992; Jacks *et al.,* 1992; Lee *et al.,* 1992). The most prominent abnormalities in these embryos are dyserythropoiesis, ectopic mitosis and apoptosis in regions of the developing brain and

spinal cord (Lee *et al.,* 1994). In contrast, the p107- and p130-deficient mice are viable, healthy and fertile (Cobrinik *et al.,* 1996; Lee *et al.,* 1996).

On the other hand, uncontrolled expression of *RB1* gene induces premature cell growth arrest and alters normal differentiation patterns in the entire animal resulting in a dwarf phenotype (Lin *et al.,* 1996).

1.8 Interaction of pRb with its Associated Proteins

pRb is able to bind and modulate the activity of several proteins. The most studied transcription factors associating with pRb are E2Fs (or RBAP2, retinoblastoma associating protein 2) (Shirodkar *et al.,* 1992; Cao *et al.,* 1992a, b; Chepallan, 1994; Sala *et al.,* 1994; Jiang *et al.,* 1995).

1.8.1 E2F Family

The first cDNA clone encoding a protein capable of binding to pRb and to the specific E2F DNA sequences was isolated by screening an expression library using the A/B pocket of pRb as a probe. This protein is now referred to as E2F1 (Helin *et al.,* 1992a, b; Kaelin *et al.,* 1992; Shan *et al.,* 1992).

E2F factors are key components of a cell cycle checkpoint that determine whether a cell will arrest in G_1 or enter into S phase. In addition, E2F factors have also been implicated in regulating growth inhibition, differentiation, apoptosis and oncogenic transformation.

Intriguingly, E2F1 exhibits properties of both an oncogene and a tumour suppressor. E2F1 knockout mice exhibit a broad range of tumours, suggesting that E2F1 would function as a tumour suppressor (Field *et al.,* 1996; Yamasaki *et al.,* 1996). It is believed that this function derives from the ability of E2F1 to interact with pRb, converting it into a transcriptional repressor (Dyson, 1998; Yamasaki *et al.,* 1998). However, E2F1 overexpression in transgenic mice promotes tumorigenesis, prompting its classification as an oncogene. In addition, E2F1 is oncogenic in transformation assays (Singh *et al.,* 1994; Xu *et al.,* 1995).

The E2F transcription factor family consists of at least seven distinct proteins: E2F1, -2, -3a, -3b, -4, -5 and E2F6, also referred to as EMA (E2F-binding site modulating activity) (Morkel *et al.,* 1997; Trimarchi *et al.,* 1998). E2F is a heterodimer composed of an E2F polypeptide and a DP polypeptide (Helin *et al.,* 1992a, b; Ivey-Hoyle *et al.,* 1993; Lees *et al.,* 1993; Beijersbergen *et al.,* 1994; Ginsberg *et al.,* 1994; Sardet *et al.,* 1995; Morkel *et al.,* 1997; Cartwright *et al.,* 1998; Gaubatz *et al.,* 1998; Trimarchi *et al.,* 1998).

All E2Fs have similar structures although E2F1, -2, and -3 are more closely related than E2F4 and –5 (see figure 1.5). E2F1-5 contain a DNA-binding domain and a C-terminal transactivation domain (Morkel *et al.,* 1997; Trimarchi *et al.,* 1998). The 70 aa DNA-binding domain found at the amino terminus represents the area of greatest homology between the five E2F species (Sardet *et al.,* 1995; Buck *et al.,* 1995; Slansky *et al.,* 1996). Adjacent to the DNA-binding domain is the DP dimerization domain. That region contains a leucine heptad repeat that is responsible for the association between E2F and DP proteins and DNA-binding (Girling *et al.,* 1993; Jordan *et al.,* 1994). The carboxy termini of the five E2F polypeptides possess defined transcriptional activation domains, which are characterized by an abundance of acidic residues (Kaelin *et al.,* 1992; Shan *et al.,* 1992). Embedded within the transactivation domain of each E2F is a region of homology involved in binding to the pocket proteins (Mayol *et al.,* 1998). An additional region of homology termed the "Marked box" lies between the dimerization and transcriptional activation domains. Although this "Marked box" motif is highly conserved among the different E2Fs, its precise function is unclear. The amino termini of E2F1, -2, and -3 contain an additional region of homology not found in E2F4, -5. This region has been demonstrated to have several functions, including binding to cyclin A protein (Krek *et al.,* 1994; Xu *et al.,* 1994). E2F6 lacks the pocket protein binding domain as well as the acidic C-terminal transcriptional activation domain. Instead it possesses a repression domain at its N-terminus. It appears to function as a repressor of E2F site-dependent transcription independently of pocket protein binding (Cartwright *et al.,* 1998; Gaubatz *et al.,* 1998; Trimarchi *et al.,* 1998; Morkel *et al.,* 1997).

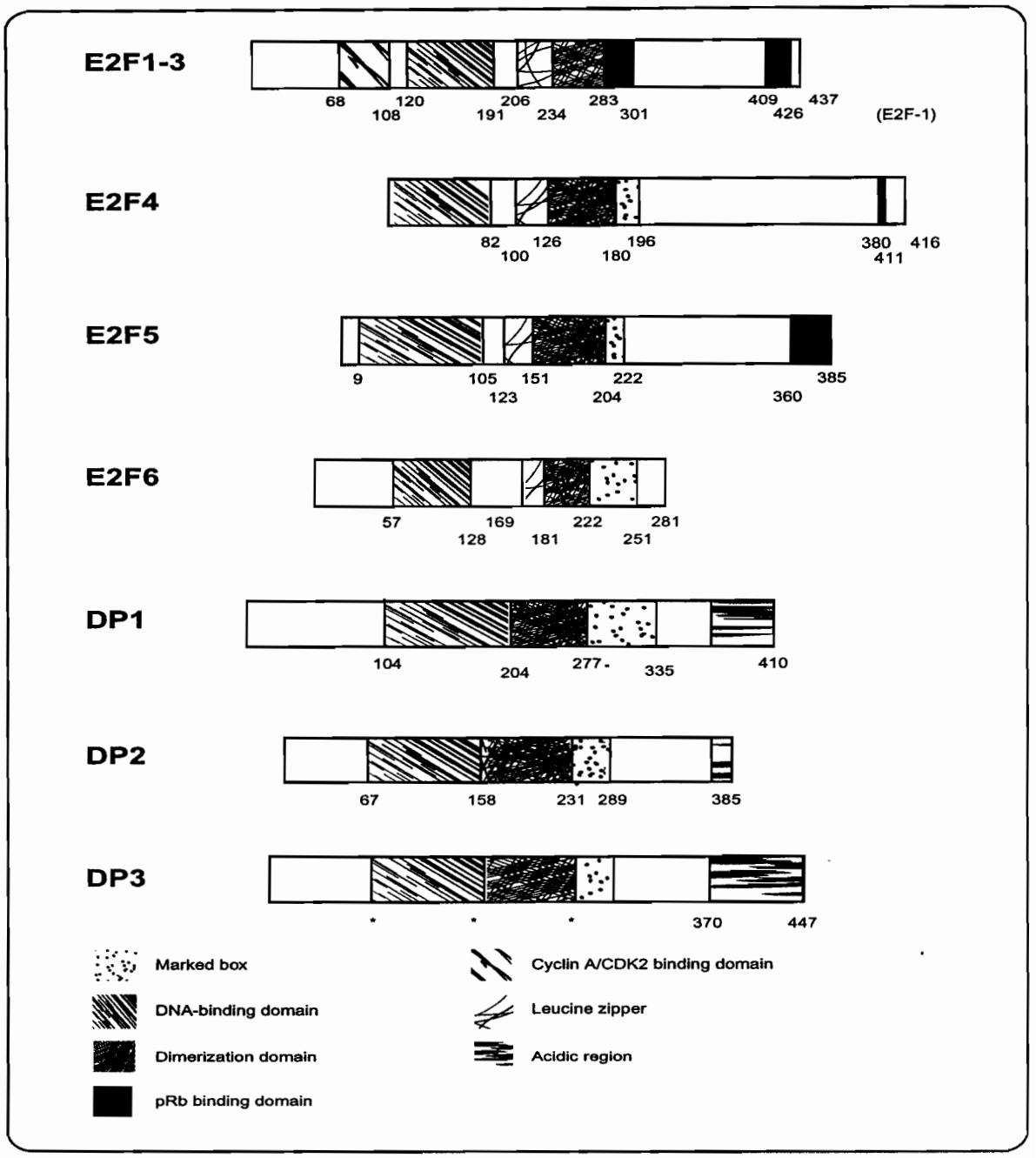


Figure 1.5 General Structure and Functions of E2F and DP Transcription Factors
The E2F family possesses 6 different members whereas the DP family has three. All E2Fs, but E2F6, act as transcriptional activator and can interact with the pocket proteins using their transactivation domain, located at the C-terminus. These proteins contain highly conserved regions, including a DNA-binding domain, a transcriptional activation domain and a leucine zipper motif. This leucine zipper motif is necessary for E2F to heterodimerize with DP. E2F1. -2. and -3 also have an N-terminal cyclin A-binding region that is absent in E2F4 and E2F5. DP3 has differents mRNAs encoding proteins of varying sizes (370, 371, 386 and 447 residues). Because of protein variations, the position of the domains are not indicated (*). The figure is drawn to scale. (Adapted from Lavia *et al.,* 1999)

Only two genes are responsible for the five DP proteins as DP2 has four splice variants (reviewed in Helin *et al.,* 1998; Dyson *et al.,* 1998, and references therein; Lavia *et al.,* 1999). DP1 is the major transcription factor partner associated with members of the E2F family (Helin *et al.,* 1993). Beside the DNA binding and the dimerization domains, DP1 and DP2 share limited homology with the E2Fs (see figure 1.5) (Helin *et al.,* 1993; Ormondroyd *et al.,* 1995; Wu *et al.,* 1995). It is the leucine zipper domain within the dimerization domain that is responsible for the association between E2F and DP proteins and DNA-binding (Jordan *et al.,* 1994). DP proteins do not contain transcriptional activation domains nor regions homologous to the pocket protein binding domains (Wu *et al.,* 1995; Zang *et al.,* 1995; Slansky *et al.,* 1996).

Both E2F and DP individually bind DNA. However, heterodimerization enhances their ability to bind DNA, potentiate the E2F activation domain, and stabilize its interaction with pRb (Helin *et al.,* 1993; Bandara *et al.,* 1994; Moberg *et al.,* 1996). DNA binding activity requires both the DNA binding domain and the dimerization domain (Helin *et al.,* 1993; Lees *et al.,* 1993; Ormondroyd *et al.,* 1995; Wu *et al.,* 1995). Each of the six E2Fs can form a complex with either DP1 or DP2 (Ormondroyd *et al.,* 1995; Helin *et al.,* 1993, Krek *et al.,* 1993; Wu *et al.,* 1995; Zang *et al.,* 1995).

pRb family members bind to the C-terminal activation domain of E2F1-5 (Flemington *et al.,* 1993; Helin *et al.,* 1993; Bandara *et al.,* 1994; Shan *et al.,* 1996). pRb binds to all E2Fs, except E2F6, while p130 and p107 preferentially bind to E2F4 and E2F5 (Helin *et al.,* 1992a, b; Kaelin *et al.,* 1992; Shan *et al.,* 1992; Lees *et al.,* 1993; Moberg *et al.,* 1996; Beijersbergen *et al.,* 1994; Ginsberg *et al.,* 1994; Sardet *et al.,* 1995; Vairo *et al.,* 1995; Moberg *et al.,* 1996).

Surprisingly, the pRb-binding sequences found in E2F family members do not contain the LXCXE sequence (Flemington *et al.,* 1993; Helin *et al.,* 1993; Bandara *et al.,* 1994; Shan *et al.,* 1996).

1.8.1.1 E2F-Regulated Genes

Several growth promoting genes possess E2F binding sites (TTTC/GG/CCGC/G) in their promoter region (Horowitz, 1993). E2F target genes fall into two categories: those whose encoded proteins are required for synthesis and replication of DNA; and those that contribute to cell cycle regulation. E2F also targets promoters of several proto-ocogenes (see table 1.3 for a partial list).

1.8.1.2 E2F Regulation

One way of regulating E2F activity is through their association with certain pRb family members. Binding to hypophosphorylated pocket proteins sequesters and actively represses their transcription activating capacity. This results in a blockade of E2F-mediated growth stimulation (Chepallan *et al.,* 1991; Schwartz *et al.,* 1993; Helin *et al.,* 1993; Mayol *et al.,* 1998; Hiebert *et al.,* 1992; Claudio *et al.,* 1994; Smith *et al.,* 1995; Vairo *et al.,* 1995; Zhu *et al.,* 1995a; Weintraud *et al.,* 1995).

The activity of E2F/DP1 is further modulated by cell cycle dependent phosphorylation of DPs and E2Fs. E2F1, -2, and -3 possess an N-terminal domain that is involved in direct binding to cyclin A (Krek *et al.,* 1994; Xu *et al.,* 1994; Dynlacht *et al.,* 1997). In S phase, cyclin A/CDK2 targets DP1 for phosphorylation, leading to a loss of DNA-binding and downregulation of E2F activities (Dynlacht *et al.,* 1994; Xu *et al.,* 1994; Krek *et al.,* 1995; Bandara *et al.,* 1994; Joss *et al.,* 1995). As well, TFIIH or CAK phosphorylation in the C-terminal region of E2F1 could mediate its degradation during S phase (Pearson and Greenblatt, 1997; Vandel and Kouzarides, 1999). E2F4, -5, and –6, which lack the cyclin/CDK2 binding motif, are possibly indirectly phosphorylated through p107 interaction with cyclin A/CDK2 through the spacer region (Pearson and Greenblatt, 1997; Vandel and Kouzarides, 1999).

Cellular localization is another way of regulating E2F activity. E2F1, -2, and -3 localize to the nucleus. In their free forms, E2F4/5 (which both lack a NLS) are found in the cytoplasm. Nuclear translocation of E2F4/5 requires co-expression of

Table 1.3 E2F-Regulated Genes				
Regulatory Genes			**DNA Biosynthesis Genes**	
pRb	p107		DNA Polymerase alpha	TS
E2F1	E2F2	E2F3a	DHFR	TK
cdc2	cdc6		Histone H2A	PCNA
cdc25A	cdc25C		ORC1	
cyclin A	cyclin E		RRM2	
B-myb	c-myc		SRP20	

Table 1.3 E2F-Regulated Genes
This table provides a partial list of E2F-responsive genes. These genes fall into two categories: regulatory genes and DNA biosynthesis genes. (Adapted from Helin, 1998, Lavia *et al.,* 1999).

their pocket protein partner (p107 or p130) or the DP2 protein, which contain NLS motifs. Bound to their pocket protein partner, they act as repressor, while bound to DP2, they act as activators (Magae *et al.,* 1996; Linderman *et al.,* 1997; Muller *et al.,* 1997; Moberg *et al.,* 1996).

Targeted protein degradation is also used to control E2F protein levels (Hateboer *et al.,* 1996; Campanero *et al.,* 1997; Hofmann *et al.,* 1996). E2F1 and E2F4 contain sequences at their C-termini that target them for degradation (Hateboer *et al.,* 1996; Campanero *et al.,* 1997). Free E2Fs are unstable and rapidly degraded by the ubiquitin-proteasome (Campanero and Flemington, 1997). E2Fs found in complexes are protected through shielding of the signal by the pocket protein (Hateboer *et al.,* 1996; Hofmann *et al.,* 1996; Campanero *et al.,* 1997).

Other mechanisms, such as acetylation by P/CAF and by p300/CBP as well as CpG methylation provide other levels of regulating E2F activity (Martinez-Balbas *et al.,* 2000; Campanero *et al.,* 2000). Acetylation by P/CAF has three functional consequences on E2F1 activity: increased DNA-binding ability, activation potential, and protein half-life (Martinez-Balbas *et al.,* 2000). This suggests that acetylation stimulates the functions of the freeform of E2F1.

1.8.1.3 E2Fs and the Cell Cycle

Quiescent cells are characterized by the presence of E2F3b/pRb complex (Adams *et al.,* 2000; Leone *et al.,* 2000). These cells also contain E2F/p130 complexes. Unlike E2F3b, which is expressed equivalently in quiescent and proliferating cells, the expression of the E2F1, -2, and -3a genes is very tightly coupled to cell growth (see figure 1.6) (Sardet *et al.,* 1995; Moberg *et al.,* 1996; Johnson *et al.,* 1994b; Hsiao *et al.,* 1994; Sears *et al.,* 1997). Little or no expression of these genes is seen in quiescent cells, whereas their transcription is rapidly induced after growth stimulation, reflecting an E2F-dependent repression mechanism in quiescent cells (Dyson, 1998; Nevins, 1998; Lavia *et al.,* 1999; Slansky *et al.,* 1996). Since these genes are expressed as p130 is degraded, it has been suggested that p130/E2F4/5complexes are responsible for their downregulation in the quiescent

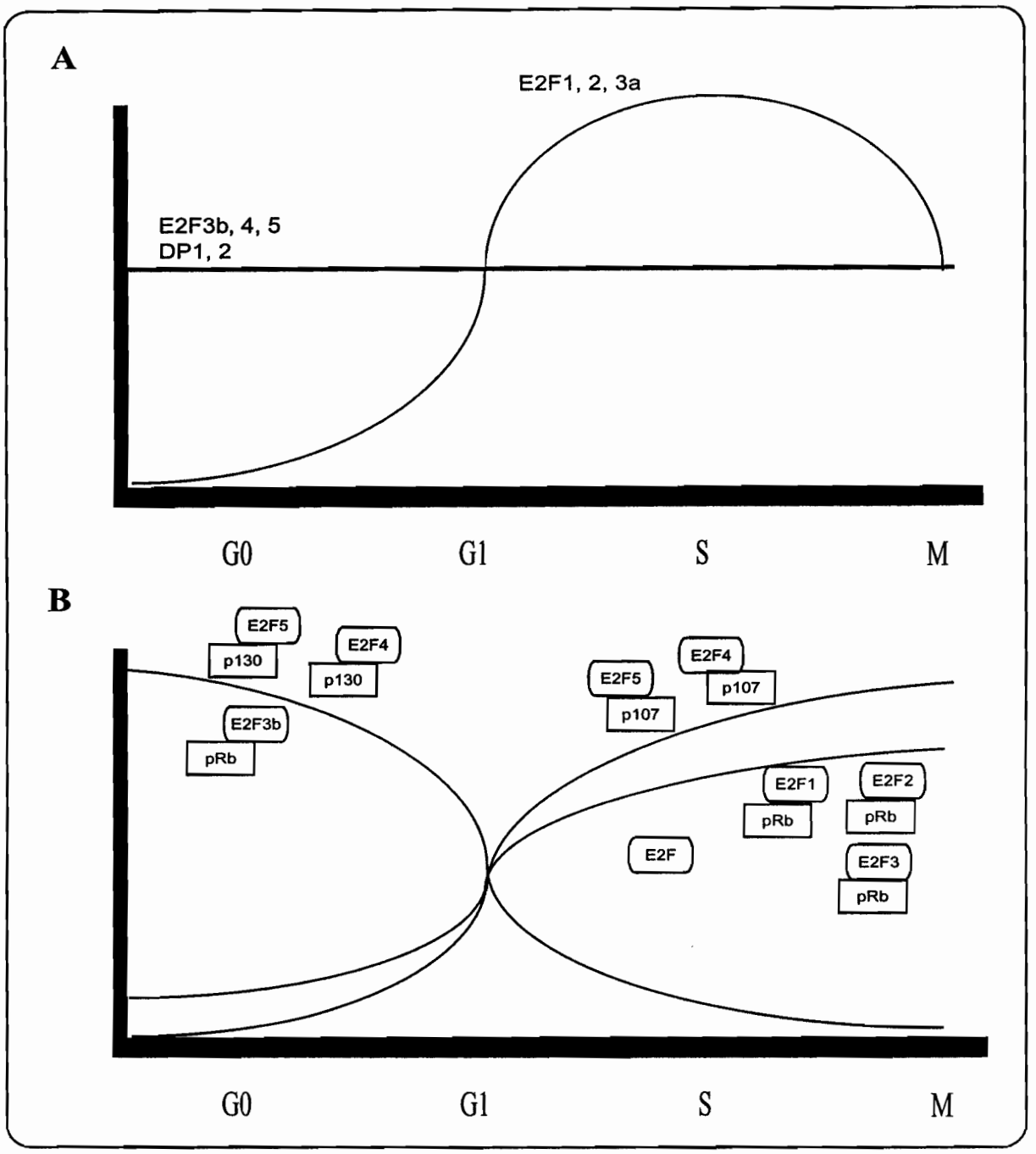


Figure 1.6 E2Fs and Pocket Proteins/E2F Complexes Formation during the Cell Cycle Panel A illustrates the expression profile of E2F and DP proteins. As mentionned in the text, expression of E2F1, 2, and 3 is the only one to be cell cycle dependent. Panel B shows pocket proteins/E2F complexes formation during the cell cycle. Notice that as cells progress through S phase, there is a switch from p130/E2F to p107/E2F complexes. Free E2F start to appear at the G1/S transition although pRb/E2F and p107/E2F complexes persist into S phase. This suggests that there is new synthesis of E2F. This figure is not to scale and the levels depicted above are not quantitative. (Adapted from Nevins, 1998)

state. p107/E2F complexes appear in G_1/S and disappear in late S upon degradation of p107. E2F4 and E2F5 protein levels are relatively constant throughout the cell cycle, exhibiting only a slight increase as cells go through mid G_1 (Ginsberg *et al.,* 1994; Sardet *et al.,* 1995; Moberg *et al.,* 1996). E2F4 is the major E2F class in G_o nuclei, but it is targeted to the cytoplasm in G_1/S (Magae *et al.*, 1996; Moberg *et al.*, 1996; Muller *et al.,* 1997; Helin, 1998).

DP1 expression overlaps with the expression of all E2F family members throughout the cell cycle. DP2 expression seems to be tissue-specific (Zhang *et al.,* 1995).

E2F family members have different S phase promoting capabilities. E2F1, -2, and –3 are the most efficient at promoting cell cycle entry (Sardet *et al.,* 1995). DP1 expression is required for E2F4 to promote cell cycle progression. This is probably due to the fact that E2F4 lacks an NLS and thus requires its binding partner to enter the nucleus (Beijersbergen *et al.,* 1994; Johnson *et al.,* 1993; Kowalik *et al.,* 1995; Shan *et al.,* 1994; Qin *et al.,* 1994; Lukas *et al.,* 1996). E2F5 does not induce S phase in quiescent cells, in agreement with the fact that it is the only E2F factor that cannot transform cells in culture (Beijersbergen *et al.,* 1994; Shan *et al.,* 1994; Xu *et al.,* 1995; Singh *et al.,* 1994; Johnson *et al.,* 1994a; De Gregori *et al.,* 1997). However, a role for E2F5 in promoting cell cycle progression is suggested as its expression is induced as cells progress from G_1 to S phase in response to serum stimulation (Sardet *et al.,* 1995).

1.8.1.4 Pocket Protein association with E2Fs in the cell cycle

pRb, p107 and p130 interact with E2Fs at different stages of the cell cycle (see figure 1.6) (Cao *et al.,* 1992a, b; Chittenden *et al.,* 1993; Cobrinik *et al.,* 1993; Lees *et al.,* 1992; Mudryj *et al.,* 1991; Shirodkar *et al.,* 1992). It is the E2F component of the heterodimer that determines which pocket protein it is going to associate with (Helin *et al.,* 1993; Krek *et al.,* 1993).

p130/E2F4, p130/E2F5 and pRb/E2F3b are the most prominent complexes in G_o and G_1 cells. As cells progress through G_1, p130 protein levels are dramatically

reduced, relieving p107 promoter from p130-mediated repression. This coincides with the appearance of p107 protein in late G_1. Because E2F4 and E2F5 are free from interacting with p130, p107/E2F4 and p107/E2F5 complexes start forming (Moberg *et al.,* 1996; Smith *et al.,* 1998; Richon *et al.,* 1997; Grana *et al.,* 1998). As cells approach the G_1/S phase transition, pRb/E2F1, -2, -3 complexes are forming and free E2Fs start to appear. The appearance of free E2F at the G_1/S transition is most probably due to the new synthesis of E2F since pRb/E2Fs and p107/E2F complexes persist into S phase although pRb phosphorylation occurs before the G_1/S transition (Sardet *et al.,* 1995; Johnson *et al.,* 1994b; Huang *et al.,* 1992; Shirodkar *et al.,* 1992; Smith and Nevins, 1995; Mudryj *et al.,* 1991). These processes coincide with the activation of E2F-dependent genes encoding proteins involved in DNA replication. Whether or not a cell will complete the cell cycle is determined by the [free E2F]/[pRb/E2F] ratio. Since E2F6 is a repressor and E2F6/DNA complexes are predominantly found at late S phase, it has been proposed that one of the role of this complex is to shut off gene expression that is up-regulated at the end of S phase (Cartwright *et al.,* 1998; Gaubatz *et al.,* 1998; Trimarchi *et al.,* 1998).

The activities of pocket proteins/E2F complexes are also regulated by cell cycle-dependent changes in cellular localization. p107/E2F and p130/E2F complexes are found in the cytoplasm and there is no obvious change in the cellular localization of these species throughout the cell cycle. However, the nuclear localization of pRb/E2F complexes (E2F4 being its major component) strongly suggests that it is the major specie involved in repressing transcription of E2F-dependent genes before the G_1/S transition. This complex is present at high levels only in G_1 and disappears as cells enter S phase, correlating with pRb phosphorylation and derepression of E2F4/5, which are then targeted to the cytoplasm. The reduction in nuclear E2F4 protein levels seen at later stages of the cell cycle is a consequence of these event (Gill and Hamel, 2000).

Because pRb is capable of interacting with each of the E2F proteins, the nature of the E2F/pRb complexes generally reflects the availability of the E2F proteins. As cells pass through G_1 after growth stimulation, pRb can be found bound to E2F1, -2

and –3. At later times, when inducible E2Fs decline, pRb can be found in complex with E2F4. Given these observations, a role for pRb as a controller of E2F accumulation is more realistic than a simple G_1/S phase switch.

1.0.0 pRb Interaction with Viral Oncoproteins

Disruption of the pRb/E2F complex is necessary for the life cycle of some DNA tumour viruses. This is consistent with a critical role played by this complex in S phase progression. Binding of pRb and sequestration of E2F by viral oncoproteins allow the expression of S phase specific genes necessary for viral DNA replication (Zamanian *et al.,* 1992; Hagemeier *et al.,* 1994). All members of the pRb family share the ability to interact with the E1A oncoprotein of adenovirus via their pocket domain (Whyte *et al.,* 1988a, b; Egan *et al.,* 1989). SV40 and polyoma large T antigens as well as the human papillomavirus type 16 E7 protein were also shown to interact with and inactivate pRb (DeCaprio *et al.,* 1988; Dyson *et al.,* 1989). Mutations inactivating the ability of these viral oncoproteins to bind the A/B pocket also inactivate their ability to stimulate cell proliferation (Egan *et al.,* 1988; Whyte *et al.,* 1989). Some cellular proteins also use the LXCXE motif, suggesting that viral oncoproteins could also displace cellular LXCXE proteins from interacting with pRb.

1.8.3 Other Targets of pRb Family Members

Apart from the E2Fs and viral oncoproteins, the pocket proteins regulate a number of other pathways involved in cell cycle regulation. At least fifty pRb-binding proteins have been identified (reviewed in Taya *et al.,* 1997).

pRb family members can interact with a large variety of cellular proteins (see tables 1.4, 1.5, and 1.6).

Table 1.4 pRb Binding Proteins

Cellular proteins	Functions
UBF	transcription factor
PP-1/PP2	phosphatase
P48	Ras regulator related
LamininC	nuclear matrix component
E2F-1,2,3	transcription factor
DP-1	partner for E2F's
c-myc, N-myc	transcription factor
elf-1	transcription factor
RBP1, RBP2	unknown function
PU.1	transcription factor
Cyclins D1, D2, D3	regulatory subunits for cdk's
Cdc2	ser thr kinase
RbAp48	unknown function possible G protein
PP1	protein phosphatase
c-abl	tyrosine kinase
MyoD related proteins	transcription factors
ATF2	transcription factor
ID-2	helix-loop-helix protein
Brm	disruption of nucleosome structure
BRG1	disruption of nucleosome structure
MDM2	oncoprotein
hsc 73	heat shock protein
RBQ-1	?
RbK	protein kinase
c-jun	transcription factor
MCM7	DNA replication licensing
HNuc	Nuclear protein
AhR	transcription factor
TAFII250/TFIID	transcription factor
TFIIIB	transcription factor
HBP1	transcription factor
p202	transcription factor
C/EBP, NF-IL6	transcription factor
NRP/B	nuclear matrix
PLH protein	transcription factor
AP-2	transcription factor
Trip 230	THR-coactivator
Chx 10	transcription factor
Mhox	transcription factor
Pax-3	transcription factor
myogenin	
Viral proteins	
E1A (Adenovirus)	transcription factor
Tag (SV 40)	transcription factor
E7 (papillomavirus)	transcription factor
IE.2 (Cytomegalovirus)	transcription factor

Table 1.4 pRb Binding Proteins
This table provides a partial list of pRb binding proteins.
(Adapted from Lipinski *et al.,* 1999, Grana *et al.,* 1998, and Whyte, 1995)

Table 1.5 p107 Binding Proteins	
Protein	**Function**
E2F-4	transcription factor
c-myc, N-Myc	transcription factor
cyclins A, D1, D2, D3, E	reg subunits for cdk's
MCM7	DNA replication licensing
MyoD	transcription factor
PLH protein	transcription factor

Table 1.6 p130 Binding Proteins	
Protein	**Function**
E2F	transcription factor
cyclins A, D1, D2, D3, E	reg. subunit for cdk's
MCM7	DNA replication licensing
PLH protein	transcription factor

Tables 1.5 and 1.6 107 and p130 Binding Proteins
These tables show partial lists of p107 and p130 binding proteins (Adapted from Whyte, 1995).

1.8.3.1 RBP1

The RBP1 product was initially identified and cloned because of its ability to bind to the T/E1A-binding region of the pRb pocket (Defeo-Jones *et al.,* 1991; Kaelin *et al.,* 1991).

RBP1 has a predicted size of 142.6 kDa. However, since it is highly charged (39 % of its total amino acids) and very acidic (21% of its total amino acids), this nuclear phosphoprotein migrates at 200 kDa on SDS-PAGE. RBP1 is phosphorylated at multiple sites, most probably by $p34^{cdc2}$ kinase as well as casein kinase II (CKII). Its human genomic sequence contains a splice site clustered within an internal exon giving rise to four splice variants: the full-length 1257-residues protein (referred to as isoform I); two variants that share the same N-termini and C-termini (RBP1-II deletes 162 bp and 54 codons and RBP1-III deletes 207 bp and 69 codons); and an isoform having a distinct 5'splice site within the same internal region, giving rise to a truncated C-terminus of 12 aa (RBP1-IV) (see figure 1.7) (Otterson *et al.,* 1991).

Each isoform is capable of specifically interacting with the small pocket of pRb. This is consistent with the presence of an LXCXE motif within the carboxy-terminus (aa 957-996) of RBP1. Using an HPV E7 peptide with only the LXCXE motif provided evidence that this region is important in mediating RBP1 interaction with pRb. In support of this idea, both deletion and point mutations at the LXCXE motif on RBP1 dramatically reduced binding to the small pocket of pRb (Lai *et al.,* 1999a).

The isoforms differ in a 207-nucleotide sequence containing many potential CKII and four $p34^{cdc2}$ phosphorylation sites (Fattaey *et al.,* 1993). The predicted peptides encoded by RBP1-II, and RBP1–III would remove two $p34^{cdc2}$ phosphorylation sites, while RBP1-IV would remove only one. This suggests that RBP1 activity could be modulated by its phosphorylation status. Using α-RBP1 antiserum, it was shown that RBP1 is present in abundant steady-state levels, though at different levels, in all cell lines examined (Otterson *et al.,* 1993). This is in contrast to the relatively low levels of mRNA detected by RNA blotting (Defeo-Jones *et al.,*

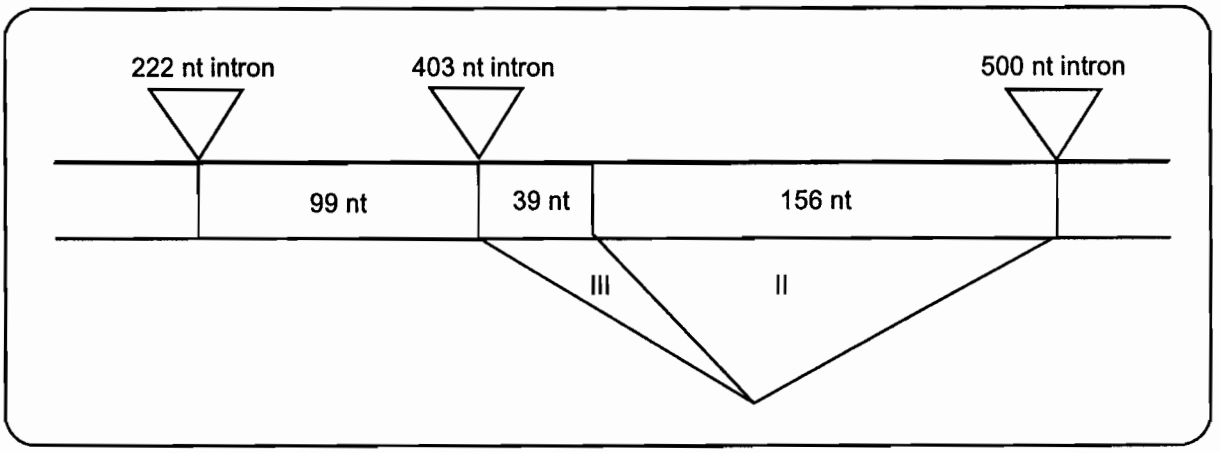


Figure 1.7 Alternative Splicing within *RBP1*
RBP1 gene has three introns. The alternative exon splicing gives rise to four RBP1 isoforms (isoform I, II, III, and IV). Several evidences suggest that this splicing is functionally relevant for RBP1 activity (see text).
(Adapted from Otterson *et al.*, 1992).

1991). RBP1-I is the predominant steady-state mRNA species, while RBP1-IV could only be detected in a bone marrow cDNA library, possibly indicating that this isoform has a tissue specific function. In accordance with previous studies, immunohistochemical analysis demonstrated that this protein localizes within the nucleus (Otterson *et al.,* 1993).

Studies on pRb family members during terminal differentiation led to the discovery that RBP1 is part of the C7 complex (novel large p130/E2F complex) and certain pRb/E2F complexes in growth arrested cells and in early G_1 cells (Corbeil and Branton, 1997). It was also demonstrated that RBP1 represses expression of the E2F1 promoter through the E2F element in a pRb/p130-dependent fashion. Consistently, colony formation assay experiments indicated that overexpression of RBP1 induced growth arrest, suggesting that RBP1 plays a role in the control of cell proliferation by inhibiting E2F-dependent transcription. Moreover, CAT assays using wild type RBP1 as well as a series of deletion and point mutants indicated that once tethered to a promoter, GAL4-RBP1 exhibits a strong repression activity (about 80-90%), suggesting that RBP1 repression activity does not depend on pRb binding (Lai *et al.,* 1999a). These results indicate that binding of RBP1 to pRb/E2F or p130/E2F complexes via the LXCXE motif bring a repressor activity to these complexes, regulating p130 and pRb-mediated growth arrest by repressing E2F promoter.

Mapping of the regions required for repression showed that RBP1 possesses two distinct repression domains, both existing apart from the pRb binding site (see figure 1.8) (Lai *et al.,* 1999a). Interestingly, neither of these domains relied on pRb interaction for repressing E2F-dependent promoter, when tethered to DNA (Lai *et al.,* 1999a).

Repression domain I (R1) comprises both the ARID region (A/T rich interacting domain) and an adjacent region having a α-helical conformation (aa 388-599). ARID is a conserved 80 amino acids stretch that was first identified as a DNA binding domain preferentially interacting with A/T-rich sequences (Herrscher *et al.,* 1995; Gregory *et al.,* 1996; Huang *et al.,* 1996). ARID regions have been found in a variety of organisms (see table 1.7). Its level of conservation ranges from 90% to 40%

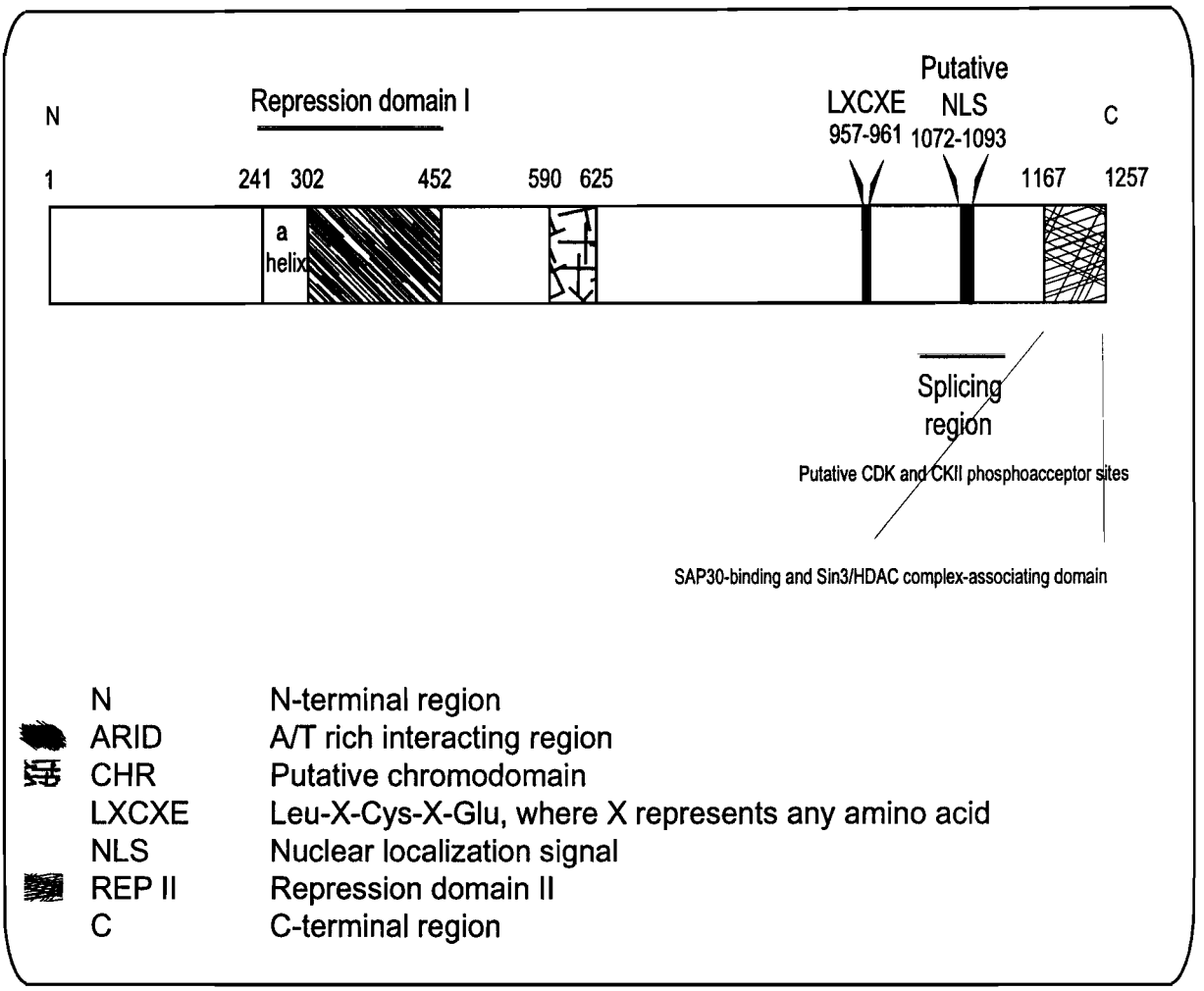


Figure 1.8 RBP1 Structure

RBP1 possesses domains that have been identified recently. Repression domain I consists of an a-helix and an ARID region. ARID is a transcriptional activation domain that can activate basal transcription. However, together with the helix, this region has been demonstrated to repress transcription in a HDAC-independent manner (see text). Repression domain II, which maps to the C-terminal portion of RBP1, represses transcription by recruiting SAP30/mSIN3/HDAC complex to the promoter. RBP1 is present in four different isoforms. The splicing region contains potential CDK and CKII sites. Bipartite nuclear localization signals are also found in that region. This suggests that the alternative exon splicing is functionally relevant for RBP1 function. RBP1 is known to associate with specialized chromatin region and the putative chromodomain could possibly mediate this activity. (Adapted from Lai et al., 1999a)

Table 1.7 ARID-Containing Proteins	
Proteins	**Organisms**
Product of dead ringer (dri)	Drosophila melanogaster
SWI1/ADR6	Yeast
B Cell Regulator of IgH Transcription (Bright)	Mouse
Modulator recognition factors (Mrf1 and Mrf2A)	Human
RBP2	Human

Table 1.7 ARID-Containing Proteins
This table shows a partial list of ARID-containing proteins. (Gregory *et al.*, 1996, Cote *et al.,* 1994; Herrscher *et al.*, 1995; Huang *et al.*, 1996; Defeo *et al.*, 1991; Kaelin *et al.*, 1992; Fattaey *et al.*, 1993; Otterson *et al.*, 1993; Kim *et al.*, 1994)

(Kortschak *et al.,* 2000). ARID-containing proteins have all been implicated in transcriptional regulation. Expression of this segment of RBP1 linked to the GAL4 DBD led to activation of basal transcription whereas ARID by itself had no effect (Lai *et al.,* 1999a). This suggests that, although RBP1 has high affinity towards DNA, this property may not be a result of the presence of ARID (Fattaey *et al.,* 1993). Our group showed that the α-helical region cannot repress transcription alone, but rather requires the presence of ARID. Such repression is independent of all classes of histone deacetylases that have been isolated to date (Lai *et al.,* 1999). The mechanism of how this region represses and activates transcription remains to be elucidated.

In addition, RBP1 also contains a 30-50 amino acids chromodomain (see figure 1.8) (Koonin *et al.,* 1995; Jones *et al.,* 2000). This motif is also found in several eukaryotic chromatin-binding proteins (see table 1.8). A common feature of these proteins is that they are implicated in transcriptional repression. Interestingly, neither of the transcriptional repression domains of RBP1 map to the chromodomain and deletion mutants analysis showed that this region is dispensible for RBP1-induced transcriptional repression. The exact function of the chromodomain is still in debate.

Some groups recently suggested that pRb-mediated repression of E2F-dependent promoters occurs via both HDACs-dependent and independent mechanisms (Brehm *et al.,* 1998; Ferreira *et al.,* 1998; Luo *et al.,* 1998; Magnaghi-Jaulin *et al.,* 1998). They suggested that HDAC1 would utilize its degenerate ICXCE motif to directly interact with the small pocket of pRb (aa 379-792) (Ferreira *et al.,* 1998; Magnaghi-Jaulin *et al.,* 1998). Mapping studies revealed that RBP1 contains separable pRb-binding and HDAC-binding domains (Lai *et al.,* 1999a). The pRb-binding domain is the region containing the LXCXE domain, whereas the HDAC-binding domain overlaps with R2 (Lai *et al.,* 1999a).

Results from binding studies suggested that RBP1 interacts with pRb in a pocket-dependent manner as such interactions were completely abolished when the assays were performed in 293T cells (Lai *et al.,* 1999a). Moreover, the same studies also demonstrated that RBP1 and HDACs interactions were not sensitive to the presence of T antigen and were thus occuring in a pocket-independent manner. These studies suggest that RBP1 acts as a bridging factor linking class I HDACs (HDAC1, 2,

Table 1.8 Chromodomain-Containing Proteins	
Proteins	**Organisms**
SWI6	Fission yeast
CHD1	Mammalian
Polycomb (PC)	Mammalian
Polycomb (PC)	Drosophila
Drosophila heterochromatin protein 1 (HP1)	Drosophila

Table 1.8 Chromodomain-Containing Proteins
This table provides a partial list of ARID-containing proteins.
(Assland and Stewart, 1995; Platero *et al.*, 1995; Messmer *et al.*, 1992; Strokes and Perry, 1995; Lorentz *et al.*, 1994).

and 3) to the small pocket of pRb in addition of providing a second HDAC-independent repression function (Lai *et al.,* 1999b).

Drugs inhibition studies revealed that RBP1 can repress E2F-dependent promoter via pRb interactions in both HDACs-dependent (via R2) and independent (via R1) manners (Luo *et al.,* 1998; Lai *et al.,* 1999b). These studies also indicated that all HDAC activity associated with the pocket proteins or the R2 (between residues 1314-1404) of RBP1 was inhibited by TSA (Lai *et al.,* 1999b). Consistently, it was previously demonstrated that pRb and RBP1-mediated transcriptional repression is only partially sensitive to TSA, supporting the hypothesis that pRb-mediated repression could also utilize the HDACs-independent repression activity associated with R1 of RBP1 (Luo *et al.* 1998; Lai *et al.,* 1999b).

This model suggests that RBP1 would be implicated in pRb-mediated repression of E2F-driven promoters by binding to hypophosphorylated pRb in growth arrested cells and recruiting HDACs.

Studies performed by other groups revealed that RBP1 is part of the mSIN3/HDAC complexes (Zhang *et al.,* 1999). This is one of the two class I HDAC complexes found in mammalian cells (Xue *et al.,* 1998; Zhang *et al.,* 1997, 1998, 1999; Wade *et al.,* 1998, 1999). The mSIN3/HDAC complex is recruited to pRb via a pocket-dependent association with RBP1. However, such interactions between RBP1 and mSIN3/HDAC most likely occurs via another bridging factor; SAP30 (Brehm *et al.,* 1998; Ferreira *et al.,* 1998; Luo *et al.,* 1998; Magnaghi-Jaulin *et al.,* 1998; Lai *et al.,* 1999c). Mapping studies revealed that RBP1 contacts SAP30 directly via the R2 repression domain whereas SAP30 interacts directly with HDAC1 and HDAC2 within the complex. Our group also determined that the relative amount of class I HDAC activity being recruited to the pocket of pRb via binding of SAP30 to R2 is about 50-60% of the total HDAC activity recruited to pRb. The rest may be accounted for by other pRb-binding proteins such as RBAP48 or c-ski (Tokitou *et al.,* 1999). Thus, pRb family members recruit the mSIN3/HDAC complex via the pocket association with RBP1.

No RBP1 mutations have been detected in cancer cells so far. However, it has recently been reported that breast cancer patients develop high titers of α-RBP1 IgG

antibodies (Cao *et al.*, 1999). These breast cancer cells overexpressed antibodies against KASIFLK peptide (aa 250-256 of RBP1) (Cao *et al.*, 1999). Interestingly, this peptide sequence is unique to RBP1. Although further work is required, this suggests that RBP1 could be involved in tumorigenesis.

1.0 pRb and Repression of Transcription

Several mechanisms have been proposed to explain how pRb represses transcription once bound to DNA. It has initially been suggested that pRb represses E2F-dependent transcription simply by masking E2F activation domain (Helin *et al.*, 1993). However, this model could not explain why deletion of certain E2F promoters led to an increase in gene expression (Dalton, 1992; Dyson, 1998). Moreover, the ability to bind E2F was not sufficient for pRb to repress transcription as some pRb mutants could bind E2F but failed to repress (Sellers *et al.*, 1998). Weintraud *et al.* suggested that pRb could block the ability of promoter-bound transcription factors, such as AP-2 and PU.1, to interact with the basal transcription complex (Weintraud *et al.*, 1995). Pocket proteins could also repress transcription by remodelling chromatin structure through interaction with proteins such as hBRM, BRG1 and HDAC1 (Dunaief *et al.*, 1994; Strober *et al.*, 1996; Trouche *et al.*, 1997; Brehm *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998). Consistent with this idea, studies have described a role for histone deacetylation in transcription repression whereby a histone deacetylase is brought to a target promoter via interactions with sequence-specific transcription factors (Laherty *et al.*, 1997; Nagy *et al.*, 1997). Indeed, we recently showed that class I HDACs are being recruited to pRb via RBP1 and that this coincides with the ability of pRb to repress transcription (Lai *et al.*, 1999b). Further experiments demonstrating that pRb represses transcription of a wide variety of promoters independently of E2F, when tethered to DNA, led to a model suggesting that pRb assembles an active repression complex that is targeted via E2F interactions (Hamel *et al.*, 1992; Weintraud *et al.*, 1992, 1995; Sellers *et al.*, 1995). In support of this idea, mapping studies revealed that pRb repression activity mapped to the small pocket of pRb, which is unable to interact with E2F.

Two classes of mammalian HDACs complexes have been identified so far (Taunton *et al.,* 1996; Yang *et al.,* 1996, 1997; Dangond *et al.,* 1998; Emiliani *et al.,* 1998; Miska *et al.,* 1999; Wang *et al.,* 1999). Class I enzymes comprises HDAC1, HDAC2, and HDAC3. These proteins have homology with yRpd3. Class II enzymes, which share homology with yHDA1, has four members; HDAC4, HDAC5, HDAC6, and HDAC7. Mammalian cells contain at least two distinct histone deacetylase complexes (mSIN3/HDAC and NURD) both containing class I HDACs (Wade *et al.,* 1998, 1999; Xue *et al.,* 1998; Zhang *et al.,* 1997, 1998, 1999). Most of the subunits in the NURD and mSIN3/HDAC complexes have now been identified. Although some subunits are common to both complexes (such as RBAP46/48, HDAC1 and 2), most subunits are distinct.

pRb has been shown to be able to repress transcription by all three eukaryotic RNA polymerases (reviewed in Dynlacht, 1997).

It has been suggested that pRb regulates RNA pol II transcription by blocking the activity of sequence-specific transcription factors by inhibiting their interaction with components of the basal transcription initiation machinery. It is also possible that pRb directly interacts with the transcription machinery itself. pRb could also regulates pol II activities by binding to $TAF_{II}250$. $TAF_{II}250$ is one component of TFIIID (Dynlacht *et al.,* 1991; Tanese *et al.,* 1991). Both of its N- and C-termini have kinase activity (NTK, CTK). Each one of these domains is capable of autophosphorylation and transphosphorylation of the Rap74 subunit of TFIIF (Ruppert *et al.,* 1995). pRb has been shown to interact with the N-terminus of $TAF_{II}250$, inhibiting its NTK activity (Dikstein *et al.,* 1996; Shao *et al.,* 1997). Moreover, pRb also interacts with $TAF_{II}250$ via a site overlaping the Rap47 binding site. It is thus possible that by binding to $TAF_{II}250$ via the central region and preventing its interaction with the Rap47 subunit of TFIIF, pRb would affect the formation of the transcription pre-initiation complex. In support of this idea, Ross *et al.* have demonstrated that after the establishment of a partial (TFIIA/TFIID) pre-initiation complex (PIC), E2F activation become resistant to pRb-mediated repression, most probably because TFIIA/TFIID recruitment to E2F masks the pRb/E2F interface. They showed that pRb may repress transcription by preventing the recruitment of basal transcription factors such as TFIIA

and TFIID. In contrast to our model (see section 1.8.3.1), these studies propose a mechanism whereby E2F activates and pRb represses transcription without the requirement for HDACs.

Recently, a mechanism involving chromatin has been proposed to explain pRb-mediated transcriptional repression of RNA pol II. These data suggest that pRb may function through the recruitment of HDAC1, which represses transcription by promoting nucleosome formation (Brehm *et al.,* 1998; Luo *et al.,* 1998; Magnaghi-Jaulin *et al.,* 1998). Interestingly, it has been reported that certain promoters were repressed in response to HDAC1 recruitment, while others were insensitive to HDAC1-mediated inhibition (Luo *et al.,* 1998). This suggests that pRb-mediated repression could occur through distinct, promoter-specific mechanisms. Indeed, it has been shown that pRb is able to repress transcription in a reconstituted *in vitro* transcription system lacking histones (Dynlacht *et al.,* 1994).

pRb growth control capabilities might be linked to the inhibition of protein synthesis. This contention is supported by the fact that pRb has been shown to repress RNA pol I activity, which is involved in transcribing genes encoding large rRNAs (Cavanaugh *et al.,* 1995). The exact mechanism remains to be elucidated but there is a correlation between pRb binding to UBF (upstream binding factor) and its capacity to modulate protein synthesis activities. Pol I transcriptional activity is stimulated by UBF (Reeder *et al.,* 1995). UBF binds to rRNA promoters and stimulates transcription by folding the DNA as well as recruiting other proteins (Reeder *et al.,* 1995). UBF can interact with pRb both *in vitro*, and *in vivo*, via the C pocket and such binding prevents formation of the initiation complex. UBF is also known to participate in pol III transcription and, by extension, pRb may play a role in modulating tRNA transcription.

RNA pol III is recruited to a promoter and positioned over its initiation site via BRF (Kassavetis *et al.,* 1990; Rigby *et al.,* 1993). BRF is an essential component of TFIIIB (reviewed in Hernandez *et al.,* 1993; Rigby *et al.,* 1993; White *et al.,* 1998). Transient transfection experiments clearly demonstrated that pRb can repress RNA pol III transcription, regulating tRNA and rRNA synthesis, by binding to and inactivating TFIIIB (Chu *et al.,* 1997; Larminie *et al.,* 1997, 1998; White *et al.,* 1997).

1.9.1 Transcriptional Repression of E2F by pRb

Whereas most E2F sites in cellular promoters act as positive regulatory elements, some E2F sites have also been shown to act primarily as negative elements. Most likely, these differences in transcriptional activity depend on the promoter context. Some observations suggest that the chromatin structure found *in vivo* and the specific sequence of the E2F site may determine if a given E2F/pocket protein complex will bind to a given E2F site (Zwicker *et al.,* 1996; Tomassi *et al.,* 1995; Zhu *et al.,* 1995a). As well, recent studies have shown that different E2F factors may be responsible for regulating different E2F target genes. Tao *et al.* have demonstrated that E2F, DP, and pRb protein each influence the selection of E2F-binding sites (Tao *et al.,* 1997).

In gene promoters such as cdc2, cyclin A, cyclin E, B-myb, c-myc, pRb1, p107, E2F1/2/3, insulin-like growth factor-1 (IGF-1) and cdc25A, E2F DNA binding sites function as negative regulatory elements (Dalton, 1992; Lam and Watson, 1993; Hsiao *et al.,* 1994; Ohtani-Fujita *et al.,* 1994; Shimizu *et al.,* 1995; Sugarman *et al.,* 1995; Zhu *et al.,* 1995a). This has been demonstrated by the fact that a mutated E2F binding site resulted in increased expression (reviewed in Helin *et al.,* 1998; Dyson *et al.,* 1998). Thus, pRb does not simply mask the E2F activation domain. Rather, pRb assembles an active repression complex that represses gene expression below basal levels. Repression activity was mapped to the small pocket of pRb. Thus, the large pocket of pRb is required for binding to E2F and the small pocket is responsible for active repressive function (Chow *et al.,* 1996; Starostik *et al.,* 1996).

1.9.2 Transcriptional Activation by pRb

Under certain circumstances, pRb enhances the transcriptional activity of transcription factors (Sellers *et al.,* 1996). Among others, positive regulation by pRb has been demonstrated in the cases of NF-IL6, SP-1 and RCE binding protein(s). pRb has also been implicated in the transcriptional activation of MyoD, mediating muscle cell commitment and differentiation (Gu *et al.,* 1993). Both pRb and MyoD are

necessary to induce expression and activation of nuclear localized MEF2 (myocyte enhancer factor-2) (see section 1.13) (Gu *et al.,* 1993). As well, pRb binds to and enhances C/EBP DNA binding and transcriptional activities, promoting adipocyte differentiation (Chen *et al.,* 1996). pRb also up-regulates glucocorticoid-receptor-mediated transcription by binding to hBRM via its LXCXE motif (Singh *et al.,* 1995).

1.10 The Role of pRb in Apoptosis

Apoptosis is a genetically controlled mechanism allowing cells to commit suicide. Deregulation of this pathway can easily lead to a cellular catastrophy. Cancer and autoimmune disease can result from inappropriate proliferation, while excessive apoptosis may contribute to developmental damage or immunodeficiency. Tight regulation of this process is thus critical to ensure proper development.

Several lines of evidence support the contention that pRb is a negative regulator of apoptosis. pRb knockout mice die *in utero* after 12-13 days of development. These mice have defects in the hematopoietic system and the central and peripheral nervous systems, the latter being accompanied by massive apoptosis in tissues known to express high levels of pRb (Lee *et al.,* 1992; Jacks *et al.,* 1992; Clarke *et al.,* 1992; Mulligan *et al.,* 1998).

Dou *et al.* have found that during the process of apoptosis, pRb first becomes dephosphorylated and then cleaved by caspases, into p68 and p48 fragments (Dou *et al.,* 1995, 1997; An and Dou, 1996; Chen *et al.,* 1997; Janicke *et al.,* 1996). In addition, pRb possesses a caspases cleavage recognition sequence at its extreme C-terminus. Upon cleavage at this particular site, pRb becomes more sensitive to degradation by other types of proteases (Janicke *et al.,* 1996; Tan *et al.,* 1997; Chen *et al.,* 1997). Degradation of pRb leads to E2F and p53 activation, both activators of apoptosis.

Although E2F2, and -3, are equally capable of inducing S phase, only E2F1 overexpression induces cells to undergo apoptosis (Qin *et al.,* 1994; Kowalik *et al.,* 1995; Lukas *et al.,* 1996; Muller *et al.,* 1997). E2F1 overexpression has been shown to induce apoptosis by both p53-dependent and p53-independent mechanisms (Qin *et*

al., 1994; Kowalik *et al.,* 1995). E2F1 levels are carefully monitored and cells having inappropriately high levels of E2F1 may trigger apoptosis by signalling p53 which then initiates apoptosis (Zindy *et al.,* 1998; Bates *et al.,* 1998; Tsai *et al.,* 1998; Pan *et al.,* 1998). It has been demonstrated, via a set of E2F1 mutants, that the transactivation and the apoptotic function of E2F1 are uncoupled. In contrast, its DNA-binding activity was proven to be essential for this particular activity (Hsieh *et al.,* 1997). Studies also showed that pRb inhibits E2F1-induced apoptosis through direct binding, but not suppression of E2F1 transactivation. Zacksenhaus *et al.* have demonstrated that pRb/E2F1 complex can actively suppress expression of genes involved in apoptosis. The loss of pRb binding results in an increase in apoptosis, induced by unrestrained E2F1, and may also allow E2F1 interaction with other factors that transactivate apoptotic genes (Zacksenhaus *et al.,* 1996).

Accumulating evidences point to a more complex role of pRb than simply one of a growth suppressor. pRb is also an inhibitor of apoptosis that can influence the decision of a cell to differentiate (Morgenbesser *et al.,* 1994).

1.11 Muscle Determination and Differentiation

Four skeletal muscle myogenic control genes have been identified in all mammalian species examined so far: MyoD, myogenin, myf-5, and MRF-4 (Davis *et al.,* 1987; Edmondson and Olson, 1989; Wright *et al.,* 1989; Braun *et al.,* 1990a, b; Rhodes *et al.,* 1989; Lassar *et al.,* 1991; Emerson, 1993; Sassoon, 1993; Weintraud, 1993; Lassar and Munsterberg, 1994; Olson and Klien, 1994). These proteins share amino acid sequence homology in the basic helix-loop-helix (bHLH) structural domain (Murre *et al.,* 1989). These proteins heterodimerize through their HLH domain with the ubiquitously expressed bHLH products of the E2-2 (ITF2) and E2-5 genes (E12, E47, and ITF1) (Murre *et al.,* 1989; Lassar *et al.,* 1991; Brennan and Olson, 1990).

HLH homo- and heterodimers bind to a consensus sequence called the E box. This DNA sequence (CANNTG) is found in the promoter of a number of cellular genes implicated in muscle differentiation such as muscle creatine kinase, desmin,

actetylcholine receptor and α–skeletal and cardiac actin genes (Johnson *et al.,* 1989; Li *et al.,* 1993; Muscat *et al.,* 1993; Simon *et al.,* 1993). Binding of these complexes to the E box activates transcription of the downstream genes (Buskin and Hauschka, 1989; Moss *et al.,* 1988; Lassar *et al.,* 1991). Upon transfection, cDNAs of these four skeletal myogenic genes dominantly convert 10T1/2 fibroblasts into skeletal muscle cells upon removal of growth signals (reviewed by Buckingham, 1992; Emerson, 1990; Olson, 1990; Weintraud *et al.,* 1991; Davis *et al.,* 1987; Wright *et al.,* 1989; Edmondson *et al.,* 1989; Miner *et al.,* 1990; Braun *et al.,* 1989a, b, 1990a, b). In addition to activating genes responsible for myogenesis, these proteins autoregulate their own expression and cross-activate one another (see figure 1.9) (Thayer *et al.,* 1989; Braun *et al.,* 1989a, b).

The differentiation program is a highly regulated process (see figure 1.9). Upon reception of appropriate stimuli, MyoD and myf-5 gets activated and trigger a pathway leading to MEF2 activation. This in turn activates the transcription of myogenin as well as other muscle-specific genes. Subsequent to the expression of myogenin, p21 becomes expressed, leading to permanent cell cycle withdrawal. Once these cells have become post-mitotic, myofibrillar protein (myosin heavy chain) and enzymatic genes (muscle creatine kinase) begin to be expressed. Myocytes then fuse into multinucleated syncytial structures called myotubes. The latter then mature into various classes of myofibers (Andres *et al.,* 1995; Havely *et al.,* 1995; Walsh *et al.,* 1997).

1.12 Regulation of Myogenic Proteins

As MyoD and myf-5 are present in proliferating myoblasts, their activities must be restrained until the differentiation program starts (Tapscott *et al.,* 1988; Wright *et al.,* 1989; Braun *et al.,* 1989a, b).

Members of the Id family (Id1, Id2, Id3, and Id4, each encoded by a different gene) contain the HLH motif, but lack the basic domain (Benezra *et al.,* 1990a, b). They act as negative regulators of myogenesis by sequestering E proteins and myogenic bHLH factors into complexes that do not bind DNA. Since Id mRNA is

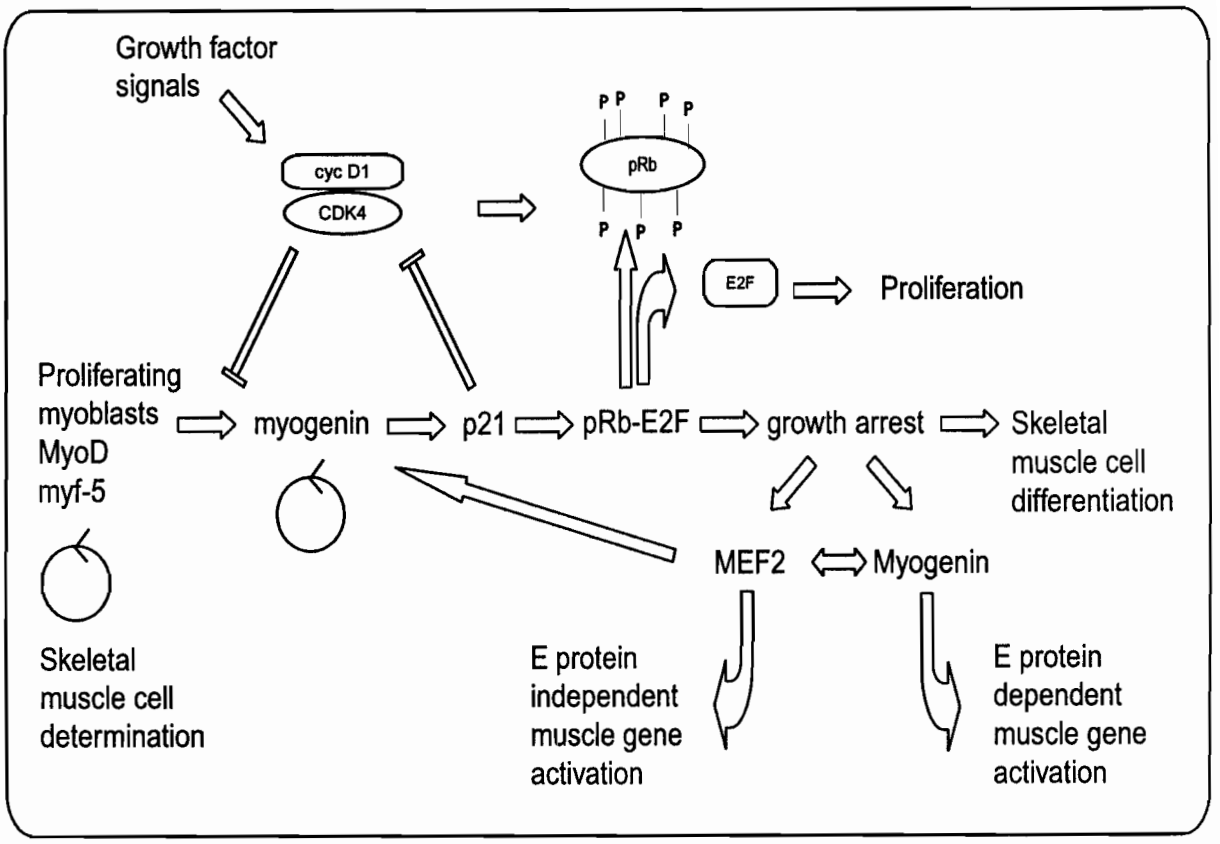


Figure 1.9 Regulatory Pathway of the Myogenic Program
Proliferating myoblasts express MyoD and myf-5. However, their activity is inhibited by cyclin D/CDK4, a G1 complex that is activated by the presence of mitogens. Unphosphorylated active pRb sequesters E2F, resulting in growth arrest. Following cell cycle withdrawal, myogenin is expressed. This protein is an important player in the pathway leading to terminal differentiation. However, cyclin D/CDK4 complex can phosphorylate pRb, liberating E2F. Free E2F stimulates cell proliferation. (Adapted from Molkenti and Olson, 1996 and Lassar *et al.*, 1994) Pointed arrows indicate positive relationships and flat-headed ones indicate negative relationship.

very unstable, Id protein levels rapidly decrease upon removal of mitogenic signals. This results in the formation of active MyoD/E12 complexes. These complexes then bind to their target enhancer sequences and activate expression of muscle protein genes.

It is likely that phosphorylation also modulates MyoD activity. This hypothesis is supported by the fact that forced expression of p21 or p16 in proliferating myoblasts triggers MyoD activation (Skapek *et al.,* 1995). Cyclin D1/CDK4 is the most likely target for p21 during differentiation since its expression correlates with MyoD phosphorylation which inhibits its function (Skapek *et al.,* 1995).

1.13 MEF2

In mammals, four MEF2 genes, MEF2A-D, encode sequence-specific DNA-binding transcription factors of the MADS-box family (MCM1 Agamous Deficiens Serum response factor) (Molkentin *et al.,* 1996a, b, c, d). MEF2 is a muscle-specific nuclear factor that activates the transcription of muscle structural genes and myogenic bHLH genes in the absence of an E box. It recognizes and binds a conserved A/T-rich DNA sequence in the regulatory regions of those genes (Gossett *et al.,* 1989). MEF2 acts relatively late in the myogenic pathway.

1.14 E2F in Differentiation

Free E2F levels are strongly reduced as cells initiate the differentiation program (LaThangue *et al.,* 1990; Corbeil *et al.,* 1995; Kiess *et al.,* 1995a; Shin *et al.,* 1995). This event is critical as ectopic E2F1 expression inhibits MyoD transcriptional activity, preventing the myogenic program to occur (Li *et al.,* 1992). It has been suggested that overexpression of E2F1 promotes the expression of growth-promoting genes, thus preventing differentiation by inhibiting myoblasts from exiting the cell cycle (Wang *et al.,* 1995).

Changes in cellular localization of E2Fs are required to prevent terminally differentiated skeletal muscle cells from re-entering S phase. Most E2F species are found in the cytoplasm in terminally differentiated myotubes (Gill and Hamel, 2000). However, E2F2 and E2F4 can partition between the nucleus and the cytoplasm. Aberrant induction of S phase occurs if E2Fs are forced to go into the nucleus (Gill and Hamel, 2000).

1.15 pRb in Differentiation

A role for pRb in differentiation was first suggested from the observation of *RB1* knockout mice (pRb-/-). These mice die *in utero* because of abnormalities in erythropoiesis and neural development (Clarke *et al.,* 1992; Jacks *et al.,* 1992; Lee *et al.,* 1992). Consistent with a role for pRb in differentiation, it has been observed that some DNA tumour virus oncoproteins inhibit myogenic differentiation through their ability to bind and inactivate the pRb family (Caruso *et al.,* 1993; Gu *et al.,* 1993). Furthermore, muscle differentiation is associated with the induced expression of nuclear hypophosphorylated pRb (Gu *et al.,* 1993). Experiments performed in pRb-/- Saos-2 cells further provided evidence that pRb expression is required for the cell cycle arrest and myogenic activities of MyoD. pRb was shown to be necessary and sufficient to induce the muscle phenotype in this pRb-/- cell line, as reintroduction of wild-type pRb induced a flat cell morphology (Sellers *et al.,* 1998). Using pRb mutants and chimeric proteins, it was demonstrated that the ability of pRb to induce myogenesis is not linked to its ability to bind E2F and repress E2F-dependent transcription (Templeton *et al.,* 1991; Hinds *et al.,* 1992; Qin *et al.,* 1992).

1.16 Cell Cycle Withdrawal is a Critical Step for Myogenesis to Occur

Skeletal muscle cells undergoing differentiation permanently withdraw from the cell cycle. In contrast to most cell types, myoblasts that exit the cell cycle to enter a quiescent state will not reinitiate cellular proliferation in the presence of growth factors (Endo and Nadal-Ginard, 1986).

1.17 HBP1

pRb is most likely to play a role in the cell cycle block by regulating E2F and HBP1. HBP1 is a transcription factor homologous to the sequence-specific HMG (high mobility group) factor family. It utilizes two LXCXE motifs to selectively interact with pRb and p130, but not with p107 (Tevosian *et al.,* 1997). A role for HBP1 in differentiation was first suggested by the observation that its protein level is drastically upregulated in the course of this process (Tevosian *et al.,* 1997).

The model suggests that MyoD induces expression of p21, leading to inactivation of multiple cyclin/CDK complexes. This, in turn, allows pRb to remain in its active hypophosphorylated form (Guo *et al.,* 1995; Havely *et al.,* 1995). Hypophosphorylated pRb binds and inactivates E2Fs, causing differentiating cells to exit the cell cycle. HBP1/pRb complexes are allowed to form as HBP1 protein accumulates, further inactivating E2F-dependent genes.

1.18 pRb as an Inhibitor of DNA Replication

p21 seems to be implicated in the regulatory pathways silencing DNA replication in myotubes (Andres and Walsh, 1996). Since p21 can inhibit cyclin/CDK2 complexes, Hengst *et al.* proposed that the activity of cyclin E/CDK2 complex in differentiated C2C12 cells could be held in check by p21 binding (Hengst *et al.,* 1998). This, in turn, would indirectly prevent the phosphorylation of key regulatory proteins important in the initiation of DNA synthesis. pRb role in differentiated muscle cells may be to target proteins involved in the temporal aspect of DNA replication. This contention is supported by the facts that an active pRb mutant devoid of phosphorylation sites can interfere with DNA synthesis in S phase-committed cells and that pRb can bind directly to the DNA licensing factor MCM7 (Knudsen *et al.,* 1998; Sterner *et al.,* 1998). Reinitiation of DNA replication in myotubes requires both activation of CDK activities and the loss of pRb (Novitch *et al.,* 1996). p21 inhibits CDK activities in myotubes so that there is no activation of

any components of the pre-RCs, which might otherwise lead to inappropriate DNA synthesis.

Although they successfully differentiate in culture, pRb-deficient myocytes are fully capable of re-entering S phase when stimulated with mitogens. However, the majority of these cells remain in S and G_2 phases and do not progress into mitosis upon serum stimulation, most probably due to the fact that p21 and p27, known to dampen CDK-dependent DNA synthesis, are expressed at high levels (Gu *et al.,* 1993; Novitch *et al.,* 1996; Schneider *et al.,* 1994). This suggests that MyoD plays a role in maintaining the non-mitotic state by positively regulating an activity that either phosphorylates cdc2 or negatively regulates genes necessary for mitosis.

The fact that cyclin A and E as well as CDK2 and cdc2 are overexpressed in pRb-deficient myocytes and that these cells are arrested in S phase might also result from a defect in E2F1/DP autoregulation. E2F1/DP complexes activate target genes, including cyclin A and cyclin E. During normal progression through S phase, the ability of E2F1/DP to bind DNA is disrupted by the phosphorylation of the DP subunit. This occurs as a consequence of the association of E2F1 with cyclin A/CDK2, suppressing E2F1/DP activity. Failure of cyclin A/CDK2 to inhibit E2F1/DP activity results in S phase arrest and subsequent apoptosis of cells. It is currently thought that the increased levels of CDIs in pRb-/- myocytes results in a decreased cyclin A/CDK2 kinase activity. This would stabilizes E2F/DP activity, thereby increasing E2F1 expression, eventually leading to a S phase arrest. Thus, pRb plays a role in blocking cell cycle progression in G_0 whereas other mechanisms are responsible for maintaining the growth arrest in M phase (Novitch *et al.,* 1996).

1.19 pRb Controls Entry into Late Stages of Differentiation

pRb-deficient myocytes express normal levels of myogenin and p21. This suggests that pRb is not required for commitment to the differentiation program nor for expression of earliest differentiation markers. In contrast, expression of late differentiation markers such MHC (myosin heavy chain) and MCK (muscle creatine kinase) requires the presence of pRb (Novitch *et al.,* 1998).

As muscle differentiation proceeds, myogenic bHLH proteins induce the expression and activity of MEF2 transcription factor family (see section 1.13) (Lassar *et al.,* 1991; Cserjesi *et al.,* 1991; reviewed in Molkentin *et al.,* 1996a, b, c, d). Along with the MyoD family, MEF2 is required for late muscle-specific gene expression (MCK, MRF-4) (Novitch *et al.,* 1999). It has recently been suggested that pRb and MyoD cooperate in the activation of MEF2. MyoD alone is sufficient to induce expression of nuclear localized MEF2 that is fully competent to bind DNA. However, both MyoD and pRb are required to stimulate MEF2 transcriptional activation. High level expression of late muscle differentiation markers requires both activation of MEF2 TAD function and G_0 arrest (Novitch *et al.,* 1996).

1.20 pRb protects Cells from Apoptosis during Myogenesis

The observation that mice deficient for pRb exhibited unique differentiation defects led to the suggestion that pRb protects cells undergoing differentiation (Jacks *et al.,* 1992; Lee *et al.,* 1992, 1994). These mice showed extensive apoptosis and defective differentiation in the nervous system and liver hematopoietic cells. Moreover, Zacksenhaus *et al.* demonstrated that cell cycle exit and apoptosis protection during myogenesis require a threshold level of pRb (Zacksenhaus *et al.,* 1996).

Precursor cells in the early stages of differentiation are vulnerable to apoptotic cell death (reviewed in Wang *et al.,* 1996). However, as myocytes permanently withdraw from the cell cycle and start expressing tissue-specific proteins, they become resistant to programmed cell death. This coincides with the accumulation of active hypophosphorylated pRb. This also correlates with the expression of p21 and the establishment of the irreversible cell cycle withdrawal. As myocytes undergo the differentiation program, p21 inhibits CDK2 and CDK4 activities, leading to pRb dephosphorylation. This ensures a complete cell cycle arrest. This is consistent with the fact that pRb expression is required for p21 to inhibit cell cycle progression (Wang *et al.,* 1996; Guo *et al.,* 1995). Both the maintenance of the active hypophosphorylated form of pRb as well as the constant expression of p21 under

conditions of serum stimulation are consistent with the irreversible cell cycle withdrawal accompanying differentiation (Wang *et al.,* 1996; Martelli *et al.,* 1994; Halevy *et al.,* 1995; Andres *et al.,* 1995).

1.21 A Role for pRb in E box Activation

pRb has been shown to stabilize MyoD/E2-2 complex when bound to the E box. Indeed, upon immunodepletion of pRb from muscle cells extracts, E box DNA binding activity is abolished or highly diminished (Gu *et al.,* 1993).

Also, pRb appears to promote myogenesis by influencing the type of myogenic factor that binds to E-box: it inhibits the binding of MyoD dimers (which cannot induce myogenesis) and promotes the binding of MyoD-E2.2 heterodimers, which can induce myogenesis (Gu *et al.,* 1993).

1.22 Regulation of pRb Phosphorylation during Myogenesis

Regulation of pRb activity during differentiation varies considerably among different tissues and cell types. pRb is present in proliferating myoblasts as well as in differentiated myotubes. In general, the total protein levels of pRb do not change upon differentiation, although the amount of hypophosphorylated active pRb increases at the onset of differentiation (Lipinski, M.M. and T. Jacks, 1999).

In myotubes, pRb is maintained in its hypophosphorylated state even in the presence of mitogens. This is consistent with the inability of these cells to re-enter the cell cycle upon growth factors stimulation. Although the precise mechanism(s) of how this happens remains to be elucidated, it has been shown that pRb is an *in vitro* substrate for $p34^{cdc2}$ kinase, whose activity is present in myoblasts and lost in myotubes (reviewed in Nurse, 1990; Lees *et al.,* 1991; Lin *et al.,* 1991; Hu *et al.,* 1992; Hinds *et al.,* 1991). In C2C12 myotubes, $p34^{cdc2}$ kinase activity is not reinduced, like pRb phosphorylation, in response to growth factor stimulation. Therefore, there is a correlation between cyclin-regulated $p34^{cdc2}$ and pRb phosphorylation during myogenesis.

Another means by which pRb phosphorylation is probably regulated during differentiation is through p21. In proliferating cells, p21 inhibits the activities of CDKs responsible for promoting S phase entry as well as those involved in pRb phosphorylation (Sher *et al.,* 1994). There is evidence suggesting that p21 may be indirectly responsible for maintaining pRb in its active form in the early stages of the differentiation program (Novitch *et al.,* 1996).

Overall, these results provide evidence that accumulation of active hypophosphorylated pRb plays an important role for maintenance of the growth arrest observed upon terminal differentiation.

1.23 pRb Family Members and Myogenesis

p107 and p130 regulation during myogenesis is different from that of pRb (Callaghan *et al.,* 1999; Ikeda *et al.,* 1996; Kastner *et al.,* 1998; Kiess *et al.,* 1995b; Paramino *et al.,* 1998; Richon *et al.,* 1997). During myoblast proliferation, p107 is phosphorylated and forms complexes with E2F4, including cyclin A/CDK2. (Ikeda *et al.,* 1996; Kastner *et al.,* 1998). Formation of these complexes does not inhibit E2F growth promoting activity. p130 levels are very low under these conditions. As cells go through terminal mitosis and begin to exit the cell cycle, there is a switch from p107/E2F4 to p130/E2F4 complexes due to the fact that p107 levels decline, while p130 increases (Kiess *et al.,* 1995b; Shin *et al.,* 1995; Corbeil *et al.,* 1995). These complexes can also include CDK4 and cyclin E/CDK2, suggesting that they could play a role in control of cyclin E/CD2 activity in differentiated cells and prevent cell cycle re-entry.

Project Proposal

Previous work on pocket proteins during myogenesis led to the discovery that upon differentiation, p107/E2F complexes disappeared, pRb/E2F complexes are maintained and p130/E2F complexes are induced (Corbeil and Branton, 1997).

Moreover, a novel p130/E2F slowly migrating complex, termed C7, was also discovered. This complex is made of E2F, pRb, p130 as well as RBP1.

RBP1 was also found to be part of pRb/E2F complexes in growth arrested cells and in early G_1 cells (Corbeil and Branton, 1997). Further studies on RBP1 revealed that its ability to repress E2F-dependent transcription and induce growth arrest following its overexpression was linked to the presence of two repression domains, both working independently from pRb. These results suggest that, by binding to pRb/E2F or p130/E2F complexes via its LXCXE motif, RBP1 regulates p130- and pRb-mediated repression of E2F-dependent transcription, inducing the growth arrest. Binding studies revealed that RBP1 acts as a linker protein between class I HDACs and pRb in addition of bringing its own repression activity (Lai *et al.*, 1999b). According to this model, RBP1 would play a role in pRb-mediated repression of E2F-dependent transcription by binding to pRb in G_0 cells and recruiting mSIN3/HDACs complexes.

One of the early step of the differentiation program is the permanent cell cycle withdrawal. pRb/MyoD complex is involved in repressing specific genes, allowing the cells to exit the cell cycle. However, as myogenesis proceeds, pRb must activate myogenic proteins (such as MEF2), that trigger the expression of structural proteins (such as MCK and MHC), leading to terminal differentiation. Since RBP1 is known to participate in pRb-mediated repression of E2F-dependent transcription by linking pRb to the mSIN3/HDAC complex (via SAP30), we studied whether or not RBP1 was involved in the differentiation pathway. We hypothesized that RBP1 plays a role in the early steps of the differentiation program by inducing the growth arrest (in concert with pRb/MyoD complex). Then, as myogenesis proceeds, RBP1 repressive functions would be turned off, allowing pRb to activate myogenic proteins necessary for terminal differentiation to occur.

Chapter 2

Materials and Methods

2.1 Design of the Peptides for the Production of Polyclonal Rabbit Antibodies against Retinoblastoma Binding Protein 1 (RBP1)

α-N-RBP1 polyclonal serum was produced commercially by injecting a peptide corresponding to the N-terminus of RBP1 (CLKQDNTTQLVQDDQVKGPLRV) which had been coupled to KLH peptide into NewZealand white rabbits (Genemed synthesis Inc). Antibodies against RBP1-II and RBP1-III were raised in the same manner against peptides corresponding to the splice junction of RBP1-II (CEDLPVLDNSNELDNMNSTER) and RBP1-III (CAAAKNEKNGTDELDNMNSTE) respectively (see figure 2.1). The resulting antibodies were immunoaffinity purified using peptide columns.

2.2 Purification of Polyclonal Rabbit Antibodies

Two rabbits were immunized per antigen; however, purification was done only on one representative serum for each antigen. 10 mg of pure peptide was resuspended in 10 ml of 1X PBS, pH 7.2 (hereafter referred to as 1X PBS, unless specified) (coupling solution). NHS-activated sepharose 4 fast flow beads (Amersham Pharmacia) were washed with 10-15 bead-volumes of cold HCL (1 mM). The beads and the coupling solution were mixed and adjusted to a pH between 6 and 8. They were then incubated overnight at 4°C with slow rotation. Beads were washed twice with 1X PBS, followed by one wash with 1 M NaCL and one wash with 1X PBS. After completion of the coupling reaction, any non-reacted group was blocked by adding 10 bead-volumes of 100 mM ethanolamine (pH 7.5) followed by incubation at room temperature for 4 hours with gentle mixing. Beads were then washed twice with 1X PBS. The beads/antigens complexes were then transferred into a column and sequentially washed with 10 bead-volumes of 10 mM Tris (pH 7.5), 10 bead-volumes of 100 mM glycine (pH 2.5), and 10 bead-volumes of 10 mM Tris (pH 8.8). 10 bead-volumes of 100 mM triethylamine (pH 11.5, prepared fresh) were then added, followed by 5 washes with 10 mM Tris (pH 7.5). The polyclonal serum was cleared of any debris by centrifugation at 13000 RPM before passing it through the column at

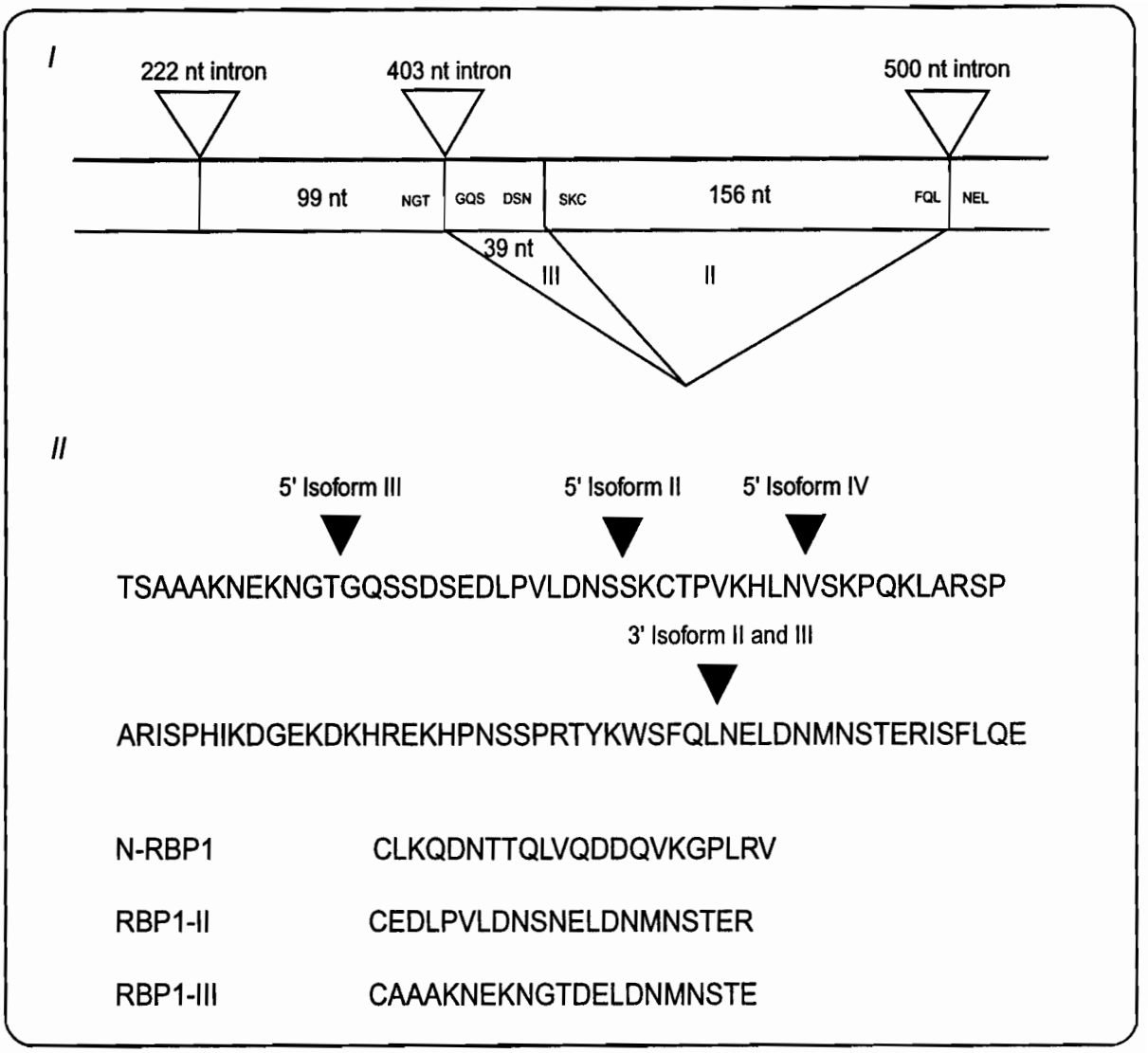


Figure 2.1 Alternative Splicing within *RBP1* and Peptide Sequence for Antibodies Production

Panel I shows the genomic structure of RBP1 and its splicing pattern. The alternative exon splicing gives rise to four RBP1 isoforms (isoform I, II, III, and IV). Several evidences suggest that this splicing is functionally relevant for RBP1 activity (see section 1.8.3.1). Panel II shows the peptide sequences that were chosen to generate the rabbit polyclonal sera. (Adapted from Otterson *et al.,* 1992).

a slow rate. Three loads were performed to ensure good binding. The column was then washed sequentially with 20 bead-volumes of 10 mM Tris (pH 7.5), and 20 bead-volumes of 500 mM NaCl/10 mM Tris (pH 7.5). Antibodies bound by acid-sensitive interactions were eluted by passing 10 bead-volumes of 100 mM glycine (pH 3.0) through the column. They were collected in a tube containing 1 bead-volume of 1 M Tris (pH 8.0). The column was washed with 10 mM Tris (pH 8.8) until the pH rose to 8.8. Passing 10 bead-volumes of 100 mM triethylamine (pH 11.5 freshly prepared) through the column eluted the antibodies bound by base-sensitive interactions. The eluate was collected in a tube containing 1 bead-volume of 1 M Tris (pH 8.0). The fractions were then dialysed against 1X PBS containing 0.02% sodium azide. Antibodies were then concentrated into 1X PBS by multiple rounds of spin-dialysis using Centricon-30 columns (Millipore, using the manufacturer's recommended protocol).

2.3 Coomassie Gel Analysis of Purified Antibodies

5 ug of protein (measured spectrophotometrically using Bradford reagent, BioRAD) was loaded on SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) containing 10% polyacrylamide. The gel was stained for 10 minutes at room temperature in filtered staining solution (90% v/v methanol:H_2O, 10% glacial acetic acid, 0.25% (w/v) coomassie staining). The gel was rinsed with water and destained 10 minutes at room temperature with destaining solution (90% v/v methanol:H_2O, 10% glacial acetic acid). The gel was destained in boiling water for 20 minutes, followed by soaking overnight in 10% glycerol. The next day, the gel was dried for 1 hour at 80°C.

2.4 Expression and Purification of Gluthathione S-Transferase Fusion Proteins

pGEX2T-N-RBP1, pGEX2T-RBP1-II and pGEX2T-RBP1-III fusion proteins as well as GST alone were expressed in competent BL21 DE3 E. *coli* bacteria

(Stratagene) using the appropriate constructs generously given by Frederic Kaye (Otterson *et al.,* 1992). The transformed colonies were inoculated in 2YT-ampicillin (100 ug/ml) and grown at 30°C with agitation for 14 hours (to get an O.D $_{600}$ of 1-1.2). They were then induced at 30°C for 60-90 minutes with 100 mM Isopropyl ß-D-thiogalactopyranoside (IPTG). Bacteria were spun down and cell pellets were frozen at –80°C. The next day, cell pellets were resuspended with 20-25 ml of GST lysis buffer (1X PBS, 1 mM DTT, 0.5 PMSF, 1% Triton X-100). Sonication on ice was used to lyse the cell followed by centrifugation at 18000 RPM at 4°C for 45 minutes. 1 ml of a 50% slurry of glutathione sepharose 4 fast flow beads (Pharmacia) was added to 20-25 ml of lysate and incubated with gentle rotation at 4°C overnight. Beads were collected and washed repeatedly with 1X PBS containing, 1% Triton X-100, 1 mM DTT and 0.5 mM PMSF over a period of 2 hours. Elution of the purified GST protein was performed using 1 ml of 20 mM reduced glutathione pH 8.5, rotating at 4°C for 15 minutes. This was repeated 3 times. GST-proteins were finally stored in 10% glycerol at –80°C. The next day, they were thawed on ice, concentrated and dialysed into 1X PBS by multiple rounds of spin-dialysis using Centricon-30 columns (Millipore, using the manufacturer's recommended protocol). Protein concentration was assayed with the Bradford assay using the BioRAD Protein assay kit and protein purity was observed by SDS-PAGE analysis followed by staining with Brilliant Coomassie Blue.

2.5 Cell Culture

CV-1 (African green monkey kidney fibroblasts) (ATCC CCL-70), human lung carcinoma-H1299 (p53-defective human large cell carcinoma) (ATCC CRL-5803) (Mitsudomi *et al.,* 1992) and L6 (rat myoblasts) (Eidelman *et al.,* 1993) were grown in monolayer culture at 37°C in a humidified atmosphere of 95% air plus 5% CO_2 using Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 units/ml streptomycin, and 0.292 mg/ml L-glutamine (GIBCO BRL, Life Technologies). NCI-H630 (human colorectal carcinoma, from ATCC (CRL-5833) were grown in RPM1 medium (GIBCO BRL,

Life Technologies) supplemented with 10% FBS and 1% PSG. C2C12 (mouse fibroblast, from ATCC CRL-1772) were maintained in D-MEM 20% FBS and 1% PSG in subconfluent culture to prevent differentiation.

2.6 Whole Cell Extracts

Tissue culture cells were washed twice with 1X PBS, and harvested by scraping followed by centrifugation. Whole cells extracts were prepared by lysing the cells on ice using RIPA buffer (50 mM Tris base, 150 mM NaCL, 1% Triton X-100, NaDoC, 0.1% SDS, 4 mM $NaVO_4$, 4 mM NaF, 1 ug/ml pepstatin, 1 ug/ml leupeptin, and 1 ug/ml aprotinin) followed by centrifugation at 4°C for 30 minutes at 13000 RPM. The supernatents were aliquoted and store at –80°C until use.

2.7 Cell Fractionation

Nuclear extracts were prepared from cells which were harvested, washed three times in ice-cold 1X PBS, and lysed in 2.5 cell volumes of lysis buffer (10 mM Tris-HCL, pH 7.5, containing 150 mM NaCL, 1 mM $MgCl_2$, and 0.08% Triton X-100 (v/v)). Nuclei were separated by centrifugation at 13000 RPM, at 4°C for 5 minutes, washed twice, and then lysed in 2 volumes of extraction buffer C (20 mM Hepes, pH 7.9, containing 25% glycerol (v/v), 420 mM NaCl, 1.5 mM $MgCL_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT) followed by incubating for 90 minutes on ice. Cells debris were removed by centrifugation at 13000 RPM, for 30 minutes and the supernatent containing the nuclear extract was aliquoted and stored at –80°C until use.

2.8 Western Blotting Analysis

Varying amount of cell extract (measured spectrophotometrically using Bradford reagent) were subject to SDS-PAGE using gels composed of 6%, 10% or 12% polyacrylamide. The proteins were subsequently transferred to a methanol activated Immobilon-P PVDF membranes (Millipore) using a semi-dry transfer

apparatus with a current density of 1.2 mA per cm^2. Following Ponceau staining to verify equal protein transfer, the membranes were blocked overnight at 4°C or for 2 hours at room temperature in PBS-T (1X TBS, 1% calf serum, 1% BSA, 0.1% Tween 20) solution containing 5% powdered non-fat milk. It was then incubated with the primary antibody for 2 hours at room temperature. The membranes were then washed with PBS-T. Reacting species were identified by addition of appropriate horseradish peroxidase (HRP)-conjugated antibodies (Jackson Immuno Research Laboratories) followed by enhanced chemiluminescence (ECL) according to manufacturer's specifications (Amersham Pharmacia).

2.9 Antibodies

Goat polyclonal antisera against actin (C-11) and immunoprecipitating antibody against HA (12CA5) were purchased from Santa Cruz Biotechnology. α-Grb-2 antibodies (3F2) were purchased froms Upstate Biotechnology, and western blotting α-HA antibodies (HA11) from Babco. LY32, LY42 and LY48 mouse monoclonal antibodies were raised by James DeCaprio and William Kaelin specially against RBP1 as described previously (Lai *et al.*, 1999a). Quale monoclonal α-MHC antibodies were kindly provided by Clifford Stanners. Rabbit polyclonal antisera against GST-HDAC were a gift from Ed Seto.

2.10 Adenovirus Infection

Cells were co-infected with adenovirus vectors expressing Tind RBP1 protein fused to HA epitope at the N-terminus and viruses expressing Ted repressor (rttA) at a total multiplicity of infection of 100 plaque-forming units per cell. The virus expressed full length RBP1 under the cytomegalovirus promoter in a vector lacking both E1A and E1B. These viruses were generously provided by GeminX Biotechnologies, Inc. All vectors were titred on 293 cells.

2.11 Immunoprecipitation-Western Studies

Immunoprecipitation assays were performed as follows. Cells were carefully washed with 1X PBS, scraped off the dish and incubated on ice for 30 minutes in 1 ml of lysis buffer (1X PBS containing 0.1% NP-40, 1X APL, 4 mM $NaVO_4$, and 4 mM NaF). The extract was then sonicated with a small-bore probe and spun at 4°C for 30 minutes at 13000 RPM. Supernatant was collected and pre-cleared with 50 ul of either protein G sepharose fast-flow or protein A sepharose fast-flow (GIBCO BRL, Life Technologies) for 2 hours at 4°C with slow rotation to eliminate non-specific interactions. Following centrifugation, the supernatant was removed and saved. 1 ml of antisera was incubated overnight at 4°C with slow rotation with protein G or A sepharose fast-flow. The bead-coupled antibodies were washed 3 times with 1X PBS containing 0.1 % NP-40. 20 ul of 50% slurry beads were then added to the pre-cleared extract followed by immunoprecipitation at 4°C overnight. Beads with bound antibody/antigen complexes were collected following centrifugation and were washed 6 times in 1X PBS containing 0.1% NP-40. Following removal of the supernatent, the beads were resuspended in 2X Laemmli sample buffer (BioRAD), and boiled for 5 minutes before resolution by SDS-PAGE.

2.12 Immunofluorescence Studies

Cells were grown in TC plates covered with autoclaved cover slips (Fisher). Culture media was removed and cells were washed once with 1X PBS. 4% paraformaldehyde (Sigma) in 1X PBS was used to fix the cell. They were then washed with 1X PBS/0.2 % Tween-20 (PBS-T). Permeabilization was performed using 1X PBS/0.5% Triton X-100 solution for 10 minutes at room temperature. 500 ul of blocking solution (1X PBS, 0.2% Tween-20, 5% horse serum and 5% goat serum) was added and incubation at 37°C for 10-60 minutes followed. Indirect immunofluorescence was performed to detect the staining pattern of endogenous proteins using antibodies against N-RBP1, RBP1-II and RBP1-III. Following removal of primary antibodies and washes with PBS-T, Alexa Fluor 488-conjugated goat anti-

mouse IgG or Alexa Fluor 594-conjugated goat anti-rabbit IgG secondary antibodies (Molecular Probes) were used to detect staining. Vectorshield-H1000 (Vector Labs) was added to prevent photobleaching and cover slips were mounted and sealed with nail polish. Cells were visualized at 100X using a light microscope (Axioplan, Ziess) to detect the presence and location of the protein. Images were captured and visualized on a PC computer using a digital output CCD camera and Metamorph Imaging System (Diagnostic Instruments).

2.13 Assay for Myogenic Differentiation and Fusion

The ability of C2C12 myoblasts to fuse into myotubes was assessed by seeding cultures at 10^4 cells per cm^2 in 100-mm plastic tissue culture petri dishes (Nunc) in 6 ml DMEM/20% FBS/1% PSG. Three days later, when the cells were confluent, the medium was changed to differentiation medium (DMEM containing 2% horse serum and 1% PSG), as already described (Eidelman *et al.,* 1993). Differentiation was allowed to occur for up to 5 days.

2.14 Hematoxylin Staining

Cells were washed with 1X PBS and were then fixed with 2.5% glutaraldehyde (Sigma) in 1X PBS for 10 minutes at room temperature. They were then washed again with 1X PBS. 5 ml of ethanol was added drop by drop and left on the cells for 2 minutes at room temperature. The same amount of water was added. Cells were washed with water and were then stained with 5 ml of Harris hematoxyllin staining solution (7.5 g/L) (Sigma) for 10 minutes at room temperature. Excess of staining was removed with water. Photographs were taken of humidified plates using a light microscope (Axioplan, Ziess) at 40X.

2.15 RNA Isolation

Cells were harvested as described previously (section 2.6) and RNA was extracted using the Rnaeasy Mini prep kit (Qiagen) according to the manufacturer's directions. 7.5 ug of total RNA was then used for reverse transcription to determine the transcript level of RBP1.

2.16. RT-PCR analysis

RT-PCR was performed using Pro STAR First-Strand RT-PCR Kit (Stratagene) following the manufacturer's directions. 7.5 ug of total RNA was reverse transcribed in a total volume of 50 ul using murine moloney leukaemia virus reverse transcriptase (MMLV-RT).

2.17 Polymerase Chain Reaction

Oligonucleotide primers for mouse glyceralgehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Biocorp. The sequence of GAPDH primers were as follows; 5` TAT TGG GCG CTT GGT CAC CA-3` (sense) and 5`-CCA CCT TCT TGA TGT CAT CA-3` (antisense). RBP1 primer sequences were as follows; 5'-GTA CAA GAG AGA GAG AGC AGA G-3' (sense) and 5'-CTC CTC CTG TCT ATG GTT GCA AC-3' (antisense). The predicted sizes of the amplified products (cDNA) were 752 bp for GAPDH and 452bp for RBP1.

PCR was performed under the following conditions with a thermal cycler (Techne). Each sample contained 5 ul of cDNA, 1X final PCR buffer, 5 mM dNTP (Pharmacia), the sense and antisense primers (10 uM for RBP1 and 2 uM for GAPDH), and 5 U/ul *Thermophius aquaticus* DNA polymerase (*Taq* DNA polymerase, GIBCO BRL, Life Technologies) in a final volume of 100 ul. PCR was carried out for 30 cycles. The following amplification protocol was used; 1st cycle with 5 minutes denaturation at 91°C, 1 minute annealing at 46°C and 2 minutes synthesis at 72°C. The following cycles consisted of 1 minute denaturation. The last

cycle extension was 10 minutes at 72°C. To monitor DNA contamination, control reactions were performed without the cDNA template. After PCR, a 10 ul aliquot of the RT-PCR (cDNA) was electrophoresed in a 1% agarose gel in Tris/acetic acid/EDTA (TAE) buffer, stained with ethidium bromide, and visualised with UV light.

Chapter 3

Experimental Results

3.1 Purification and Characterization of Rabbit Polyclonal Antibodies

We possess several efficient monoclonal antibodies (DeCaprio, unpublished) against RBP1: LY11 and LY31, which are precipitating antibodies, as well as LY32, LY42 and LY48, which are blotting antibodies. LY11 recognizes the C-terminus of RBP1 and as R2 maps to this region, we need a second immunoprecipitating antibody. Rabbit polyclonal serum against the N-terminus as well as rabbit polyclonal sera specific for the second and the third isoform were prepared using synthetic peptides. NewZealand white rabbits were immunized with the corresponding peptides (see figure 2.1), which express high antigenicity fragments of RBP1. The antigenicity profile of RBP1 was determined using the Protean sequence analysis software (DNAStar suite, Lasergene).

3.1.2 Determination of Binding Activity of Crude Antisera

NewZealand white rabbits were immunized with peptides representing a small portion of N-RBP1, RBP1-II and RBP1-III (see figure 2.1). The immunoblotting potential of the generated antisera was assayed. H630 cells, which are known to express all four isoforms of RBP1, were used to generate whole cell extract (Otterson *et al.,* 1992). The extract was separated by SDS-PAGE in one-well preparative gels. The gels were transferred to methanol-activated PVDF membranes, which were used to test the various antibodies in western blotting by means of a multiscreen apparatus (BioRAD). As a positive control for RBP1 migration, the first two lanes of the multiscreen apparatus were blotted with mouse monoclonal LY32 and LY42 already described in (Lai *et al.,* 1999a) (see figure 3.1, panel *I*). A series of dilutions ranging from 1:10 to 1:5000 of the antisera was used to determine the best conditions for visualization of RBP1 isoforms proteins on western blotting. As demonstrated in panel *II*, four bands appeared at the expected size of RBP1 for both α-N-RBP1 (each one coming from a different rabbit). α-RBP1-II from rabbit #6197 was the only one recognizing a lower form of RBP1 (panel *III*) while both α-RBP1-III antisera

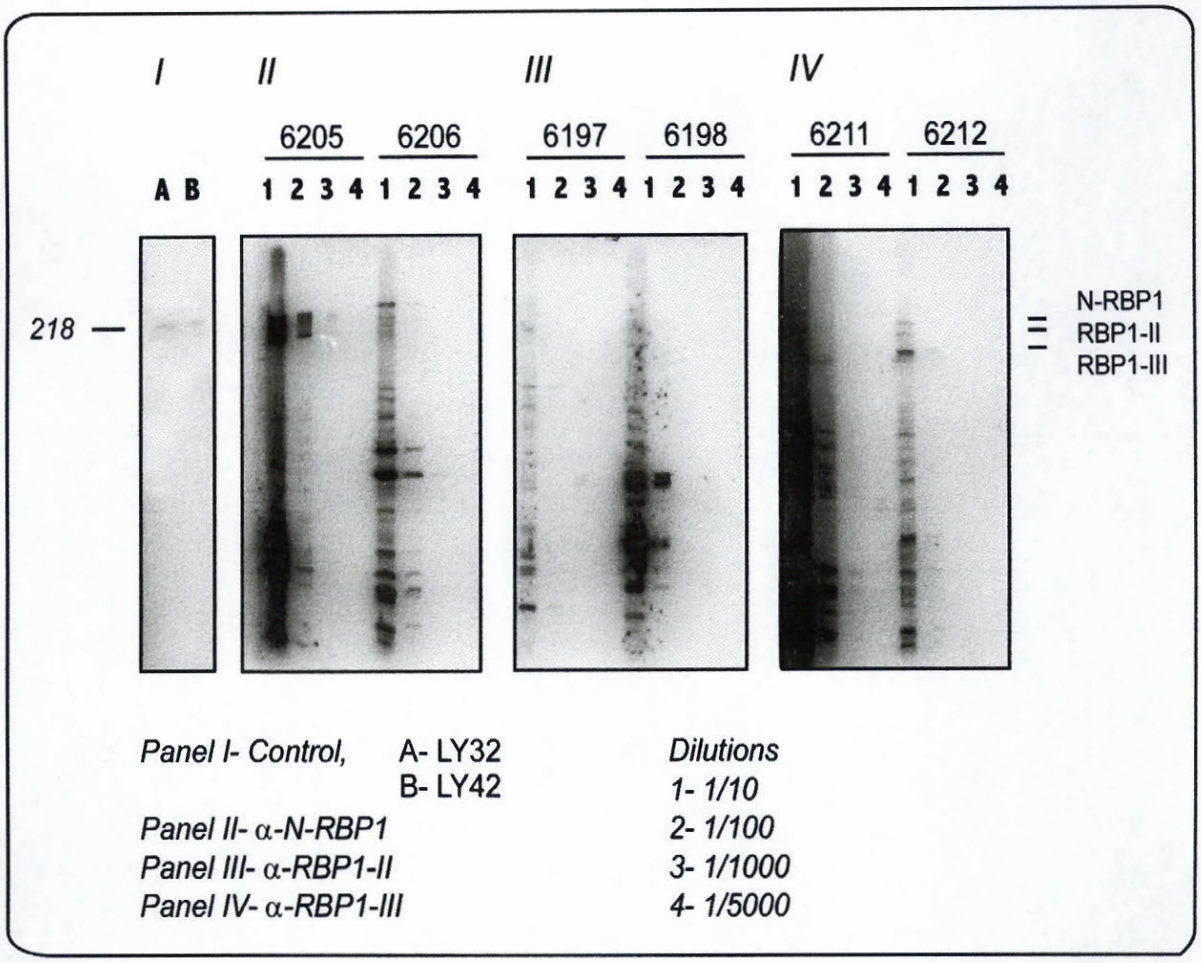
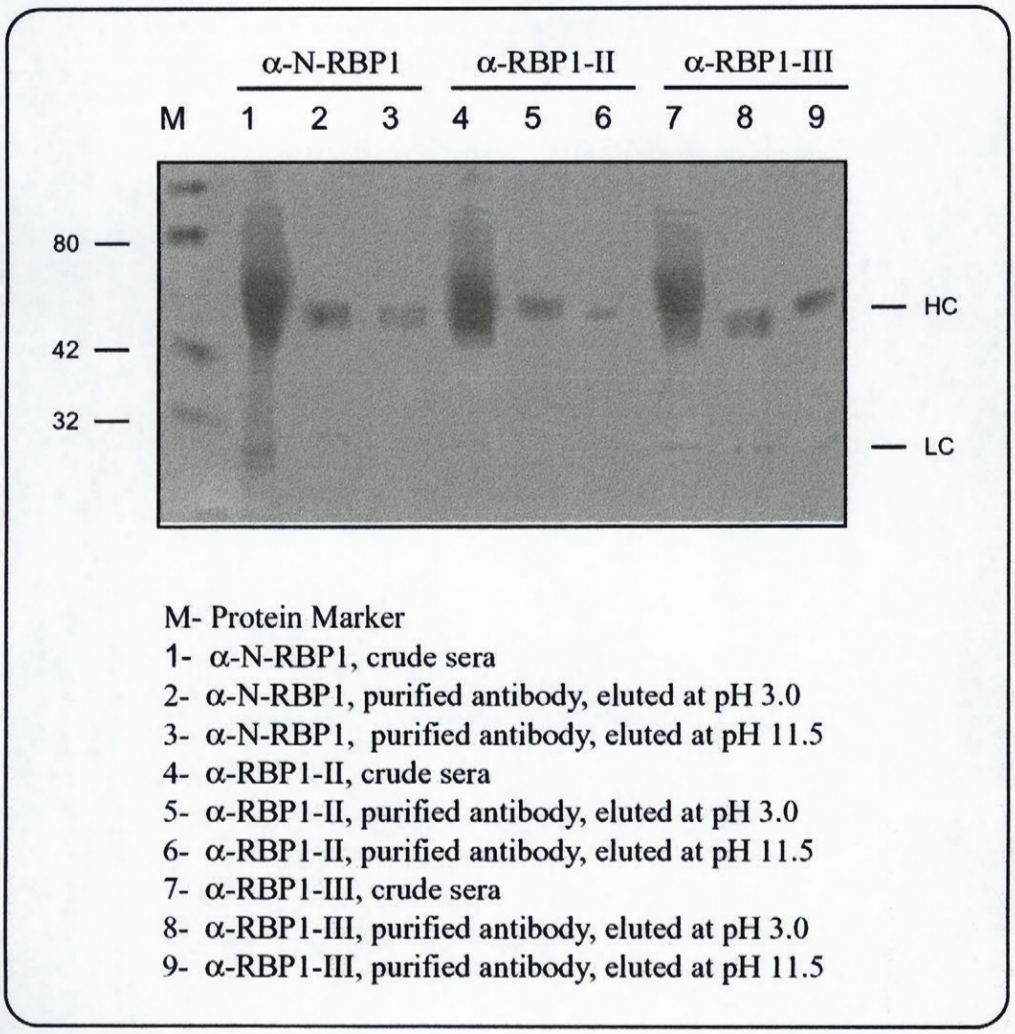


Figure 3.1 Western Blotting using α-RBP1 Antibodies
One-well preparative gels were used to separate 500 ug of whole cell extracts from H630. α-RBP1 antibodies were tested at four different dilutions ranging from 1/10 to 1/5000. Panel II was blotted with rabbit # 6205 and rabbit #6206 α-N-RBP1 at the noted dilutions. Dilutions 1/10 and 1/100 worked best for rabbit #6205 while rabbit #6206 gave signal only at dilution 1/10. Panel III was blotted with α-RBP1-II from rabbit # 6197and #6198. In both cases, only dilution 1/10 gave signals. Panel IV was blotted with rabbit # 6211 and # 6212 α-RBP1-III. A strong signal corresponding to the lower species was seen at dilution 1/10. Mouse monoclonal LY32 and LY42 were used as positive controls for RBP1 migration although it is not known if they are specific to any of the isoform. Appropriate HRP-conjugated secondary antibodies were used to visualize recognized proteins by ECL. Immunoblotting was done as described in section 2.8. Sizes are expressed in kDa.

recognized the three RBP1 isoforms with a predominance for the third species (panel *IV*). On every blot, non-specific background bands of varying intensity were seen. α-N-RBP1 from rabbit #6205 produced a recognizable signal at dilutions of up to 1:1000 while the signal from all other antibodies was clearer at a dilution of 1:10. These crude antisera generated too much background to be useful. In an effort to increase their specificity and lower their non-specific activity, affinity purification was carried out.

3.1.3 Coomassie Staining of Crude and Purified Antisera

Affinity purification of the antisera was performed in order to increase the specificity of the antibodies. Concentration of the purified antibody solutions were performed by multiple rounds of spin-dialysis using Centricon-30 columns (Millipore) using the manufacturer's recommended protocol. The easiest method to determine the purity of an antibody solution is to run an aliquot on a SDS-PAGE. In our case, the gel was then stained with Coomassie blue (sensitivity 0.1-0.5 ug/band). 10 ug of both crude and purified antibodies were loaded on a 10% SDS-PAGE followed by coomassie staining. As can be seen in figure 3.2, lane M contains a broad-range protein size marker (New England Biolabs). Lane 2 and 3 contain the low pH and high pH elution fraction of antibody #6205 respectively. The heavy chain of the antibody, which runs at ~ 55 kDa can easily be seen for the all of the antibodies. However, the light chain, which runs at ~ 25 kDa, is more difficult to visualize. The α-RBP1-II eluted at high pH (pH 11.5) seem to have been lost either in the purification procedure or during the concentration step (lane 6) as the bands seen are much lighter. After purification and concentration of the antibodies, immunoblotting on whole cell extract was performed (see figure 3.5) and the specificity was greatly enhanced.



M- Protein Marker
1- α-N-RBP1, crude sera
2- α-N-RBP1, purified antibody, eluted at pH 3.0
3- α-N-RBP1, purified antibody, eluted at pH 11.5
4- α-RBP1-II, crude sera
5- α-RBP1-II, purified antibody, eluted at pH 3.0
6- α-RBP1-II, purified antibody, eluted at pH 11.5
7- α-RBP1-III, crude sera
8- α-RBP1-III, purified antibody, eluted at pH 3.0
9- α-RBP1-III, purified antibody, eluted at pH 11.5

Figure 3.2 Coomassie Gel Analysis of Purified Antibodies
Antibodies obtained from NewZealand white rabbits were affinity purified using column-bound RBP1-peptides. The purified antibodies were quantified and 10 ug were loaded in a 10% polyacrylamide gel. Following SDS-PAGE, the gel was stained by coomassie blue. M: protein marker. Lanes are as indicated in the figure. Sizes are expressed in kDa. HC- Heavy chain, LC- Light chain

3.2 Verification of GST-Protein Stability

10 ug of purified GST-protein corresponding to the C-terminal portion of RBP1 as well as a portion of RBP1-II, RBP1-III and GST alone were loaded on a 10% SDS-PAGE to verify their integrity after their production. As seen in figure 3.3, lane M contains a broad-range protein size marker (New England Biolabs). Lane 1 corresponds to the GST protein alone, and migrates at the expected size of ~ 27 kDa. Lanes 2, 3 and 4 correspond to GST-C-RBP1, GST-RBP1-II and GST-RBP1-III which migrate at their predicted size of 75 kDa, 63 kDa and 61 kDa respectively. Degradation products were visible but they did not alter the interpretation of results.

3.2.2 Verifying Specificity of the Antibodies

Purified GST-proteins were used to verify the specificity of the isoform specific antibodies. In order to verify that the antibodies were specific for RBP1, 200 ng of purified GST proteins corresponding to each of the isoforms were loaded on a 12% polyacrylamide gel and were probed with the crude (figure 3.4a) and the purified antibodies (see figure 3.4b). Note that the GST-C-RBP1 is a truncated protein that does not possess the N-terminal portion and can thus be used as a negative control for specific binding. As can be seen on figure 3.4a, crude sera has no specificity and all isoforms are being recognized by each antibody, even the N-terminal truncated protein. In contrast, after purification, (figure 3.4b), the N-terminal truncated protein is not being recognized by any of the antibody (as expected). Both α-RBP1-II antibodies (see figure 3.4, panel *II* and *V*) are specific to their related protein. Unfortunately, none of the α-RBP1-III antibodies were specific for the third isoform. α-RBP1-III eluted at low pH is not specific since it recognizes all three isoforms and the one eluted at the highest pH seems to have specificity toward isoform II and III (see figure 3.4b, panel *III* and *VI* respectively). This could be explained by the fact that part of the epitope to which the α-RBP1-III antibody was raised is present in all

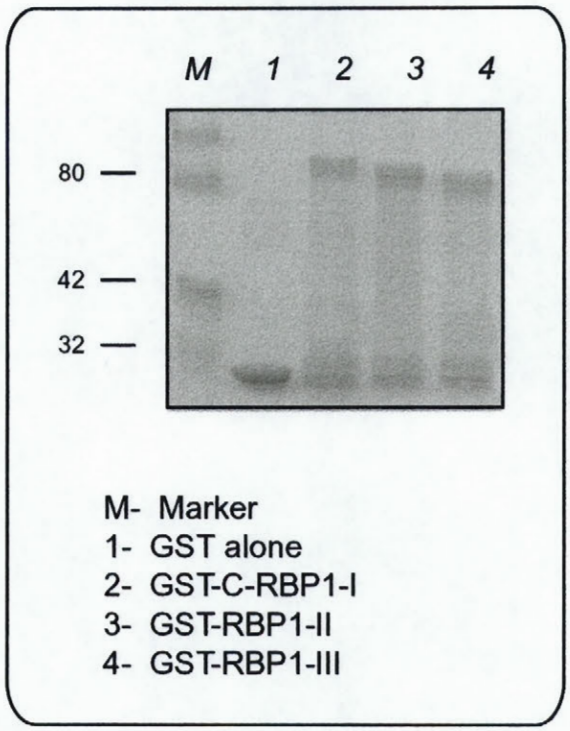


Figure 3.3 Expression and Purification of GST-RBP1 Proteins
BL21 DE3 E. *Coli* were transformed with pGEX2T-RBP1 (isoform I, II, and III) plasmids (generously given by Frederic Kaye). Protein production and purification were performed as described in section 2.4. 10 ug of protein was resolved by electrophoresis in a 10% polyacrylamide gel, separated by SDS-PAGE and stained by coomassie blue. M: Protein weight marker. Lanes are as indicated in the figure. Sizes are expressed in kDa.

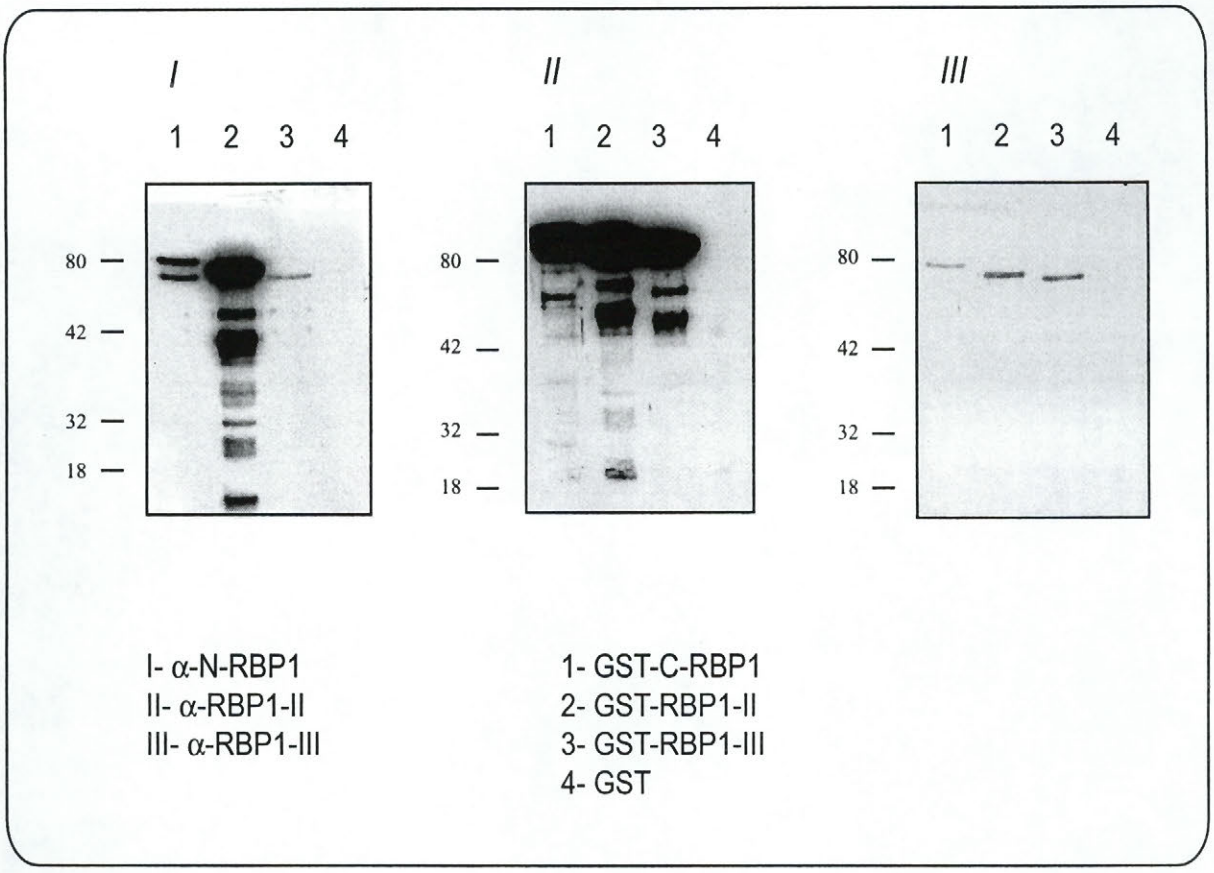


Figure 3.4a Western Blot Analysis on Purified GST-Proteins using Crude Sera
In order to verify if the antibodies were specific to their related proteins, GST-RBP1 proteins corresponding to the C-teminal portion of RBP1 as well as to RBP1-II and RBP1-III were produced, purified and loaded on a 12% polyacrylamide gel. Western blotting using the non-purified rabbit polyclonal sera followed. As can be seen, none of the antibody was specific to its related protein as each antibody recognized every proteins.

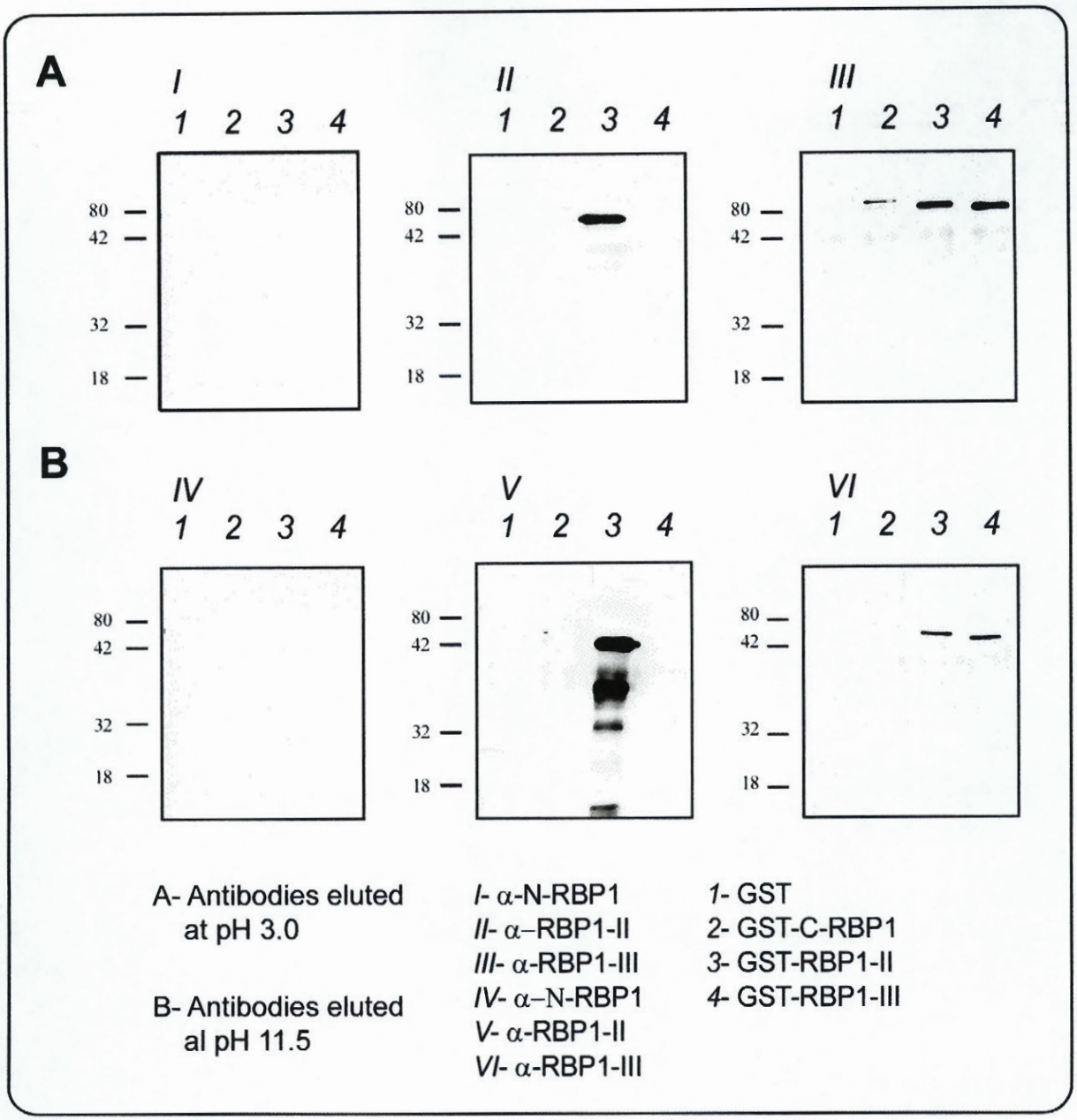


Figure 3.4b Western Blot Analysis on Purified GST-Proteins using α-RBP1 Antibodies after their Purification

Following purification, the α-RBP1 antibodies show a much more specific activity toward their respective proteins. The α-N-RBP1 did not recognize the C-RBP1, as expected and the α–RBP1–II are specific for the second isoform. However, the α–RBP1-III antibodies were not specific as they recongnized all three isoforms (in the case of the antibody eluted at low pH) and RBP1-II as well as RBP1-III (in the case of the antibody eluted at high pH). A second round of purification is necessary to achieve higher specificity.

the isoforms. However, since the antibody was raised against the splice junction, we expected a better specificity (see figure 2.1). In order to achieve a higher specificity, we could performed a second round of affinity purification. This purification step would utilize a column containing peptides corresponding to N-RBP1 and RBP1-II isoforms. The α-RBP1-III antibody preparation would be passed through the column, enabling binding of antibodies recognizing N-RBP1 and RBP1-II peptides, and only the eluate (containing α-RBP1-III specific antibodies) would be collected. This would ensure that any antibody reacting to the first and second isoform would be trapped in the column, getting a higher concentration of antibodies specific for the third isoform in the eluate.

3.3 Western Blotting using α-RBP1 Antibodies

H1299 and H630 whole cell extracts were subject to western blot analysis to verify that the purification procedure cleaned up most of the background. Each antibody was eluted at two different pH (to collect acid-sensitive as well as base-sensitive antibodies). As can be seen in figure 3.5, α-N-RBP1 and α-RBP1-III, both eluted at pH 11.5 gave the strongest signal (see figure 3.5, panel C, *VI*, *VIII* respectively). α-RBP1-II eluted at pH 3.0 shows a very weak signal, whereas the antibody eluted at pH 11.5 does not have any activity (see figure 3.5, panel B, *IV*, *VII* respectively). α-N-RBP1 recognizes many forms of RBP1. It is clearly seen that the highest migrating form is the most prominent (see figure 3.5, panel C, *VI*). In contrast, α-RBP1-III displays three bands with the second one being the most prominent (see figure 3.5, panel C, *VIII*). There is a large difference between the blot using the crude antibody compared to the one using the purified antibody (compare figure 3.1 to figure 3.5). There is much more background on the western blots using the non-purified antibody preparations compared to those using the purified antibodies. The purification procedure eliminated most of the non-specific background.

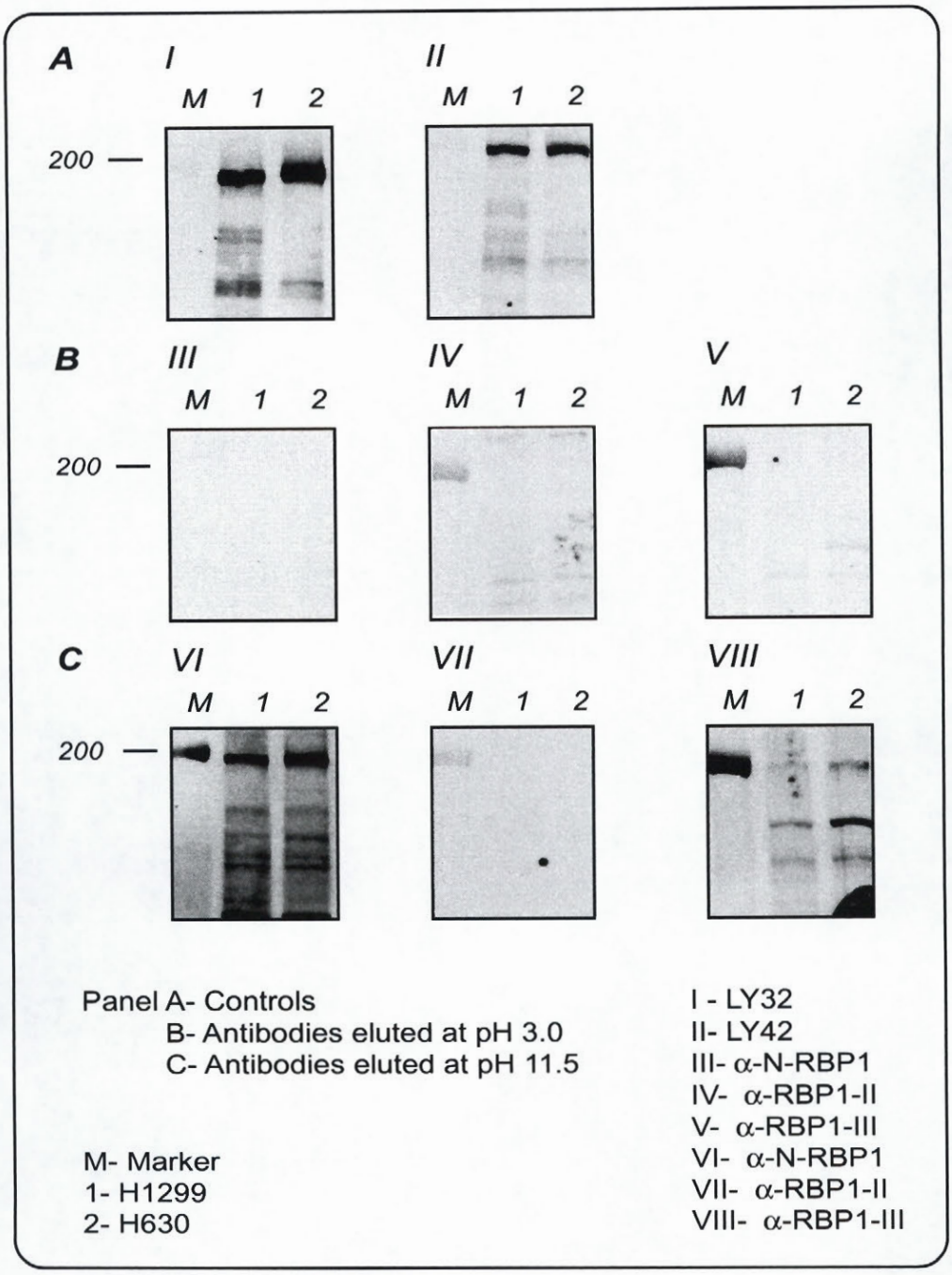


Figure 3.5 Western Blot Analysis on Whole Cell Extracts using the Isoforms Specific Antibodies after their Purification

H630 as well as H1299 whole cell extracts were subject to SDS-PAGE. Westen blot analysis using the purified isoforms specific antibodies followed. As demonstrated in this figure, α-N-RBP1 and α-RBP1-III antibodies eluted at high pH gave the strongest signals, whereas α-RBP1-II antibodies eluted at low pH worked best. In order to lower the non-specific background, a second round of purification should be carried out.

3.4 Overexpression of RBP1 via Adenovirus

H630 were infected with a replication defective HA-RBP1 expressing adenovirus. Since the protein is encoded by the cDNA, and the virus does not perform splicing, only the first isoform of RBP1 is expressed. Immunoprecipitation against the HA epitope (using the α-HA antibody 12CA5 from Santa Cruze) was performed followed by western blotting with the three isoform specific antibodies (purified) as well as LY42, as a positive control for RBP1 migration and expression. As can be seen on figure 3.6, there is a signal only in the lane in which cells infected with Ad-RBP1 were loaded as well as in the one containing the whole cell extracts from H630 (used as a positive control for position of RBP1). In lane 5 of all panels, several bands can be seen. This is surprising since the virus is known to produce only the first isoform. Moreover, α-N-RBP1 and α-RBP1-II were determined to be quite specific according to the western blot analysis performed on the purified protein (see figure 3.4b). This result could be explained by the fact that RBP1 can oligomerize (Albert Lai, personal communication). HA-RBP1-I would have come down with α-HA antibodies as a complex also containing the other isoforms. In the case of α-N-RBP1, it most probably means that this antibody recognizes all three isoforms, as expected. However, in the case of α-RBP1-II and α-RBP1-III antibodies, it suggests that these antibodies are not specific since they recognize all isoforms (also demonstrated by the fact that there are multiple bands in lane 7 of panels *II* and *III*, which contains whole cell extracts from H630 cells). The lower bands seen in the LY42 blot seem to be due to non-specific proteins bound to the beads as they are present in lanes 1, 3, and 5.

3.5 Species Specificity of the Purified Antibodies

It was of interest to determine if the isoforms specific antibodies could recognize RBP1 from a variety of species since certain LY from Dr. DeCaprio were only specific to either mouse or human proteins. Whole cell extract as well as the nuclear and cytoplasmic fractions of human H1299 and H630, mouse C2C12, rat L6

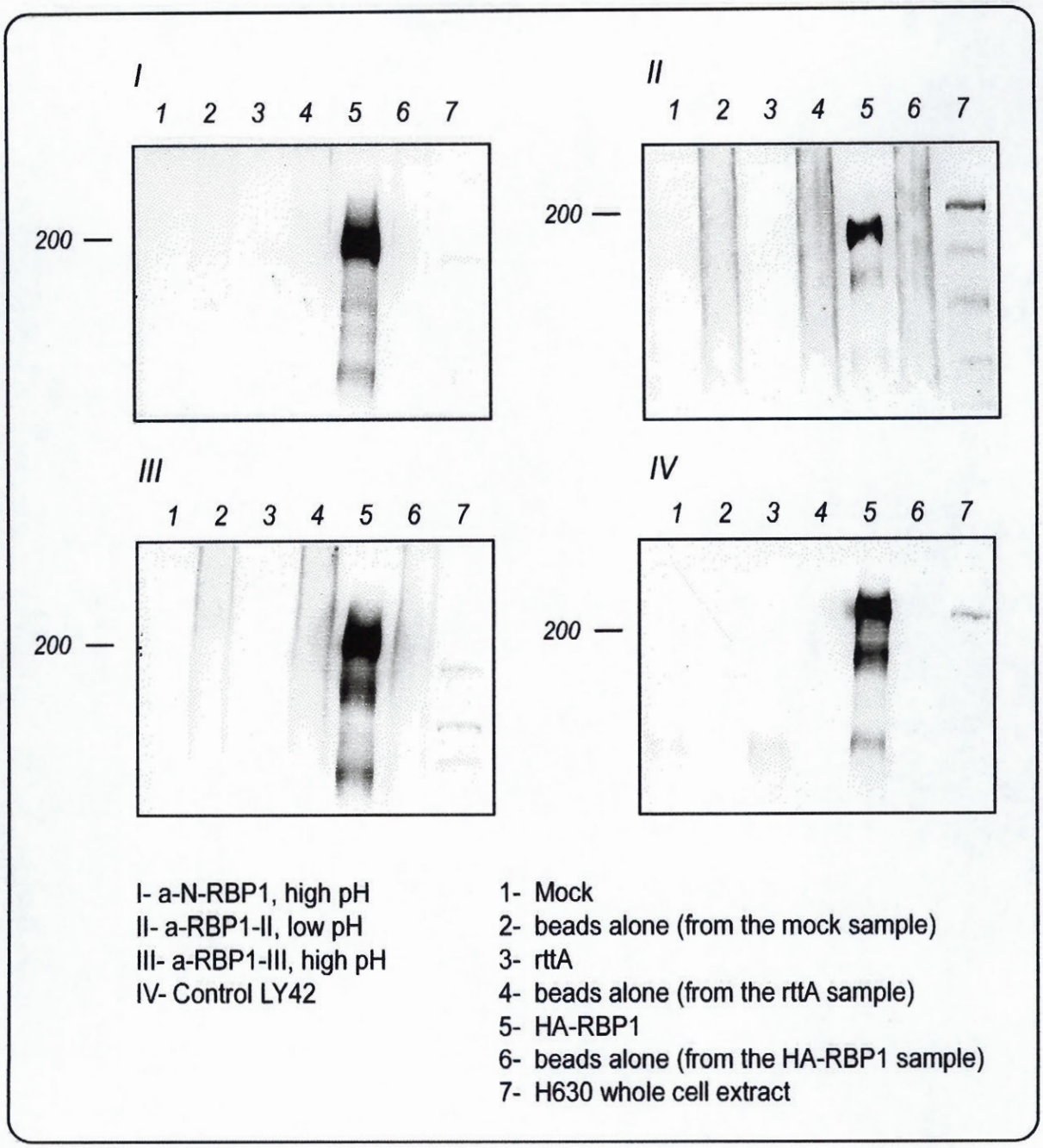


Figure 3.6 Immunoprecipitation-Western Blot Analysis of Overexpressed HA-RBP1
H630 cells were infected with HA-RBP1 expressing virus. Following harvesting, the cells were lysed and immunoprecipitation against the HA epitope was carried out. Western blot analysis using the purified antibodies preparations was then performed. Since only the first isoform is encoded by the virus, our results suggest that RBP1 forms oligomers containing the isoforms and that the antibodies are not specific to their corresponding protein.

and monkey CV-1 were used to verify the activity of the antibodies toward RBP1 from different species. 40 ug of whole cell extract, 90 ug of nuclear fraction and 90 ug of cytoplasmic fraction were loaded on a 6% SDS-PAGE and western blotting was performed as described in section 2.8. As shown in figure 3.7a and 3.7b, each antibody recognized RBP1 in all cell lines tested. It can be noted that there was a variation in the migration pattern from species to species. This variation could be due to different post-translational modifications of RBP1 from species to species. RBP1 is known to be a nuclear protein (Otterson *et al.*, 1992). As can be seen in figure 3.7b, there is a strong signal when the nuclear portion was used and no signal at all when the cytoplasmic fraction was used. This also suggests that, in the cytoplasm, no protein is being recognized non-specifically by any of the antibodies.

3.6 Immunofluorescence Studies

Mouse C2C12 cells were used instead of human H630 for immunofluorescence as the latter have a morphology that is much more difficult to analyse. As shown in figure 3.8, the α-N-RBP1 displayed the strongest signal while α–RBP1-II displayed the weakest. Cells were visualized using a fluorescent light microscope (Axioplan, Ziess) and pictures were taken using a CCD camera and Metamorph Imaging System (Diagnostic Instruments) at 100X magnification.

In order for the isoform specific antibodies to be useful, a second round of purification is necessary to achieve a higher specificity (see discussion). Once the antibodies are specific toward their respective protein, they could be used in western blot analysis to determine the pattern of expression of each isoform during the cell cycle and during myogenesis. They could also be used in immunofluorescence analysis to study the cellular localization of each isoform at different stages of the cell cycle or of the differentiation program. Unfortunately, these antibodies did not have immunoprecipitation activity. However, if the second round of purification yields antibodies that can do so, it would

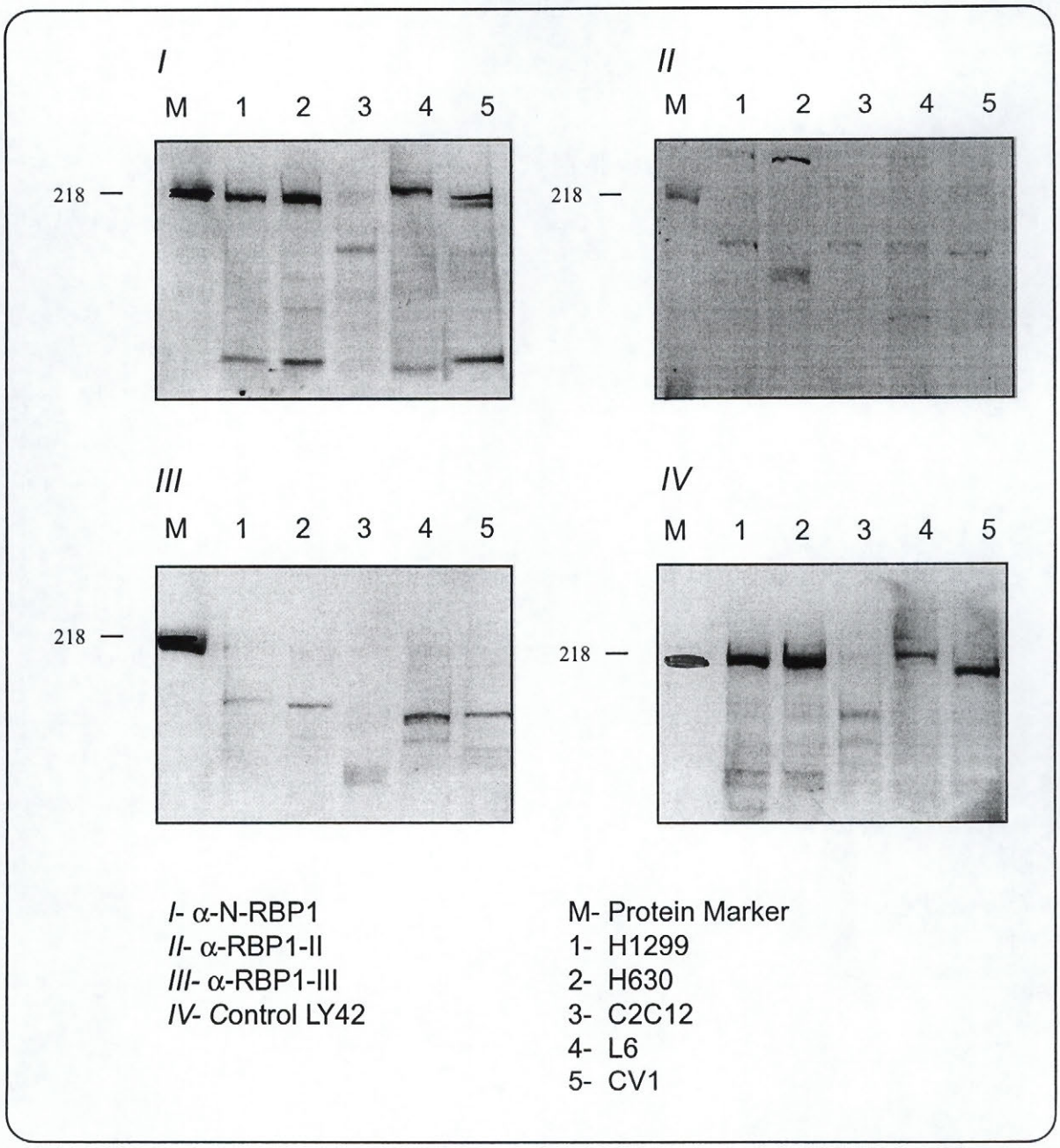


Figure 3.7a Western Blot Analysis using Purified α-RBP1 Antibodies on Whole Cell Extracts from Different Species

Whole cell extracts from different species were collected and western blot using the purified antibodies was carried out. As demonstrated, all α-RBP1 antibodies recognize RBP1 in a variety of species. The variation in the migration pattern could be due to post-translational modifications that vary from species to species.

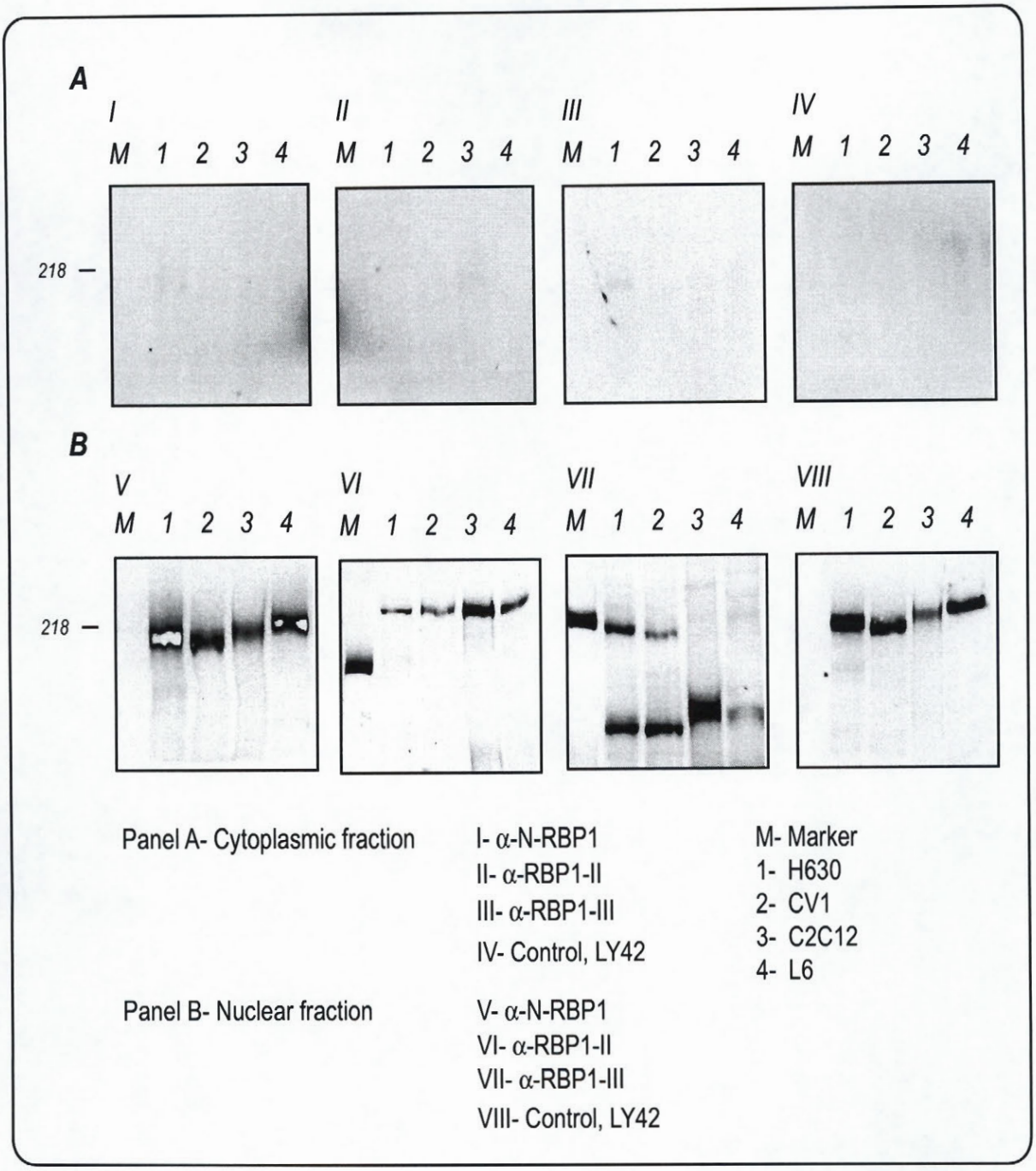


Figure 3.7b Western Blot Analysis on Cytoplasmic and Nuclear Fractions from a Variety of Species using the Purified α-RBP1 Antibodies

Cellular fractions from different species were collected and loaded on 6% polyacrylamide gel. They were then subject to western blotting using the purified α-RBP1 antibodies. As can be seen in panel A, none of the antibody recognize proteins in the cytoplasmic fraction (very light background bands can be seen on long enposure). This suggests that no proteins is being recongnized non-specifically in the cytoplasmic portion. However, as shown in panel B, the antibodies recognize RBP1 in the nuclear portion coming from all species tested.

Figure 3.8 Immunofluorescence Analysis using Purified α–RBP1 Antibodies

Immunofluorescence analysis was conducted using the purified isoforms specific antibodies. As can be seen, all of the antibodies generated a signal. As expected, the signal is mostly nuclear, as indicated by the fact that it colocalizes with that from pRb. (The intensity is not quantitative).

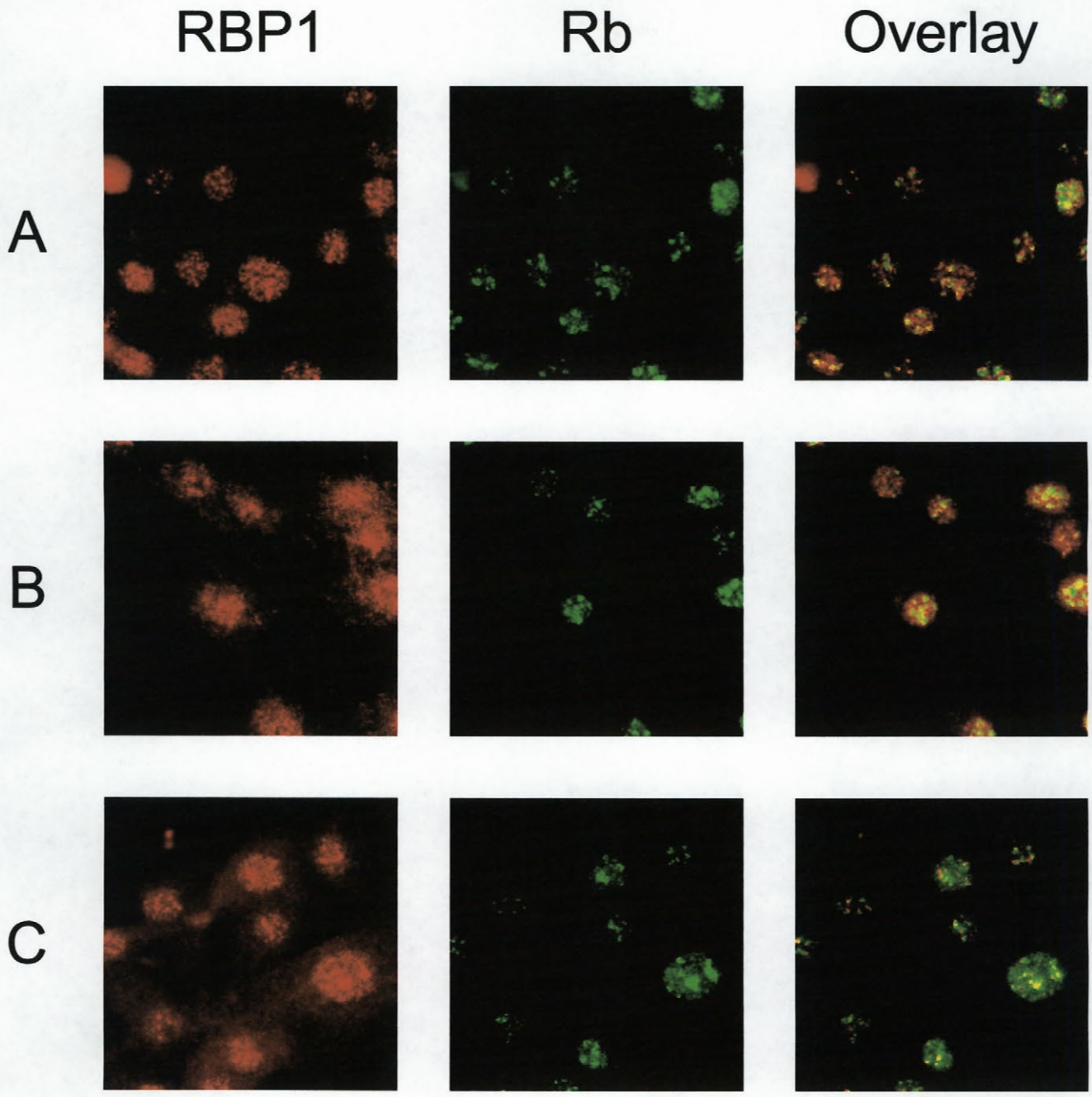


Figure 3.8 Immunofluorescence Analysis using Purified α-RBP1 Antibodies

Legend: **A : α-N Term**
B : α-RBP1-II
C : α-RBP1-III

be interesting to study the composition of complexes containing the different isoforms and look for variation.

3.7 Implication of RBP1 in Myoblast Differentiation

RBP1 has been shown to associate with p130/E2F and pRb/E2F complexes during growth arrest (Lai *et al.,* 1999a). Our group also demonstrated that RBP1 overexpression both inhibited E2F-dependent transcription as well as cellular proliferation (Lai *et al.,* 1999a). Our initial model suggests that RBP1 would be implicated in the induction of the growth arrest, early in myogenesis. As differentiation proceeds, RBP1 repressor function would be turned off, enabling pRb to activate proteins necessary for terminal differentiation to occur. In an effort to verify the accuracy of that model, RT-PCR and western blot analysis were first conducted to determine patterns of mRNA and protein levels at various times during C2C12 muscle cells differentiation program. C2C12 were chosen as these cells easily undergo differentiation when deprived from serum.

3.8 C2C12 Myotubes Formation

The ability of C2C12 to form multinucleated myotubes was evaluated using Harris Hematoxyllin staining and light microscopy. C2C12 myoblasts were seeded at 10^4 cells per cm^2 and allowed to grow in 20% FBS media for 3 days. They were then switched to DMEM containing 2% horse serum (HS) and allowed to differentiate for 4 days. Following Hematoxyllin staining, cells were examined at 40X using light microscopy. As demonstrated in figure 3.9, about 24 hours after switching to the differentiation media, cells started to align and formation of multinucleated myotubes took place as early as 2 days after the medium switch.

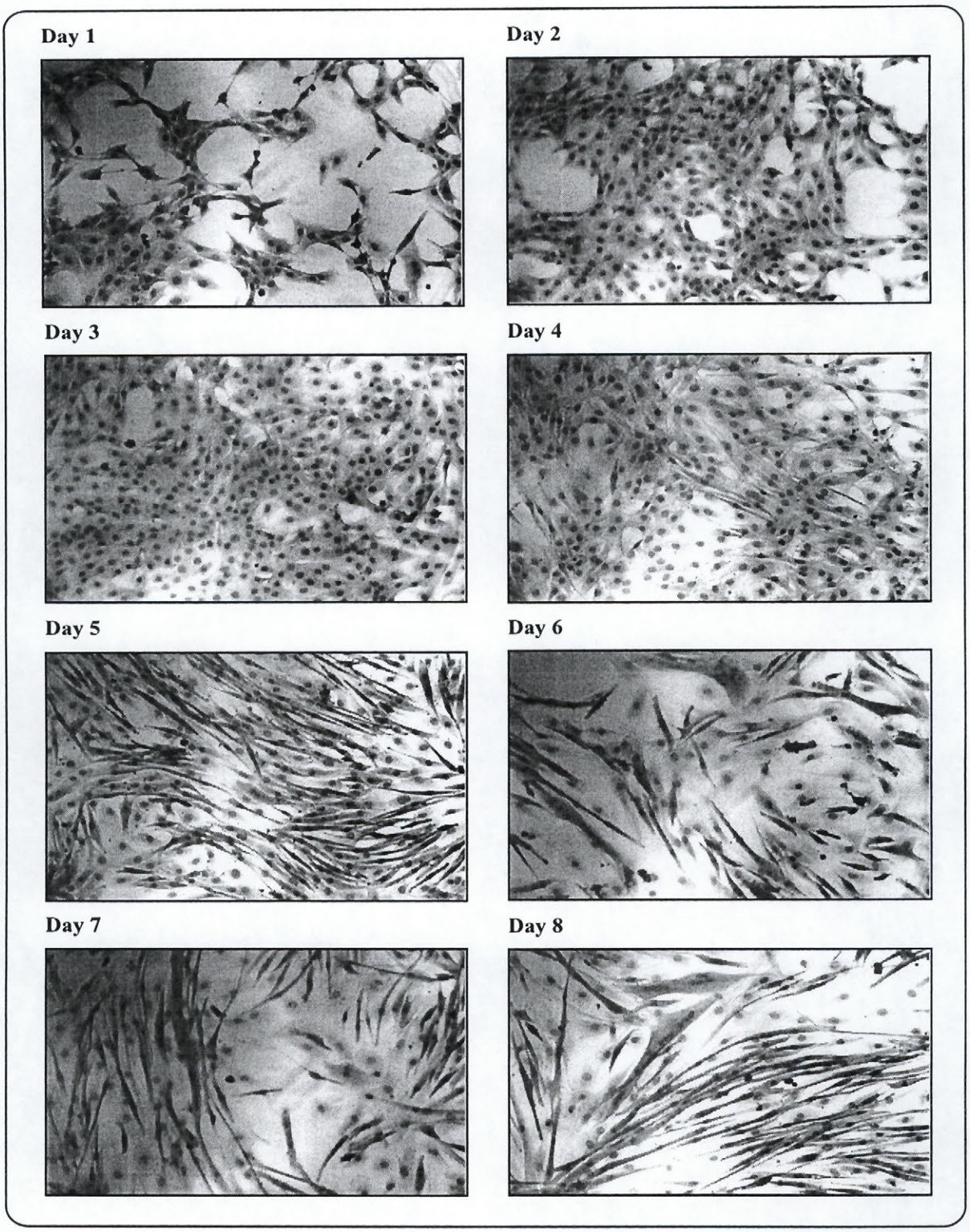


Figure 3.9 Analysis of C2C12 Morphology during the Differentiation Program
C2C12 cells were induced to differentiate followed by staining with Harris hematoxyllin solution at every day of the differentiation program. Their morphology was then looked at by light microscopy and the presence of multinucleated myotubes was analyzed. All photos show cells at an identical magnification.

3.9 Expression Levels of Control Proteins

To analyse the cells during the differentiation program, immunoblotting was performed on C2C12 extracts. Sera against actin was used as a control for protein loading. As can be seen in figure 3.10, panel A, equal amounts of extracts were loaded. Grb-2 and HDAC1 were used to verify that the cytoplasmic and the nuclear portion were free of cross-contamination. As can be seen in panel B, there is a little bit of cytoplasmic proteins in the nuclear fraction, but this did not alter the interpretation of the results. As shown in panel C, the cytoplasmic fraction was free of nuclear contaminant as demonstrated by the absence of signal in the cytoplasmic portion when probed with α-HDAC antibodies. Western analysis demonstrated that MHC protein (which is induced at relatively late times in the skeletal muscle differentiation program) accumulated to high levels in myotubes, indicating that these cells have entered the differentiation program (Gunning *et al.,* 1987: Lin *et al.,* 1994: Andrea and Walsh, 1996).

3.10 RT-PCR analysis of RBP1 mRNA Levels during Myogenesis

To further examine the synthesis of RBP1 during myoblast differentiation, total RNA was isolated from myoblasts as well as myotubes and analysed by RT-PCR. As shown in figure 3.11, panel *II*, myoblasts express transcripts of 452 kb, similar to the size of RBP1 transcript, described previously (Otterson *et al.,* 1992). No change in the amount of RBP1 transcript was observed at any stage of the differentiation program. The amount of transcript for GAPDH, used as a control for variation, is also shown in the figure 3.11, panel *I*. The lower panel *(III)* shows an ethidium bromide stain of the agarose gel, indicating that relatively equivalent amounts of total RNA were loaded. Together, these results clearly demonstrate that RBP1 is continuously being transcribed during myogenesis.

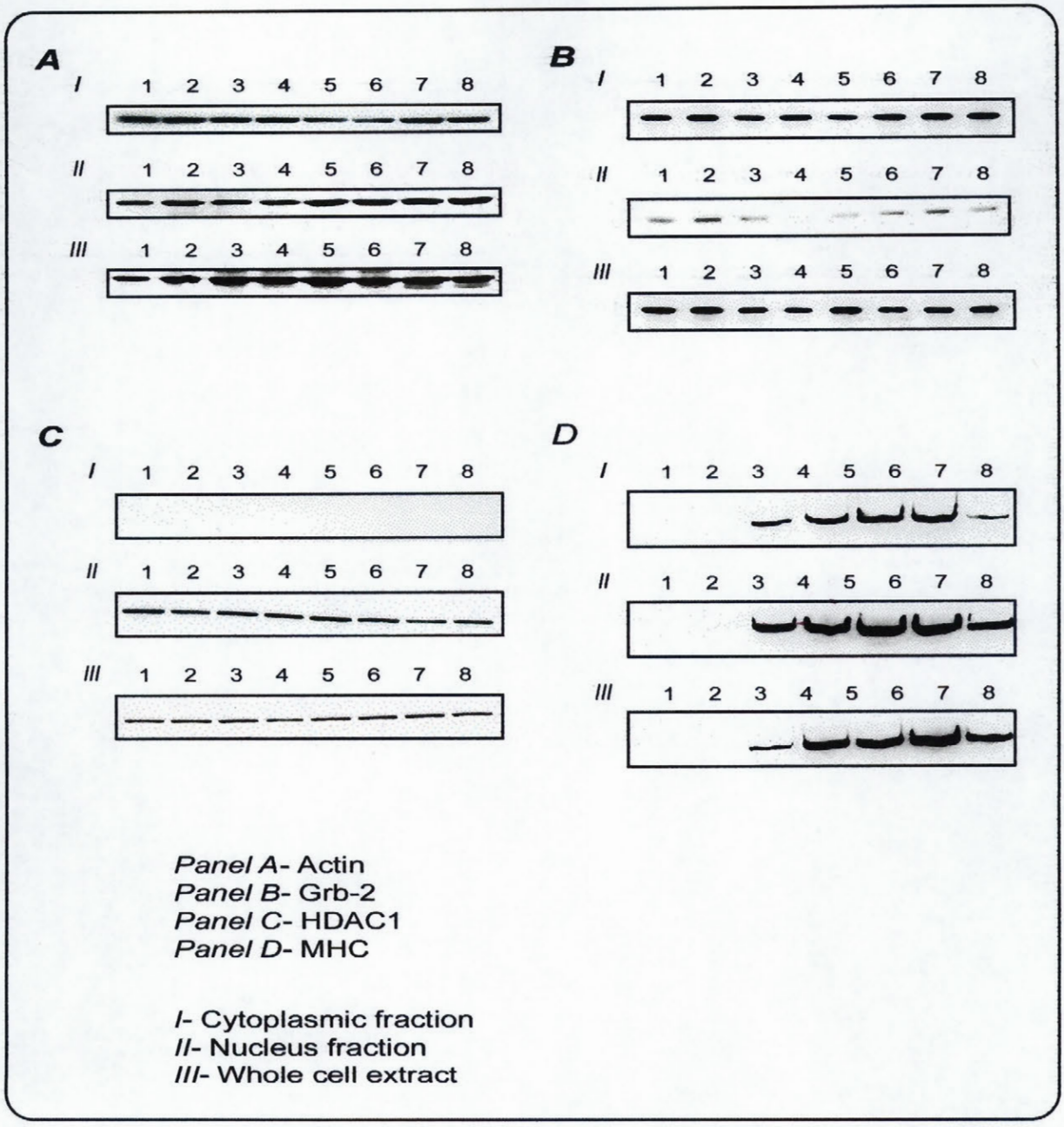


Figure 3.10 Expression Levels of Control Proteins
Total cell extracts as well as cytoplasmic and nuclear fractions were prepared from C2C12 at every day of the differentiation program (referred above as 1, 2, 3,...8). They were then separated by SDS-PAGE and subject to western blotting analysis involving sera specific for Actin (loading control), Grb-2 (cytoplasmic marker), HDAC1 (nuclear marker), and MHC (MF-4) (differentiation marker).

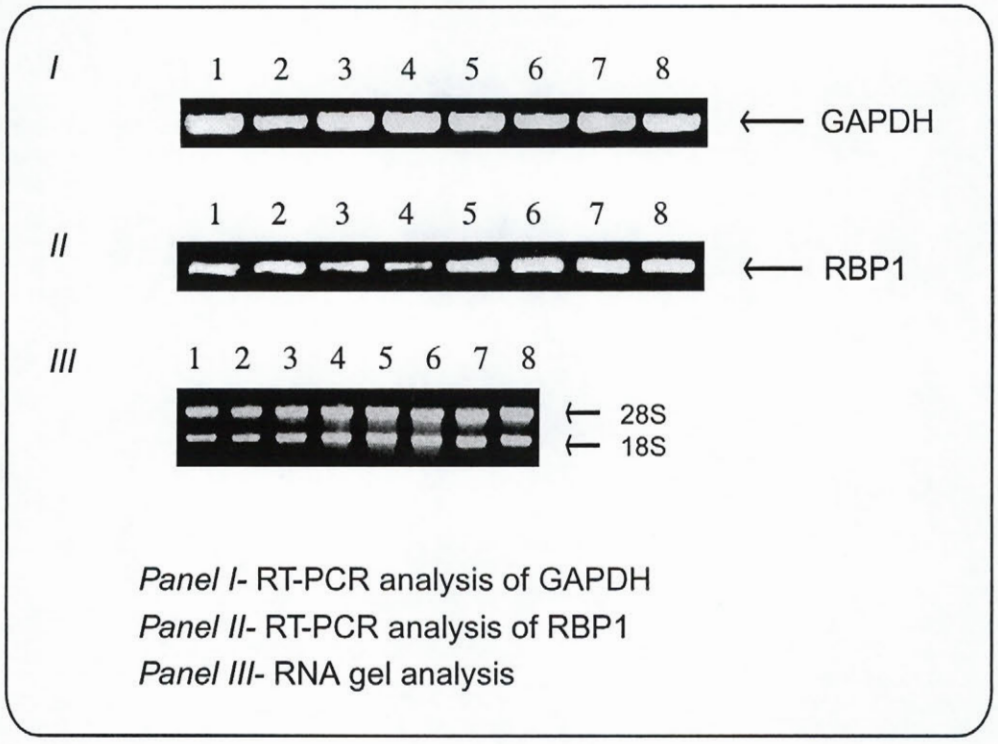


Figure 3.11 RBP1 mRNA Levels in Differentiating C2C12
RT-PCR analysis was performed on differentiating C2C12 in order to verify if RBP1 is being transcribed during myogenesis. Total RNA was isolated from cells at every day of the differentiation program (referred above as 1, 2, 3,...8). RBP1 mRNA was detected at every stages of myogenesis. GAPDH was used as an RNA loading control. Panel *III* shows an ethidium bromide stain of of the samples used for RT-PCR analysis (rRNA).

3.11 RBP1 Protein is Expressed in Differentiating C2C12

We have also examined the expression pattern of RBP1 in C2C12 during the differentiation program. To determine if C2C12 myoblasts synthezise RBP1 protein while they are differentiating, myoblasts undergoing myogenesis were harvested every day and whole cell extracts were prepared and subsequently subject to SDS-PAGE. Upon western blot analysis using a monoclonal antibody against RBP1 (LY42), it was determined, as shown on figure 3.12, panel *I*, that proliferating myoblasts as well as differentiating myotubes are synthezising RBP1. These results indicate that RBP1 is synthezised in mouse C2C12 myoblasts as well as in C2C12 myotubes.

3.11.2 Nuclear RBP1 is Shuttled to the Cytoplasm during Differentiation

In order to study RBP1 localization during differentiation, cell fractionation followed by western blot analysis was conducted on C2C12 undergoing myogenesis using RBP1 monoclonal antibodies (LY42). As shown in figure 3.12, panel *II* and *III*, there is a progressive downregulation of nuclear RBP1 as soon as myotubes are forming. Interestingly, this downregulation correlates with the appearance of RBP1 in the cytoplasmic fraction. This could be a mechanism of regulating RBP1 function.

This result is very significant as it proposes a mechanism by which pRb transcriptional repression functions could be regulated during myogenesis. As already mentionned (sections 1.15 to 1.23), pRb is implicated in many steps of the differentiation program. In the early stages, pRb/MyoD complex induces the cells to withdraw from the cell cycle. Since RBP1 is present in high amount in the nuclear fraction in the early stages of myogenesis (figure 3.12), and that it has already been demonstrated to repress E2F-dependent transcription and induce growth arrest (Lai *et al.*, 1999a), we suggest that RBP1 could be part of this complex, mediating the cell cycle withdrawal. Furthermore, pRb/MyoD complex is also involved in stimulating MEF2 transcriptional activity, allowing expression of late markers of differentiation (such as MCK and MHC) (Novitch *et al.*, 1996). Interestingly, our results clearly demonstrate that RBP1 shuttles from the nucleus to the cytoplasm as myotubes start

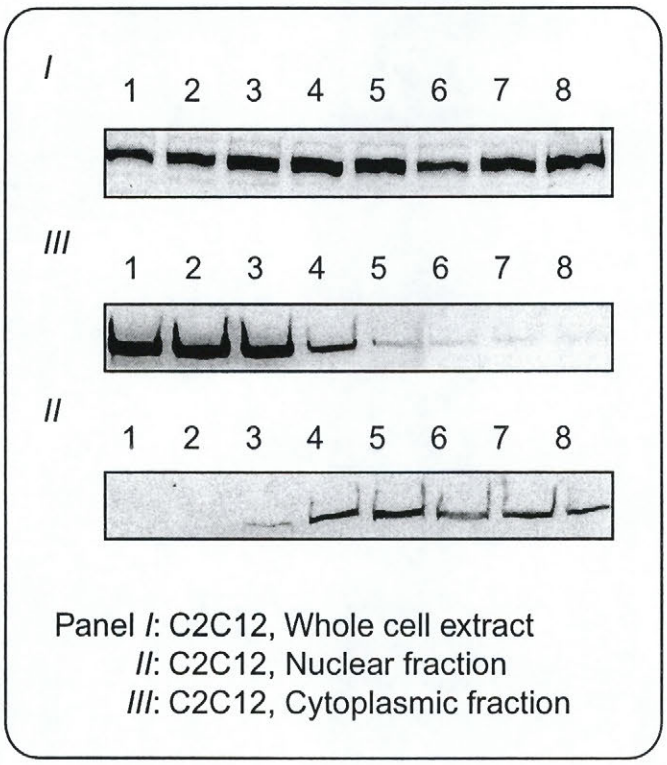


Figure 3.12 Western Blot Analysis of RBP1 Protein Levels during the Differentiation Program

C2C12 cells undergoing the differentiation program were harvested every day (referred to as 1, 2, 3,...8) and the whole cell extract, the nuclear fraction as well as the cytoplasmic fraction were prepared. Following SDS-PAGE, western blot analysis was performed using LY42 antibody (specific against RBP1). As shown above, RBP1 is continuously being synthesized during myogenesis. However, RBP1 is shuttle from the nucleus to the cytoplasm as soon as myotubes start forming (day 3-4).

forming (figure 3.12). We propose a mechanism whereby this shuttling would be responsible for regulating pRb transcriptional activity during differentiation. In support of our hypothesis, RBP1 has recently been shown to act as a linker protein between pRb and HDACs complex (Lai *et al.*, 1999b). Its translocation to the cytoplasm would disintegrate the pRb/RBP1/HDAC repression complex. Since pRb is not very efficient at recruiting HDACs directly, its transcriptional repression activity would be drastically reduced, permitting the activation of myogenic proteins.

Chapter 4

General Discussion

And

Future Work

Pocket proteins participate in processes such as cellular differentiation and inhibition of apoptosis during development (Cobrinik *et al.,* 1996). A role for pRb in differentiation was first suggested from the observation that *RB1* knockout mice (pRb-/-) exhibited unique differentiation defects and died *in utero* after 12-13 days of development (Clarke *et al.,* 1992; Jacks *et al.,* 1992; Lee *et al.,* 1992). This also led to the suggestion that pRb protects cells from apoptosis during myogenesis (Jacks *et al.,* 1992; Lee *et al.,* 1994; Lee *et al.,* 1992; Riley *et al.,* 1994).

Although pRb is primarily known as a transcriptional repressor, it has also been shown to enhance the transcriptional activity of certain transcription factors (Sellers *et al.,* 1996; Nead *et al.,* 1998; Weintraud *et al.,* 1995). For instance, pRb induces myogenesis by stimulating the transcriptional activity of MyoD protein, mediating muscle cell commitment and differentiation (Gu *et al.,* 1993). pRb has been shown to be implicated at many other stages of the differentiation process. By regulating E2F and HBP1, active hypophosphorylated pRb helps to maintain the growth arrest necessary to initiate myogenesis (Endo *et al.,* 1992; Gu *et al.,* 1993; Thorburn *et al.,* 1993). pRb is also playing an important role in late myogenesis by stimulating the expression of late differentiation markers such as MHC and MCK.

Although it was originally believed that pRb could directly recruit HDAC1 and HDAC2 to its pocket via their degenerate IXCXE motif, our group has recently demonstrated that pRb recruits class I HDACs via bridging factors such as RBP1 (Qian *et al.,* 1995; Nicolas *et al.,* 2000; Lai *et al.,* 1999b).

Considering the fact that the RBP1/pRb complex acts as a transcriptional repressor, we were interested in studying how pRb repressor activities are regulated during myogenesis. C2C12 cells were used to study the cell biology of RBP1 during muscle differentiation because these cells easily differentiate under low serum conditions.

Based on the facts that RBP1 overexpression both inhibited E2F-dependent gene expression and suppressed cell growth (Lai *et al.,* 1999a), we propose a model in which RBP1 participates in the induction of the irreversible cell cycle withdrawal mediated by pRb/MyoD complex. Consistent with this idea, our lab has previously demonstrated that RBP1 associates with p130/E2F and pRb/E2F complexes

specifically during growth arrest (Lai *et al.,* 1999a). Our hypothesis is also in accordance with the fact that free E2F levels are strongly reduced as cells initiate the differentiation program (LaThangue and Rigby, 1987; LaThangue *et al.,* 1990; Shivji and LaThangue, 1991; Corbeil *et al.,* 1995; Kiess *et al.,* 1995a; Shin *et al.,* 1995; Gill *et al.,* 1998). Moreover, it has recently been demonstrated that RBP1 acts as a linker protein between pRb and HDACs complex (Lai *et al.,* 1999b). Our model proposes that RBP1 serves as a switch that regulates pRb transcriptional activities during myogenesis. In the presence of RBP1, pRb/RBP1/HDACs complex are forming, leading to transcriptional repression. However, without RBP1, these complexes cannot form, enabling pRb to potentiate the transcriptional activities of myogenic protein such as MEF2, leading to terminal differentiation. This is just speculation and further work is required to confirm this hypothesis.

Our results showed that RBP1 protein is being synthesized at a constant level throughout the differentiation program (as shown in figure 3.12). Interestingly, as differentiation proceeded, the nuclear levels of RBP1 protein were dramatically reduced. Moreover, this reduction correlated with the appearance of RBP1 in the cytoplasm (see figure 3.12). This shuttling of RBP1 from the nucleus to the cytoplasm suggests that the switch in pRb activity from being a repressor to an activator could be due to the fact that RBP1 is no longer part of the repression complex. However further work is required to confirm this model. To verify the accuracy of our model, identification of signalling sequence(s) for shuttling within RBP1 structure is necessary. Generation of dominant negative of RBP1 to prevent its shuttling could be used to block differentiation by trapping RBP1 into the nucleus.

C2C12 undergoing differentiation were subject to immunofluorescence in order to confirm our results and also to identify some binding partners of RBP1 in the cytoplasm. α-N-RBP1 as well as the isoforms specific antibodies could not be utilized as further purification is necessary to ensure their specificity. Unfortunately, only one of the other α-RBP1 antibodies available to us recognize mouse protein (LY42) in immunofluorescence (Albert Lai, personal communication). However, this antibody yielded too much background to generate concluding results (data not shown).

In an effort to confirm our model, RBP1 was overexpressed in C2C12 by adenovirus infection. However, no results came out of that study since our negative control (rttA alone) inhibited the differentiation program (data not shown). It has also been reported that, upon transfection of MyoD into C3H10T1/2 cells, myogenesis occured when the cells were cultivated in appropriate conditions (Davis *et al.,* 1987). However, if a repressor of differentiation is cotransfected (for instance Mist1), the myogenic program is prevented and no formation of syncitia occurs (Lemercier *et al.,* 1998). This method was also tested using construct expressing RBP1. Unfortunately, we did not get a high enough expression of RBP1 by transfection (data not shown). Different transfection methods should be tried until a good expression is achieved. It is also of interest to study whether or not certain genes get up-regulated or down-regulated when RBP1 is overexpressed. Does RBP1 overexpression inhibit the differentiation program? If so, what is the mechanism? Does it inhibit the expression of certain myogenic proteins? This would be easily verified by performing western blot analysis using sera against myogenic proteins on cells overexpressing RBP1 cultured under differentiation conditions. It would also be interesting to study the ability of C2C12 RBP1-/- cells to undergo myogenesis. Are certain genes up-regulated, or down-regulated ? Is the pRb/MyoD complex still able to induce the growth arrest in these cells ?

RBP1 possesses four splice variants (Otterson *et al.,* 1992). Several characteristics point to a role for the alternative splicing pattern of RBP1. For instance, RBP1 isoforms were detected in cell lines of various origin (Otterson *et al.,* 1992). Moreover, RBP1-I, RBP1-II and RBP1-III share the N- and C-termini and only differ within an internal exon containing potential casein kinase II and $p34^{cdc2}$ phosphorylation sites. This also suggests that the activities of each isoform is differently regulated by phosphorylation. It has also been demonstrated that each isoform is capable of binding pRb (as they all retain their LXCXE motif) and that they also retain all the functional regions (Otterson *et al.,* 1992; Lai *et al.,* 1999a). We thus generated α-RBP1 antibodies against synthetic peptides representing a region specific to the N-terminal portion of RBP1 as well as to regions specific for RBP1-II and RBP1-III (see figure 2.1). We then studied the abundance and distribution of RBP1-I,

RBP1-II and RBP1-III within the cell as well as their range of expression within different animal species.

Since a polyclonal serum contains antibodies raised against the immunogen as well as all those normally present in the animal before the immunization, purification was required in order to recover our specific antipeptide antibodies. An antigen-affinity column was used as a method to bind, purify, and concentrate the antipeptides antibodies. The purification procedure removed lots of contaminating proteins that scored in the western (see figure 3.2) and also lowered the non-specific background (compare figure 3.1 to figure 3.5). Following antigen-affinity purification, a much clearer signal was given on western blotting, both on purified proteins and on whole cell extracts (compare figure 3.4a to 3.4b and figure 3.1 to 3.5).

Cells from different animal sources were also used to verify if the antibodies could recognize RBP1 from different species. Although there was a variation in the migration (which could be due to post-translational modifications), all three antibodies recognized RBP1 in the species tested, meaning that there is no major change in the amino acid sequence or structure of RBP1 from species to species. As well, only the nuclear fraction yielded a signal (as expected), meaning that no non-specific proteins are being recognized in the cytoplasm (see figure 3.7). However, α-RBP1-II (both in the whole cell extract and in the nuclear portion) gave a very weak signal, although the concentration of the antibody was similar to the other antibodies (data not shown). This could be due to the fact that there is not much RBP1-II being synthesized in those cells, or simply because the antibody does not bind strongly to the protein. However, we cannot be sure that the proteins being recognized are really RBP1 unless immunoprecipitation-western studies are carried out.

HA-RBP1 was overexpressed via adenovirus infection and IP was carried out using α-HA, followed by western blotting using the isoform specific antibodies. Surprisingly, all of them scored positive. This was unexpected since the virus was constructed using the cDNA and thus, none of the isoform, but HA-RBP1-I, should be expressed by the virus. This result can be explained by the fact that RBP1 can form oligomers (Albert Lai, personal communication). In this case, HA-RBP1 would have

come down with the α-HA antibodies as a complex containing the other isoforms. These proteins would then have been recognized on western by their corresponding antibodies. However, if this was the case, only one band should have showed up in the α-RBP1-II and α-RBP1-III blots (see figure 3.6). The fact that there are many bands could be an indication of protein modifications. However, since the banding pattern is similar in between the different blots, it most likely means that the antibodies are not specific to their corresponding proteins.

The antibodies were also tested for their immunoprecipitation capabilities. Unfortunately, neither of the antibodies preparations (before and after the purification) generated signals. The antigen-affinity purification method requires harsh conditions to elute the antibodies. These conditions could have damaged the antibodies reducing their activity . Since the cell line that was used is known to express all four RBP1 isoforms, we excluded the possibility of these proteins not being expressed (Otterson *et al.,* 1992). Although we chose a segment of the protein which was highly antigenic, it is also possible that, since the antibodies were generated against a peptide, this portion of the protein is hidden when it is in its natural conformation so that the antibody does not have access to it. Given this possibility it was not surprising to see that these antibodies gave a signal in immunofluorescence studies (see figure 3.8). The nuclear signal colocalized with the one generated by pRb, as expected. This can be explained by the fact that the procedure used to prepare the cells for immunofluorescence uses harsh conditions that could have denatured the protein, exposing the targeted sequence and making it possible for the antibodies to recognize their respective proteins.

In order to get antibodies that are more specific to their corresponding protein, a second round of purification could be performed. In this step, α-RBP1-II (for instance) would be passed through a column containing N-RBP1 and RBP1-III peptides, and only the flow through, not the eluate, would be collected. This would ensure that no antibody in the α-RBP1-II preparation would recognize N-RBP1, or RBP1-III, leading to a more specific activity.

In conclusion, our results suggest that RBP1 is implicated in myogenesis. Its precise role remains to be elucidated, but based on previous studies, we suggest that RBP1 could be involved in the induction of growth arrest. Further work is required to determine the mechanism of RBP1 shuttling as well as its importance in differentiating myoblasts.

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