To the Graduate Council:

I am submitting herewith a thesis written by Huo Li entitled "Interaction of Retinoblastoma Gene Product with Transcription Factors ATF-a and ATF-2." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry.

Wesley D. Wicks, Major Professor

We have read this thesis and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor and
Dean of The Graduate School

INTERACTION OF RETINOBLASTOMA GENE PRODUCT WITH TRANSCRIPTION FACTORS ATF-a AND ATF-2

A Thesis

Presented for the

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Degree

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Huo Li

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DEDICATION

This thesis is dedicated to my parents

Mr. Zhong-Qun Li

and

Mrs. Hong-Jing Gao

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ABSTRACT

The retinoblastoma gene product (pRb) plays an important role in constraining cellular proliferation and in regulating the cell cycle. pRb's regulation of the expression of transforming growth factor β2 (TGF-β2), an inhibitory growth factor gene, was suggested to be mediated by Activating Transcription Factor 2 (ATF-2)(Kim et al., 1992), Factor a (ATF-a) (Gong et al., 1995), and their DNA target element (ATF site: CGTCA) in the TGF-β2 promoter. The differential regulation of TGF-β2 promoter activity (first observed by Gong et al., 1995) by pRb has been explored in more depth and the functional interaction sites of ATF-2 and ATF-a on pRb have been mapped and compared in this study.

Optimal transient transfection conditions using lipofectamine as transfection inducer for the introduction of a foreign TGF-β2 CAT reporter construct into CHO cells have been selected. Under these conditions, the activation by co-transfected ATF-a, ATF-2 and the differential regulation mediated by pRb on the TGF-β2 promoter have been explored further in terms of varying input DNA concentrations and the time course for the expression of the regulated TGF-β2 CAT. Although the regulatory pattern was similar on days 1, 2 and 3, the greatest response was observed 48 hr after transient transfection.

The functional interaction sites for both ATF-2 and ATF-a on pRb were probed by cotransfecting cells with a series of truncated pRb mutants and ATF-2 or ATF-a. Our results demonstrate that ATF-2 and ATF-a share the same interaction sites on pRb *in vivo*: primarily in the so called A/B pocket domains of pRb, since deletions of amino acid sequences in these domains caused dramatic decreases in the ability of pRb to affect the TGF-β2 promoter with or without the overexpression of ATF's. The interaction sites also appear to involve the C-terminal domain of pRb (referred to as the C pocket), although the degree of effect was less substantial than that seen with the A/B pocket mutants.

The levels of expression of foreign wild type (wt) and mutant (mt) pRb's in the presence or absence of overexpression of ATF-2 and ATF-a have been monitored using an antibody against the HA tag located on the C-terminus of pRb. All mutant forms of pRb have been shown to be expressed at levels similar to wt pRb and overexpression of ATF-2 and ATF-a did not effect the levels of expression of wt and mt pRb's.

We also studied the differential regulation of ATF-2's and ATF-a's transcriptional activations by pRb in an alternative cell line, the human uterine mesodermal tumor cell line SKUT-1 which lacks endogenous pRb. The introduction of foreign pRb does not cause cellular apoptosis in SKUT-1 cells. The same regulatory pattern as in CHO cells has been observed, although there was minor variation in the degree of stimulation or inhibition.

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LIST OF ABBREVIATIONS

ATF-2	Activating Transcription Factor 2
ATF-a	Activating Transcription Factor a
BSA	Bovine Serum Albumin
bZip	basic-Leucine-Zipper
CAT	Chloramphenicol Acetyltransferase
cdk	cyclin-dependent kinase
CRE	Cyclic Adenosine 3', 5'-Monophosphate Response Element
DMEM	Dulbecco's Modified Eagle's Medium
E1A	Adenovirus Early region 1A oncoprotein
EDTA	Disodium Ethylenediamine Tetraacetate
EGTA	Ethylene Glycol-bis(β-Aminoethylether)N,N,N',N',-Tetraacetic Acid
ESPG	EDTA-Sodium-Phosphate-Glucose
HA	Hemagglutinin

HB	Homogenization Buffer
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HMG	High Mobility Group protein
JNK	c-Jun N-terminal Kinase
mt	mutant
NP-40	Nonidet P-40
PMSF	Phenylmethylsulfonyl Fluoride
pp1α	protein phosphatase catalytic subunit
pRb	Retinoblastoma gene product
SAPK	Stress-Activated Protein Kinase
TBP	TATA-Binding Protein
TGF-β2	Human Transforming Growth Factor β2
wt	wild type

CHAPTER I
INTRODUCTION

The retinoblastoma gene product, pRb, was first identified as a suppressor of tumor formation and, since then it has been implicated in the regulation of several cellular processes, including cell-cycle arrest, differentiation and apoptosis. The tumor suppressing function of pRb was first suggested by the observation that homozygous loss of functional pRb correlates with the development of retinoblastoma in humans (for reviews, see Wang et al., 1994; Riley et al., 1994).

Fundamental functions of the tumor-suppressor protein pRb in vivo have been demonstrated in mice in which the pRb gene was inactivated in the germ line (Lee et al., 1992 and 1994). In these animals, many pRb-deficient cells, most notably fetal neurons and hematopoietic precursors, fail to exit the cell cycle and continue to divide in aberrant locations. Some nondividing cells also fail to differentiate terminally and others undergo unscheduled apoptosis. Reintroduction of functional pRb into pRb-deficient retinoblastoma cells resulted in reduced tumorigenicity in nude mice (Huang et al., 1988). The tumor-suppressing function of pRb was suggested to be tissue-specific by observations made in mice with heterozygous disruption of the pRb gene. These pRb +/-

mice do not develop retinoblastomas, but have an unusually high incidence of pituitary tumors (Jacks et al., 1992).

Mutations of the pRb gene have been found not only in retinoblastomas (Fung et al., 1987; Lee, W.-H., 1987), but also in osteosarcomas, bladder carcinomas, small cell lung carcinomas, and a prostate carcinoma (Wang et al., 1994. review).

Expression of wild type pRb, via retrovirus-mediated gene transfer, suppresses the tumorigenicity of several neoplastic cell lines that contain pRb mutations (Wang et al., 1994). In addition to the suppression of growth, pRb has also been suggested to play a role in cellular differentiation during development (Chen et al., 1996). pRb has been implicated in the regulation of apoptosis. For example, pRb is inactivated in the retinas of transgenic mice, but rather than developing retinoblastomas, the retinal cells undergo apoptosis at a time when they normally would be undergoing terminal differentiation (Howes et al., 1994).

The biochemical mechanism by which pRb suppresses tumor formation is unclear; however, it has been suggested that pRb inhibits tumorigenesis by inhibiting cell-cycle progression (Templeton et al., 1991; Pushkareva et al., 1995). Introduction of functional pRb into certain pRb-deficient tumor cell lines results in G1 arrest (Templeton et al., 1991). This pRb-mediated growth arrest also displays cell type specificity, being seen in certain tumor cells while not in other cell types (Fung et al., 1993; Dbaibo et al., 1995).

The full length human pRb cDNA spans 4757 nucleotides. It has an open reading frame that encodes a protein of 928 amino acids (Riley et al., 1994, review). The pRb gene product is a 110-kD nuclear phosphorylated protein that is phosphorylated on both serine and threonine residues (Wang et al., 1994). These modifications occur in a cell cycle-dependent manner, and were suggested to be important in negatively regulating oncoproteins and transcriptional factors.

Nonfunctional mutants of pRb, present in defective alleles of the pRb gene isolated from two spontaneously arising tumors were found to be defective in phosphorylation, viral oncoprotein association and nuclear tethering (Templeton et al., 1991).

Hypophosphorylated pRb associates with the nuclear matrix during early G1, and is thought to bind peripheral matrix proteins lamin A and C (Mancini et al., 1994). pRb is distributed widely throughout the nuclear matrix, particularly concentrated at the nuclear periphery and in nuclear remnants. Phosphorylated or mutant pRb in tumor cells does not associate with the matrix (Mancini et al., 1994).

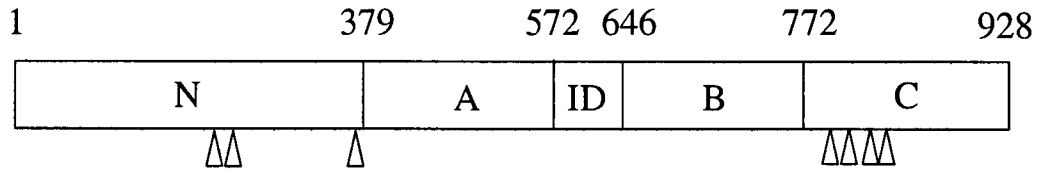
During the G1 phase of the cell cycle, pRb is hypophosphorylated and is able to repress cell proliferation. At some point later in G1 in cycling cells, it becomes hyperphosphorylated, likely by cyclin-dependent kinases (cdks), and loses its growth suppressing function. Dephosphorylation of the pRb protein occurs during mid-M phase, reactivating the protein prior to the next cell cycle. Evidence strongly implicates

the type 1 protein phosphatases (pp1) as being critical for this process (Ludlow et al., 1993).

pRb contains several functionally distinct domains, which have been found to be important for pRb's biological functions (Figure 1). It has been suggested that there is an N-terminal domain, A and B pockets and a C-terminal domain (C pocket).

The binding domains within the pRb protein have been delineated, by use of a series of in vitro site specific mutations and naturally occurring mutants, as two noncontiguous regions designated domains, A and B pockets, which are interrupted by a spacer sequence (Huang et al., 1990). The A and B pockets are critical for pRb phosphorylation during the G1 phase of cell cycle, binding to transcription factors, such as E2F, ATF-2 etc., tethering to nuclear structures and growth suppression in cell culture (Templeton et al., 1991). Table 1 shows the A and B Pockets of pRb bind directly or indirectly to a large number of oncoproteins and transcription factors and regulate their activities (Adapted from Wang et al., 1994, review).

The importance of the pocket structure was further underscored by the recent observation that the conserved C21ding protein (TBP) has significant homology with the pocket A of pRb and can bind transcription factors in common with pRb (Hagemeier et al., 1993a). It has been proposed that pRb mimics the binding surface of TBP to block transcriptional activation (Hagemeier et al., 1993b). These findings imply a central role for the pocket-binding activity of pRb and



Δ Mapped in vivo phosphorylation sites in pRb: Ser 249, Thr 252, Thr 373, Ser 807, Ser 811, Thr 821, Thr 826.

Figure 1. Domain structure and phosphorylation sites of pRb. The human pRb protein contains 928 amino acids that are organized into several domains: the N terminal region (amino acids 1-378), domain A (A, amino acids 379-572), the insert domain (ID, amino acids 573-645), domain B (B, amino acids 646-772) and the C terminal region (C, amino acids 773-928). There are 7 Ser/Thr sites in pRb (indicated by arrow heads), which have been shown to be phosphorylated potentially by the cyclin-dependent protein kinases in vivo.

Table 1. List of pRb associated proteins

Protein	Function	Interaction sites in pRb	Consequence of interacting with pRb
Large T Ag	Oncoprotein	A/B	Contributes to viral transformation
E1A	Oncoprotein	A/B	Contributes to viral transformation
E2F-1,-2,-3	Transcription factor	A/B	Inhibits activity
ATF-2	Transcription factor	A/B	Activates activity
ATF-a	Transcription factor	A/B	Inhibits activity
c-Abl	Tyrosine kinase	C	Inhibits tyrosine kinase
D1, D2, D3	Cyclins	Large A/B	Targets pRb for phosphorylation?
cdk2, cdk4	Ser/Thr kinase	?	pRb phosphorylation?
pp1a	Phosphatase	Large A/B	pRb de-phosphorylation?

suggest that understanding the properties of pRb-associated proteins is necessary to define tumor suppressor pathways.

On the other hand, pRb-associated proteins use a variety of motifs to bind; for example, T antigen of SV40, adenovirus E1A oncoprotein and other oncoproteins bind pRb via a conserved Leu-x-Cys-x-Glu motif (Kim et al., 1994); transcription factor E2F1, in contrast, uses a unique 18-amino acid motif to interact with pRb; and pp1α uses a different, large, C-terminal region to bind pRb (Lee et al., 1994).

The C terminal region outside of the A/B pocket, referred to as the C pocket, has been shown to be functionally distinct, and it interacts with the nuclear c-Abl tyrosine kinase (Welch and Wang, 1995).

It has been shown that a 73-kD heat shock cognate protein can bind directly to an N-terminal region in pRb (residues 301-372 adjacent to the N-terminal boundary of the A pocket), and phosphorylation of pRb inhibits this association in vitro and perhaps in vivo (Inoue et al., 1995). Recently, functional roles for the N-terminal region of pRb in growth suppression have been emerging. Deletion studies indicate that amino terminal sequences are necessary for hyperphosphorylation of the full length molecule (Qian et al., 1992). It was found the N-terminal domain of pRb binds a novel 84 kD nuclear protein (p84) both in vivo and in vitro, and the association is preferentially with the functionally active, hypophosphorylated form of pRb. It was suggested that

pRb may function in part to facilitate the binding of growth promoting factors at subnuclear regions where p84 is located and actively involved in RNA processing (Durfee et al., 1994).

The most established mechanism of active transcriptional regulation by the retinoblastoma repressor gene product is that pRb represses E2F transcriptional activation (Weintraub et al., 1995). E2F was first defined as a transcriptional factor that activates the E2 promoter of adenovirus genes and has since been found to activate a variety of G1/S-specific genes (Nevins, 1992, review). When E2F is bound by pRb, it is transcriptionally inactive. The increase in pRb phosphorylation before the onset of S phase of the cell cycle, brought about by cyclin D/cdk4 protein kinase which accumulates at this stage, causes pRb to dissociate from E2F, allowing it to activate S-phase genes (Weinberg, 1995, review).

More recently, other functional roles of pRb in growth repression have appeared (Adnane et al., 1995; Nasmyth, 1996). pRb has been implied to regulate ribosomal RNA transcription mediated by pol I, which provides a hint as to how pRb might influence cellular biosynthesis as well as gene expression in S phase (Cavanaugh et al., 1995). Also, pRb was found to repress pol III-mediated transcription in vitro and in cultured primary mouse fibroblasts (White et al., 1996). Therefore, it was suggested that pRb might be a more general regulator of transcription needed for cell proliferation.

pRb inhibits the transcription of genes involved in growth control (Wagner and Green, 1991) and can regulate expression of

several genes, including transforming growth factor β1 and β2 (TGF-β1 and TGF-β2)(Kim et al., 1992). Because TGF-βs inhibit proliferation of many cell types and arrest growth in the late G1 phase of the cell cycle, induction of TGF-βs by pRb may play an important role in cell cycle control. It has been demonstrated that pRb activates expression of the human transforming growth factor-β2 gene (TGF-β2) through ATF-2 (Kim et al., 1992), and pRb also positively regulates its own promoter through an ATF site (Kim et al., 1994). Although ATF-a shares a large degree of homology with ATF-2, pRb inhibits ATF-a's transcriptional activation on the expression of the same TGF-β2 gene (Gong et al., 1995).

ATF-a and ATF-2 are ATF/CRE-binding proteins, a subfamily which belongs to a large super-family of DNA-binding proteins known as the "bZip" (basic-leucine-zipper) proteins (Meyer and Habener, 1993, review). Members of this family have a basic region in their C-terminus, rich in positively charged amino acids, flanked by a leucine zipper motif. The basic region is responsible for sequence-specific DNA recognition and binding. The leucine zipper is a coiled-coil structure with leucines at every seventh position (heptad repeats) responsible for protein dimerization.

ATF-2 and ATF-a have a striking similarity to each other at the protein primary structure level, and both bind to the same DNA target element, the widely distributed CRE/ATF site, CGTCA. The abilities of ATF-a and ATF-2 to stimulate transcription are augmented by direct interaction with the adenovirus E1A protein,

and they do not mediate cAMP responses, but they are differentially modulated by the retinoblastoma gene product (Gong et al., 1995).

Both ATF-2 and ATF-a act not only to form DNA binding homodimers, but also efficiently heterodimerize with numerous members of the Jun/Fos family of factors (Hai et al., 1989; Benbrook et al., 1990). Such dimerization may result in ATF-2 or ATF-a being a constituent of factor complexes that have subtle differences in DNA binding specificity and regulatory potential.

Both ATF-2 and ATF-a were cloned from Hela cell cDNA libraries (Gaire et al., 1990) by reverse transcription PCR (RT-PCR) and Northern blot hybridization. The expressions of ATF-2 and ATF-a are both tissue-specific. The putative mRNA for ATF-2 has been found in various human and monkey tissues and many cell lines (Maekawa et al., 1989; Taketa et al., 1991). ATF-2 mRNA was found to be relatively more abundant in the brain tissue than any other tested human tissues, including lung, liver and kidney tissues (Maekawa et al., 1989). The highest mRNA level for ATF-a was found in skeletal muscle; and ATF-a was also expressed in human heart, brain, placenta and lung tissues, but no detectable signal was obtained from liver tissue (Pescini et al., 1994).

Therefore, the tissue distributions of ATF-2 and ATF-a are different. Although both ATF-2 and ATF-a are expressed in some particular tissues, for example, lung tissue; it seems the expression of ATF-2 is more involved in the differentiation of the central nervous system in the brain (Takada et al., 1991). This suggestion

was consistent with the observation of ATF-2 gene knock-out mice (Reimold et al., 1996). It has been shown that mice carrying a germ line mutation in ATF-2 suffered wide-spread abnormalities; thus, ATF-2 was demonstrated to be absolutely required for skeletal and central nervous system development.

In addition, there has been considerable interest in the role of ATF-2 because this transcription factor binds several viral proteins, including the oncoprotein E1A (Liu and Green, 1990; Hagmeyer et al., 1995). ATF-2 also interacts with pRb (Kim et al., 1992), the high mobility group protein HMG I(Y) (Du and Maniatis, 1994). These protein-protein interactions lead to modified transcriptional activity. The function of ATF-2 may therefore be determined by its interaction with these proteins.

It has also been suggested that ATF-2 is a direct target of a signal transduction pathway (Karin and Hunter, 1995, review). ATF-2 was found to be phosphorylated by the c-Jun N-terminal kinase (JNK) (Gupta et al., 1995) and Stress-activated protein kinases (SAPK's) (Livingstone et al., 1995; van Dam et al., 1995) on two closely spaced threonine residues (Thr at positions 69 and 71) within the N-terminal activation domain. The replacement of these phosphorylation sites with alanine inhibited the transcriptional activity of ATF-2 and ATF-2-stimulated gene expression mediated by pRb and the adenovirus early region 1A (E1A) oncoprotein (Gupta et al., 1995). Consistent with their sensitivity to phosphorylation in vitro, the activity of the N-terminal domain of ATF-2 is stimulated by UV irradiation and serum, which are

known factors stimulating SAPK's activities (Livingstone et al., 1995). SAPKs are a subfamily of the mitogen-activated protein kinases, which are minimally activated by growth factors but markedly activated by inflammatory cytokines, heat shock, inhibitors of protein synthesis.

ATF-2 DNA binding activity is highly regulated in the intact animal in response to the extreme, albeit common, pathophysiologic stress of reperfusion of the ischemic kidney, and is presumably regulated by phosphorylation by an ischemia-activated ATF-2 kinase (Derijard et al., 1994), probably a SAPK. Phosphorylation may serve to target ATF-2 to various promoters, and this targeting may be an important initial step in the transcriptional responses necessary for repair and proliferation of cells that have survived the ischemic insult to restore tissue functional integrity (Morooka et al., 1995).

ATF-a and its variants were initially reported to have weak transactivation functions (Chatton et al., 1993); and it was suggested that the N-terminal activation domain of the ATF-a protein is functionally masked by the C-terminal region of the protein (Chatton et al., 1994). Later, however, ATF-a has been found to stimulate gene expression mediated by the ATF/CRE site located in the TGF-β2 promoter in CHO cells (Gong et al., 1995) and mediated by the similar site in the E-selectin promoter (Pescini et al., 1994). Mutation of these ATF-binding sites abolishes ATF-a's transactivation. ATF-a appears to mediate promoter induction by the E1a proteins of adenovirus (Chatton et al., 1993); and ATF-a's

transactivation was found to be inhibited through overexpression of pRb (Gong et al., 1995).

It has been shown that ATF-a protein can interact through its basic-leucine-zipper (bZip) element with either Jun or Fos proteins with equal efficiency and that resulting heterodimers bind to ATF/CRE recognition site with affinities different from those of the corresponding homodimers. However, while heterodimers with the members of the Jun family are transcriptionally competent, associations with c-Fos are not. It has been suggested that the ATF-a moiety provides an anchoring, rather than activating functions in these heterodimers, and heterodimerization alters transcriptional properties of both ATF-a and c-Fos (Chatton et al., 1994).

Transforming growth factor type-β (TGF-β) refers to a complex family of genetically distinct polypeptides that can act as multifunctional regulators of cell growth and differentiation (Roberts and Sporn, 1990, review). The expression of TGF-β2 is spatially and temporally regulated during the earliest stages of mouse development (Rizzino, 1995). TGF-βs regulate cell differentiation, cell growth, cell function, and are the most potent growth-inhibitory polypeptides known for a wide variety of cell types including most normal and transformed epithelial, endothelial, fibroblast, lymphoid and hematopoietic cells. Although TGF-βs arrest growth in the late G1 phase of the cell cycle, the mechanism by which they inhibit cell proliferation is virtually unknown (Silberstein and Daniel, 1987).

Previous studies have indicated links between TGF-β2-mediated G1 growth arrest and the activity states of cyclins, cyclin-dependent kinases (CDKs) (Okamoto et al., 1994) and pRb (Koff et al., 1993). Overexpression of human cyclin D1 reduces the growth inhibition by TGF-βs. Treatment of cells with TGF-β appears to prevent phosphorylation of pRb and retains pRb in the hypophosphorylated, growth-suppressive state.

Four regulatory elements in the TGF-β2 promoter regions, including a critical CRE/ATF element, help control the expression of the TGF-β2 gene during early mammalian development (Kim et al., 1991; Scholtz et al., 1995). It was suggested pRb activates transcription of the human TGF-β2 gene through ATF-2 (Kim et al., 1992). The promoter element responsible for pRb-mediated transcriptional regulation is a binding site for ATF proteins.

Since ATF-a shares a certain degree of sequence homology with ATF-2 (Figure 2), especially in the zinc finger domain which has been suggested as the basis for transcriptional activation and interaction with proteins, it was of interest to test whether or not ATF-a has functions similar to those of ATF-2. In previous experiments in Dr. Wicks lab (Gong et al., 1995), it was found that both ATF-a and ATF-2 were able to activate the TGF-β2 gene promoter through its CRE/ATF site in CHO cells, and the expression of pRb differentially modulated their activities on this promoter. My project was aimed at addressing the functional significance of ATF2-pRb and ATFa-pRb interactions. I have analyzed the effects of truncated mutations in different domains of pRb on ATF-2 and

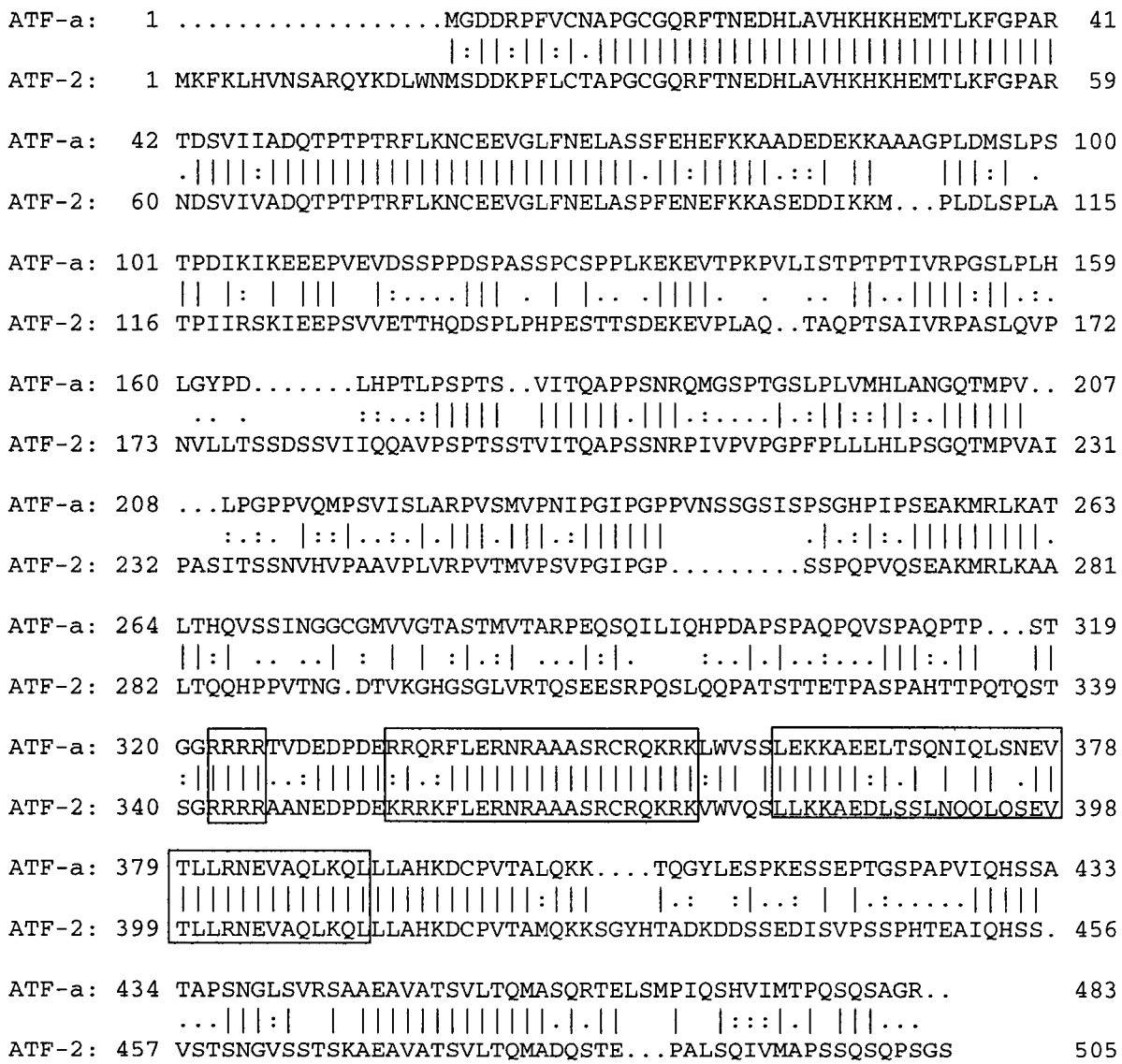


Figure 2. Amino acid sequence and homology comparison between ATF-a and ATF-2. The box areas correspond to the basic region (ATF-a: 322-325 and 334-354; ATF-2: 342-347 and 356-376) and Leucine Zipper domain (ATF-a: 360-391; ATF-2: 380-411).

ATF-a transcriptional activation. We also wanted to study further the differential regulation of ATF-2 and ATF-a's transcriptional actions by pRb in an alternative cell line and have used the human uterine mesodermal tumor cell line SKUT-1 which lacks endogenous pRb.

CHAPTER II
MATERIALS AND METHODS

Plasmids

Expression vectors for ATF-a were kindly provided by Dr. C. Kedinger. pATF-2 was a gift from Dr. M. Green, and the CAT reporter construct pB_2-77 was provided by Dr. S. Kim. Constructs of full-length and truncated pRb expression vectors, the pRbWT3HA/SVE series, were provided by Dr. D. J. Templeton. The pRb expression vectors encode proteins identical to the wild-type or truncated mutants of pRb but there are an additional 10 amino acids appended to their carboxyl terminus. These amino acids (YPYDVPDYAK) comprise an epitope that is recognized by the monoclonal antibody (MAb) 12CA5, which is directed against the hemagglutinin (HA) antigen of influenza virus. The CAT reporter construct TK CAT and pGaltkCAT series, containing Gal4 binding site(s) in the promoter region, were given by Dr. J. Koontz. Plasmids were transformed into JM101 cells and prepared by the following procedures.

Preparation of competent bacterial cells and Transformation

Five ml LB medium (10 g bactotryptone, 5 g yeast extract, 5 g NaCl per liter) was inoculated with JM 101 cells, and was shaken overnight at 37 °C. One hundred ml of LB medium was inoculated

with 2 ml of the overnight culture. The culture was shaken at 37 °C until the OD_{600} of the culture reached 0.4. After centrifugation for 5 min at 7,000 g, the bacterial pellet was gently resuspended in 50 ml of sterile and ice-cold 50 mM $CaCl_2$. After incubation in ice for 20 min, the bacterium were gently resuspended in 10 ml of 50 mM $CaCl_2$ and incubated overnight at 4 °C.

One hundred µl TCM (10 mM Tris, 10 mM $CaCl_2$, 10 mM $MgCl_2$, pH8.0), 50 µl competent cells and 0.1 µg plasmid DNA were mixed and incubated on ice for 30 min. The competent bacterial cells were heated at 42 °C for 2 min and left at room temperature for 10 min. The transformed cells were dispersed with 3 ml ampicillin-containing prewarmed top agar onto an agar plate. The bacteria were allowed to grow at 37 °C overnight.

Plasmid preparation

Plasmids were prepared by the typical phenol/chloroform extract protocol. PZ 523 columns (from 5 prime --> 3 prime, Inc.) were used to further purify the double-stranded plasmid DNA.

A fixed volume (1.8 ml) of pre-purified plasmid samples was applied onto the top of the resin bed, and the column/ collection tube assembly was centrifuged at 1,100 g in a swinging bucket rotor for 24 min. The column effluent containing purified plasmid DNA was recovered, precipitated by isopropanol and washed twice with 70% ice cold ethanol.

Agarose gel electrophoresis for plasmid DNA samples

Agarose gels were poured by dissolving 0.4 g agarose in 50 ml 0.5X TBE (100 mM Tris-borate, 2 mM EDTA). The gel was run at 100 volts for about 60 min in 0.5 X TBE. After electrophoresis, the DNA in the gel was stained in 1 μg/ml ethidium bromide for 30 min, and then visualized and photographed under ultraviolet light.

Cell culture

Two cell lines, CHO and SKUT-1, were used in these experiments. The CHO cell line was cloned from Chinese hamster ovary, and the SKUT-1 cell line was isolated from a human uterine mixed mesodermal tumor. The CHO cells have a generation time of approximately 12 hr, and the SKUT-1 cells take a little longer to double. CHO cells were grown in Ham's F12 medium (Sigma) supplemented with 10% fetal bovine serum. SKUT-1 cells were maintained in Dulbecco's modified Eagle's Medium (DMEM, Sigma) containing 10% fetal bovine serum. Both cell lines were grown as monolayers in filter sterilized medium on various sized plastic culture plates (Corning Glass Works) at 37 °C.

Growing cells were continuously maintained by serial culture every two to four days. Removal of cells from plates was normally accomplished with a solution of 0.025% trypsin in EDTA-Sodium-Phosphate-Glucose (ESPG) [0.15 M (8.8 g/liter) of sodium chloride, 0.01 M (1.7 g/liter) of potassium phosphate, 1.2 g/liter of glucose, 1 mM (0.37 g/liter) of disodium ethylenediamine tetraacetate

(EDTA), pH 7.5]. The term "trypsinization" will be used to denote a procedure with the following steps:

1. The old medium is removed from the plate of cells.
2. The cells are washed with ESPG solution.
3. The cells are incubated for five to ten min in trypsin solution.
4. The cells are resuspended in fresh medium.

Transfection

Cells were grown to 50-80% confluence (the area of the plate's bottom covered with cells) in 35 or 100 mm culture dishes. The following solutions were prepared in sterile tubes:

Solution A: For each transfection, 1-2 μg of DNA was diluted into 100 μl serum-free medium. (OPTI-MEM I Reduced serum Medium, Gibco BRL)

Solution B: For each transfection, 3 μl of lipofectamine (Gibco BRL) was diluted into 100 μl the same medium.

The two solutions were combined, mixed gently, and incubated at room temperature for 30 min to allow DNA-liposome complexes to form. While complexes were forming, the cells were rinsed once with 2 ml of serum-free medium. For each transfection, 0.8 ml of serum-free medium (no antibacterial agents are added to the medium, because the cellular membrane is very sensitive in the process of transfection) was added to the tube containing the complexes. After gently mixing, the diluted complex solution was overlaid onto the rinsed cells in a 35 mm dish.

The cells were then incubated for 6 hr at 37 °C in a CO_2 incubator. Following incubation, 1 ml of growth medium containing twice the normal concentration of serum was added without removing the transfection mixture. The medium was replaced with fresh, complete medium at 24 hr following the start of transfection. The cells were usually harvested 54 hr after the start of transfection, and lysed by three freeze-thaw cycles and clarified by centrifugation.

Protein assay

CHO cell extracts were subjected to the Bradford assay to determine the concentration of protein. Five µl of cell lysate was added to 200 µl of Coomassie bright blue dye (Sigma), and the volume of samples was brought to 1 ml by adding distilled water. After incubation at room temperature briefly, the absorbance of samples was read at 595 nm in a spectrophotometer. A standard curve was prepared with known concentrations of Bovine Serum Albumin (BSA) and used to determine the protein concentration of transfected cell extracts.

CAT activity assay

CAT activity of cell extracts was assayed by a modification of Sleigh's method (Sleigh et al., 1986). The clarified cell extracts were heated at 65 °C for 10 min to inactivate the inhibitor of CAT activity prior to incubation with 1.0 mM chloramphenicol (Sigma), 0.1 mM acetyl-CoA and 0.05 µCi [acetyl-1-^{14}C] acetyl CoA (DuPont

NEN) at 37 °C for 4 hr. The product, ^{14}C-labeled acetyl-chloramphenicol was extracted with ethyl acetate, 1 ml of Econofluor^{TM} was added and the samples were counted in a scintillation counter. The specific activities of CAT were determined by calculating the ratio of CPM of radioactive product to the protein amount present in the cell extract used.

Preparation of nuclear extracts

Nuclear extracts were prepared by a modification of the procedure described by Borgmeyer et al. (1994). Cells were homogenized and lysed in 2 volumes of homogenization buffer (HB): 10 mM N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), pH 7.5/0.5 mM spermidine/0.15 mM spermine/5 mM EDTA/0.25 mM Ethylene Glycol-bis(b-Aminoethylether)N,N,N',N',-Tetraacetic acid (EGTA) /50 mM NaF/7 mM 2-mercaptoethanol/1 mM Phenylmethylsulfonyl Fluoride (PMSF) containing 0.5 M sucrose and 0.1% Nonidet P-40 (NP-40), and the nuclei were sedimented by centrifugation at 12,000 g for 30 min. After two washes in HB containing 0.35 M sucrose, nuclei were extracted with HB containing 0.05 M NaCl and 10% (v/v) glycerol for 1 hr, and clarified by centrifugation at 12,000 g.

SDS-PAGE and Immunoblotting

One-tenth of each nuclear extract (about 50 µg of protein was present in each loaded lane) was analyzed by sodium dodecyl sulfate (SDS)-polyacryamide gel electrophoresis (PAGE). Ten

percent polyacrylamide was used as the separating gel. A 10 kD standard ladder (Gibco BRL) was used as a molecular weight standard in the coomassie blue stained gel. A prestained molecular weight marker (Sigma) was used (run and transferred) on the nitrocellulose membrane. After SDS-PAGE, the samples were electroblotted onto nitrocellulose membranes, which were then blocked by incubation in 5% dry milk at room temperature for an hour to prevent non-specific binding. The transfected CHO cell extract blots containing introduced full length and truncated pRb mutants were probed with 10 μg/ml of monoclonal antibody 12CA5 (from Berkeley Antibody Company), which recognizes the carboxyl-terminal HA (influenza hemagglutinin) epitope (YPYDVPDYAK) present in all of the foreign pRb species. Peroxidase-conjugated goat anti-mouse IgG and its substrates, H_2O_2 and 4-chloro-1-naphthol were used to reveal the presence of the HA epitope. The pRb bands were scanned with a densitometer, and the densities of the bands representing pRb mutants were normalized to that of extracts containing wt pRb, which was included in every blot.

CHAPTER III
RESULTS AND DISCUSSION

Optimization of transfection and assay conditions

Transfection inducers

Different kinds of transfection inducers, including calcium phosphate, Lipofectamine^{TM} and Lipofectace^{TM}, were used to transfect the TK CAT control vector into CHO cells. TK CAT expressed high levels of CAT activity after being transfected into CHO cells, thus it provided a strong signal in the transfection process. Transfection efficiencies were estimated by the activities of CAT enzyme expressed in the various CHO cell extracts.

Figure 3 shows that lipofectamine was more efficient than lipofectace (over 150 cpm/μg vs. 40 cpm/μg for optimal CAT activity), and required only 2 to 5 μl for efficiently transfecting 35 mm-dish of CHO cell (vs. 10-15 μl for lipofectace). I tried twice to use calcium phosphate as a transfection inducer, but it failed to work. As a result, lipofectamine was chosen as the transfection inducer. Since both lipofectamine and lipofectace are expensive, we tried to use the minimal amount of lipofectamine (3 to 4 μl per 35 mm dish of cells, in other words, 0.3% to 0.4% of transfection mixture) and got optimal transfection efficiency in subsequent experiments.

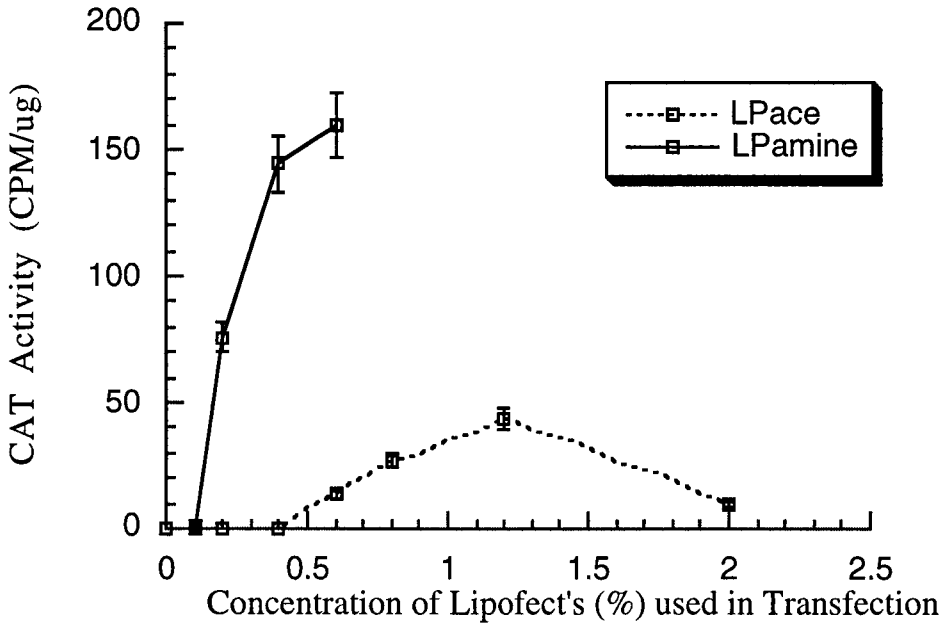


Figure 3. Optimization of lipofectamine and lipofectace for transfection of CHO cells. Two μg of the TK CAT plasmid was transfected into CHO cells (35 mm dish) by varying amounts of either lipofectamine or lipofectace. After incubation for 48 hr, the transfected CHO cells were harvested and lysed. CAT activities were measured and expressed as CPM of ^{14}C-Acetyl-chloramphenicol per μg of cell lysate protein. The concentration of lipofectamine/lipofectace was calculated and expressed as percentage [the volume of lipofectamine/lipofectace X 100 divided by the total volume of the transfection mixture]. Each determination was repeated at least three times.

Linearity of CAT assay

To confirm that the CAT activities of lysate samples are in an appropriate linear range, the dependence of CAT activity was studied as a function of the amount of lysate added. CHO cells transfected by TK CAT DNA expressing high levels of activity were used as a CAT-containing cell extract pool, which were mixed with untransfected CHO cell extract, which contained similar protein concentrations and components except for CAT activity, to give different levels of CAT activity and to maintain other factors in the cell extract as a constant.

The results in Figure 4 show that the CAT assay is in a good linear range at least up to 12,000 cpm, and thus, the fold stimulation of CAT activity within this range can be safely calculated.

Time course of CAT assay

To amplify the radioactive signal of the CAT assay, I prolonged the incubation time. Various amounts of CAT-expressing cell extract were added to CAT reaction mixtures and incubated for different times (2, 4-1/4, 6 hr) to study the time course of the expressed CAT product accumulation.

The results in Figure 5 show that CAT activities are reasonably linear for at least 4-1/4 hours with the usual amount of cell extracts used. As a result, the incubation time for the CAT assay was prolonged to 4 hr for higher radioactive signal.

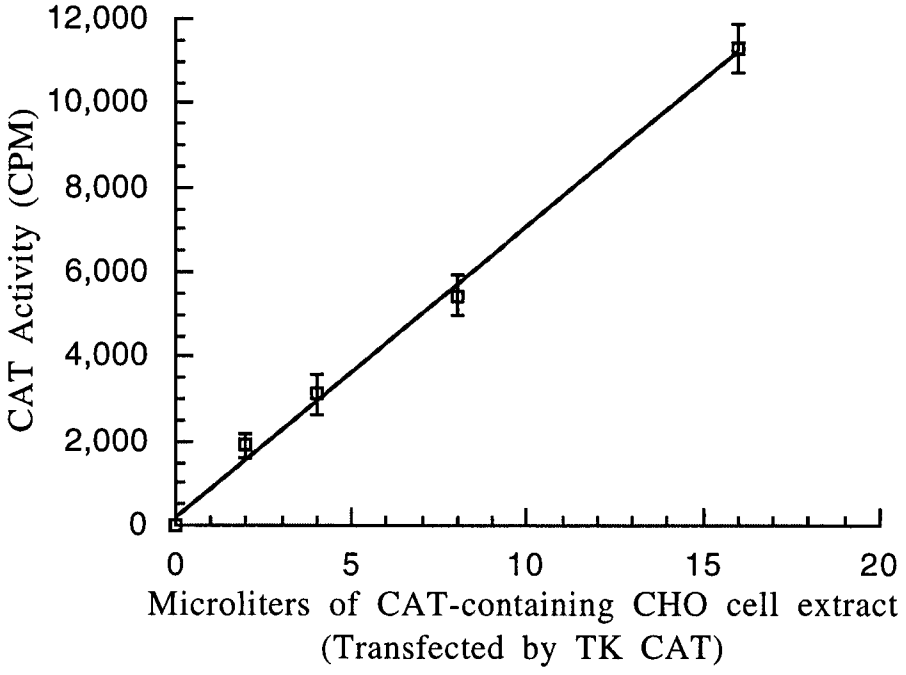


Figure 4. Linear range of CAT assay. CHO cells transfected by TK CAT DNA and expressing high levels of CAT enzyme were used as a CAT-containing pool. Different amounts of cell extract from the pool were diluted in untransfected CHO cell extracts, the final volume of all samples was 100 µl of cell extracts. The final concentration of CAT reaction mixture components used in this and following experiments was: 1.0 mM chloramphenicol, 100 µM Acetyl-CoA and 0.1 µCi/ml [Acetyl-1-^{14}C] Acetyl-CoA. The cell extracts containing different levels of CAT activity were subjected to the CAT assay, and the resulting CAT activities were plotted against µl of CAT-containing CHO cell extract. This experiment has been repeated twice and shown similar results.

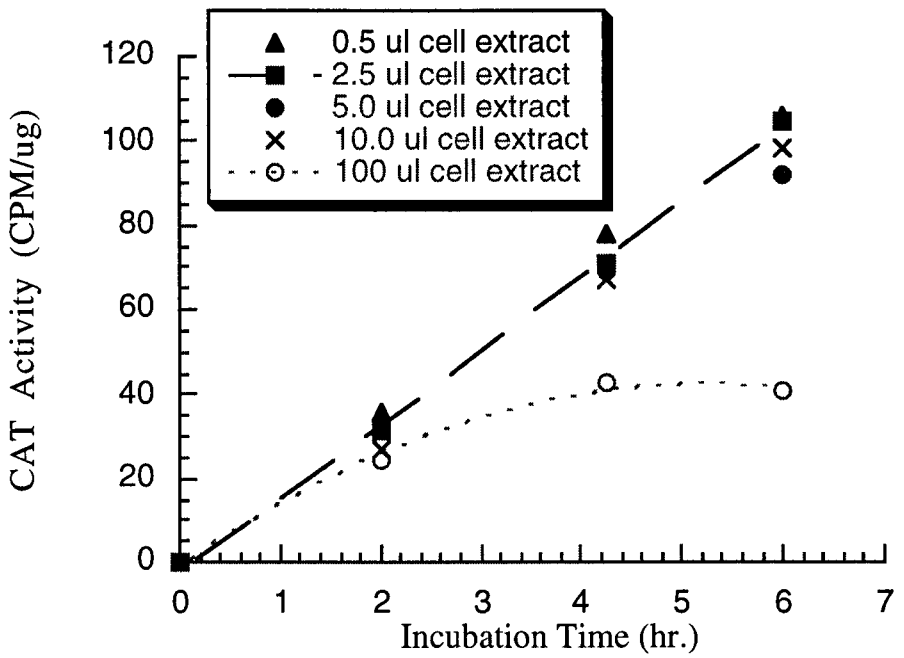
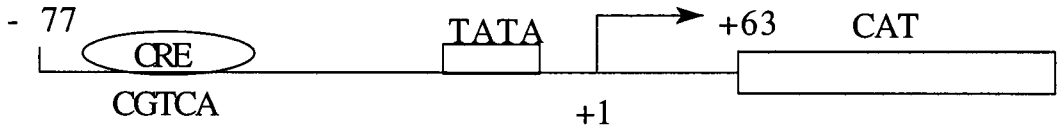


Figure 5. Time course of CAT assay. Various amounts (0.5, 2.5, 5, 10 and 100 µl) of CAT-expressing CHO extracts (transfected by TK CAT) were added to the standard CAT reaction mixture (see Figure 4), and incubated for 2, 4-1/4 and 6 hr. The radioactivities were measured, and CAT activities were calculated as CPM per µg of protein. Each point is the average of three samples.

Titration of basal CAT expression with TGF-β2 CAT DNA in CHO cells

To study basal CAT expression with TGF-β2 CAT DNA (pB_2-77) in the CHO cells, varying amounts of the pB_2-77 vector DNA were transfected into CHO cells. Figure 6 shows the structure of pB_2-77 CAT reporter construct. The resulting CAT specific activities were plotted against the input amounts of TGF-β2 CAT DNA (pB_2-77) in Figure 7.



pB_2-77

Figure 6. Schematic structure of pB_2-77 construct

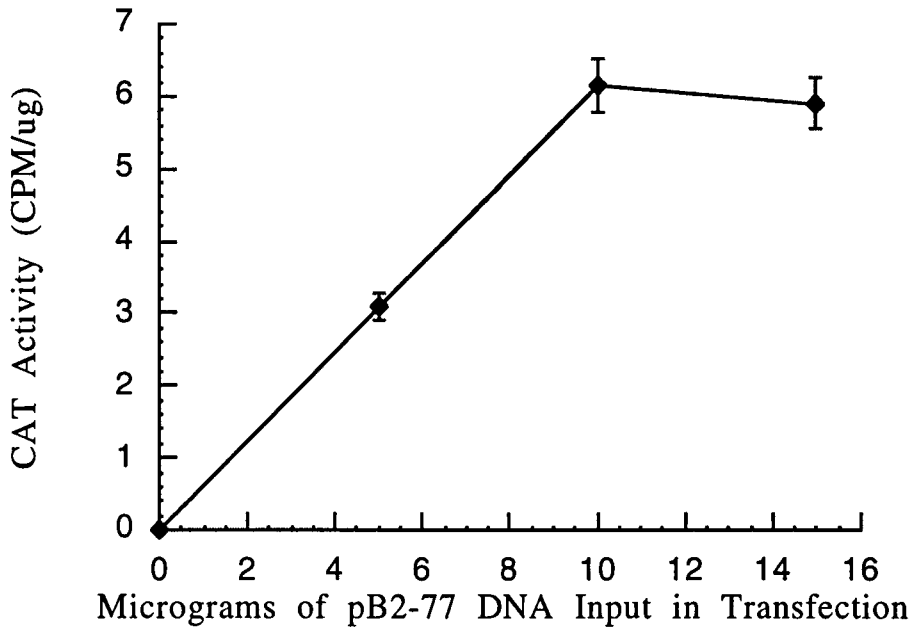


Figure 7. Titration of basal CAT expressing with TGF-β2 CAT DNA in CHO cells. Varying amounts of TGF-β2 CAT DNA, pB_2-77, (0, 5, 10 and 15 μg) were transfected into 100 mm dishes of CHO cells with 20 μl lipofectamine each. pUC 18 plasmid DNA was used to maintain the total DNA input at a constant 15 μg. After incubation for 48 hr, the transfected CHO cells were harvested, lysed and assayed as before. CAT activities were calculated as before and plotted against the amount of input pB_2-77 DNA. The experiment was repeated and also reproduced in smaller dishes (35 mm) of CHO cells.

The results (Figure 7) show that basal CAT expression is dependent on the amount of input TGF-β2 CAT DNA up to 10 μg of TGF-β2 DNA transfected in 100 mm dishes. Consequently, I chose 10 μg TGF-β2 CAT DNA for 100 mm dishes of CHO cells, and proportionally, 1 μg DNA for 35 mm dishes in the subsequent experiments [the area of a 35 mm dish is about one tenth of that of a 100 mm dish: $\pi(17.5)^2 \cong 1/10\ \pi(50)^2$]

Titration of ATF-a plasmid DNA with different amounts of reporter plasmid DNA

To study the ability of ATF-a to stimulate TGF-β2 CAT activity, ATF-a plasmid DNA was cotransfected with different amounts of the TGF-β2 CAT reporter plasmid DNA (pB_2-77) into CHO cells.

The results in Figure 8 show that ATF-a activated the expression of the TGF-β2 promoter in CHO cells. The maximal stimulation in this experiment was about 5 fold above the basal CAT activity, which was similar to that reported by Qing-qing Gong (6.6 ± 1.7 fold, Gong et al. 1995). The ATF-a-mediated activation of CAT expression from the TGF-β2 promoter was found to depend on either ATF-a or TGF-β2 input plasmid DNA amount. With higher amounts of both DNA's, a phenomenon like squelching appears to be occurring in the CHO cells. Consequently, the peak amounts of input ATF-a (5 μg) and pB_2-77 (5 μg) plasmid DNA were chosen for future experiments.

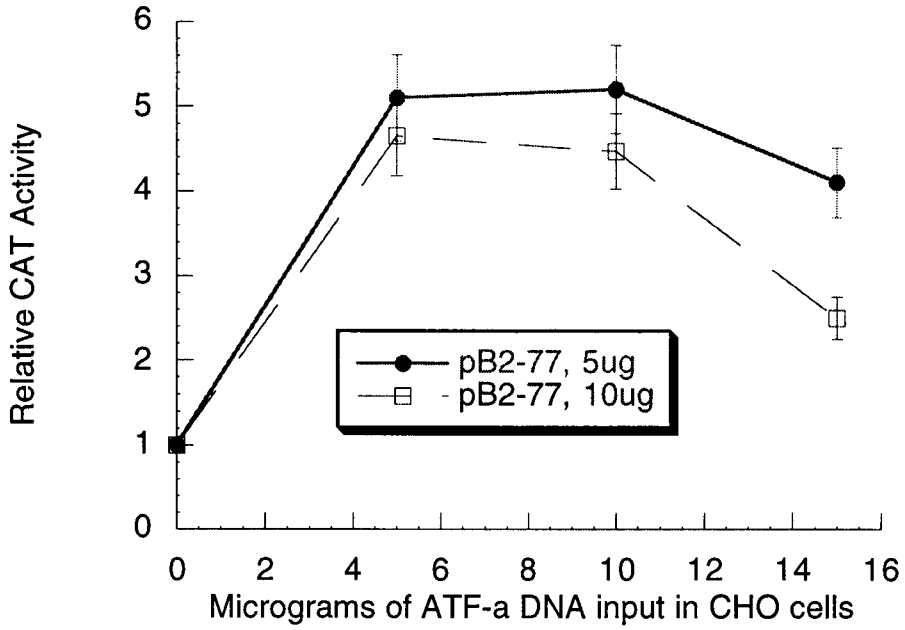


Figure 8. Titration of ATF-a plasmid DNA with different amounts of reporter plasmid DNA. Varying amounts of ATF-a plasmid DNA (5, 10, 15 μg) were cotransfected with different amounts of TGF-β2 CAT reporter plasmid DNA (pB_2-77) into 100 mm dishes of CHO cells with 20 μl lipofectamine each. After incubation for 48 hr, CHO cells were harvested, lysed and subjected to the CAT assay as before. The relative CAT activities are the ratio of CAT activities of test samples to the average of basal CAT activities (i.e. no ATF-a), and were plotted against the amount of input ATF-a DNA. pUC 18 was added to keep the total DNA input constant. This experiment was repeated at least three times in this format or in the smaller dishes (35 mm).

We also observed the activation by ATF-2 (about 5 fold) of the TGF-β2 promoter in CHO cells, which is similar to what Dr. Kim found in other cell lines (Kim et at, 1992). The pattern of activation of CAT expression by ATF-2 is very similar to that seen with ATF-a. Based on these results, it is possible that both factors have similar mechanisms of actions.

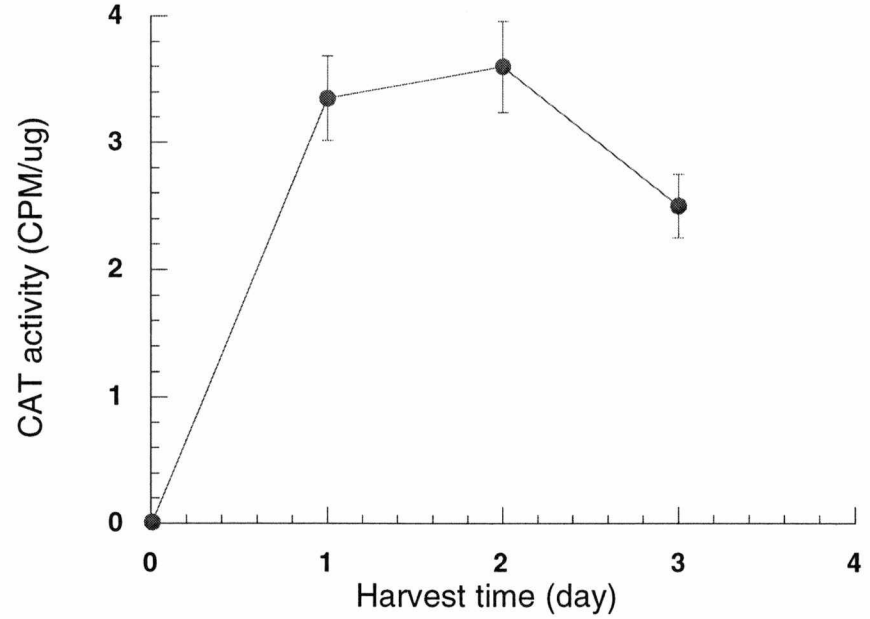
Time course of CAT expression after transient transfection

To further optimize transfection and assay conditions, we studied the time course of CAT activity after transient transfection with pB_2-77 with or without ATF-a or ATF-2/pRb combinations. The results were plotted against harvest time as shown in panel A of Figure 9. The absolute CAT expression level was roughly comparable on days 1 and 2, reached its peak on day 2, but it decreased by day 3.

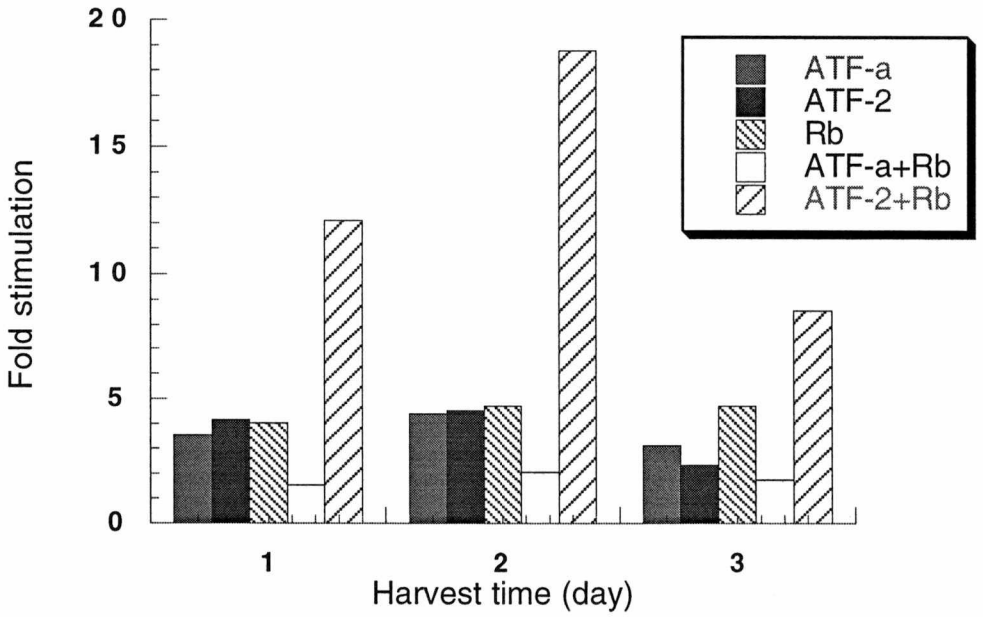
Furthermore, we studied the time course for the stimulated expression of pB_2-77 CAT by co-transfecting different combinations of ATF-a or ATF-2 and pRb (panel B of Figure 9). Basically, we found the highest CAT activities on day 2 in each case. With the combination of ATF-2 and pRb, which showed the highest stimulation, CAT activity was obviously higher on day 2 than on any other day. However, for the sets with ATF-a plus pRb and pRb alone, there were no significant differences with different harvest times. Based on all of these results, we chose day 2 (i.e. 48 hr) as the optimal incubation time after transfection.

Figure 9. Time course of CAT expression after transfection. A. Effect of varying harvest times on CAT activity in CHO cells transfected with the pB_2-77 reporter construct. One μg of pB_2-77 vector was transfected into each of 35 mm dishes containing 50 to 80% confluent CHO cells. After transfection, the cells were placed in the medium containing 10% fetal bovine serum, and incubated for varying times (24, 48 and 72 hr) before harvest. CAT activities from each cell lysate were measured as before and converted into cpm/μg by dividing by the protein amount present in the assay. **B. Time course of regulation of CAT expression after transfection.** One half μg of ATF-a/ATF-2/pRb plasmid DNA was cotransfected with 1.0 μg of TGF-β2 CAT reporter plasmid into 35 mm dishes of CHO cells. After incubation in a medium containing 10% fetal bovine serum for varying times (24, 48, 72 hr), CHO cells were harvested, lysed and subjected to the CAT assay as before. Fold stimulations are the ratio of CAT activities of test samples to the average of basal CAT activities. pUC 18 was added to keep the total DNA input constant. This experiment was repeated and each point is the average of at least four samples.

A. Effect of varying harvest time on CAT activity in pB2 77-transfected cells



B. Time course of CAT expression after transfection



Differential modulation of ATF-a and ATF-2's activities by pRb.

ATF-a and ATF-2 are highly homologous and both have high binding affinities for the ATF site (CGTCA); however, previous research work in Dr. Wicks' lab found that pRb can differentially modulate ATF-a and ATF-2's transcriptional activating effects on the TGF-β2 promoter, which implied a functional difference exists between them.

As shown in Table 2, I was able to reproduce the results reported by Gong et al. The relatively minor quantitative differences observed may be due to subtle differences in culture conditions.

Mapping the functional interaction sites for ATF-a and ATF-2 on pRb.

To map the functional interaction sites for ATF-a and ATF-2 on pRb, we purified a series of pSVE vectors expressing full length and truncated pRb mutants as well as the empty vector (Figure 10). We overexpressed ATF-a or ATF-2 with or without full length and truncated pRb mutants into CHO cells to examine changes in pRb's differential modulation of ATF-a and ATF-2, and by interference to deduce their interaction sites on pRb (Figure 11).

Table 2. pRb differentially regulates the ability of ATF-a and ATF-2 to stimulate the activity of the TGF-β2 promoter in CHO cells

Expression Constructs	*Fold Increase in CAT Activity*	*Results from Gong et al.*
ATF-2	4.3 ± 0.3 (12)	8.4 ± 1.0 (4)
ATF-a	4.0 ± 0.3 (12)	6.6 ± 1.7 (6)
pRb	4.2 ± 0.5 (22)	2.2 ± 0.4 (4)
ATF-2 with pRb	18.4 ± 1.2 (22)	22.2 ± 4.6 (4)
ATF-a with pRb	1.9 ± 0.3 (22)	3.9 ± 1.3 (8)

Note: The pB_2-77 CAT reporter construct was cotransfected with each expression vector into 50-80% confluent CHO cells. The values shown represent the average CAT activity ± SD relative to that observed with cells transfected with reporter construct alone. The number of observations is given in parentheses.

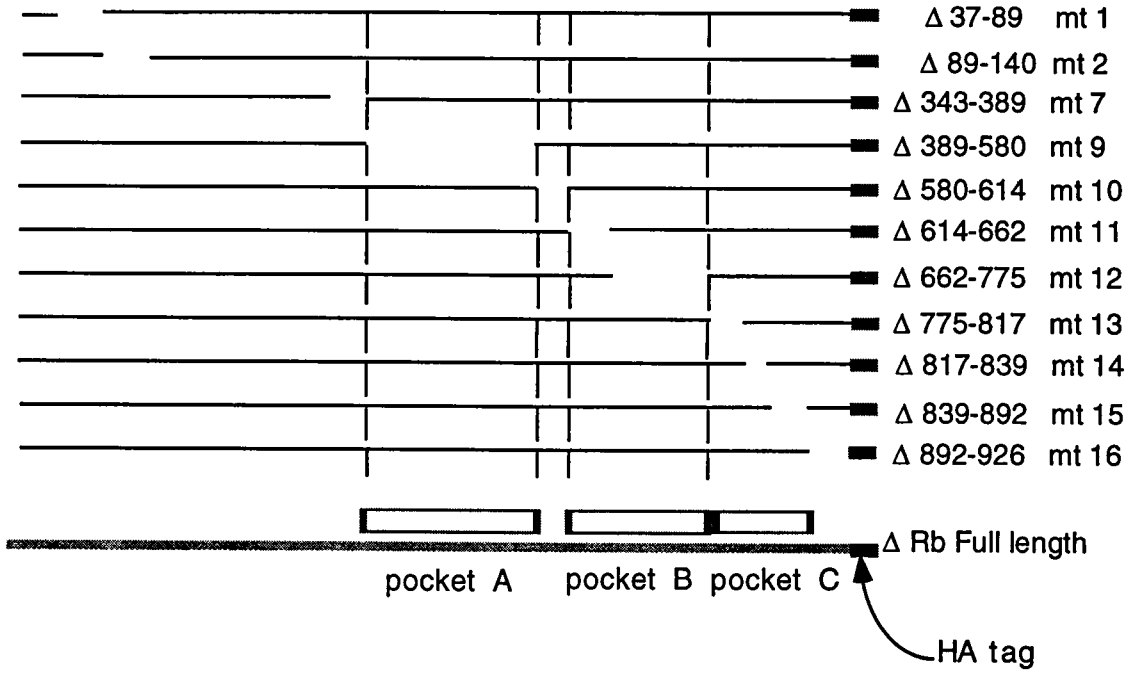
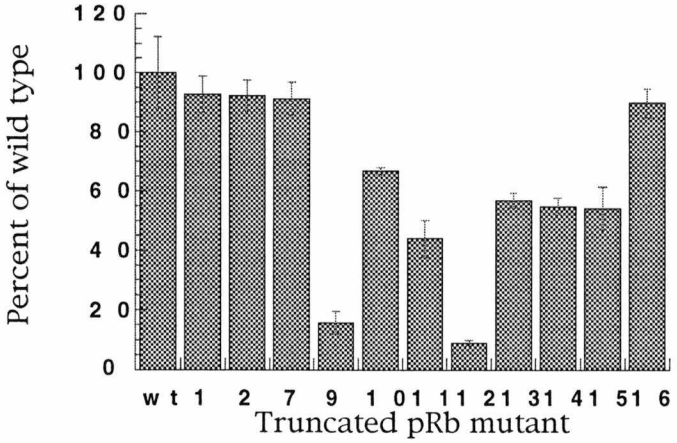


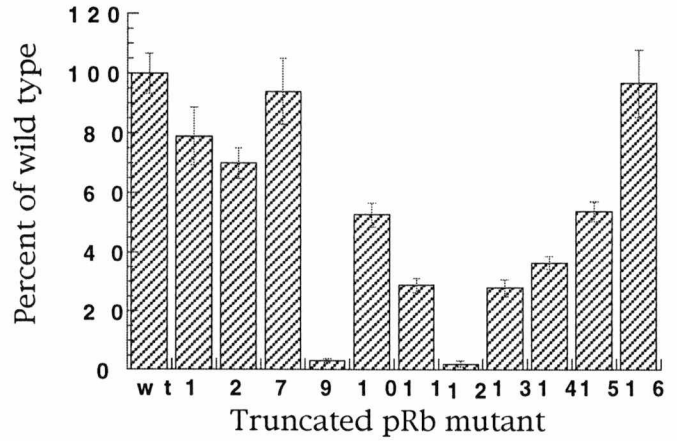
Figure 10. Schematic representation of deletion mutants of pRb expression vectors used in this study. The coding region of pRb is represented by the line at the bottom, locations of pockets A, B and C are represented by the bottom boxes, and the synthetic epitope tag is represented by the shaded box at the carboxyl terminus. Each mutant is depicted as a gapped line, with the size and location of the gap proportional to the site of the mutation. Each mutant is designated by the location of the first of the two codons that were converted into an XhoI restriction site (CTCGAG) prior to deletion of sequences between adjacent XhoI mutant sites.

Figure 11. Comparison of the activities of a series of truncated pRb mutants with wild type pRb on TGF-β2 CAT expression in CHO cells. A. The effect of truncated pRb mutants on basal CAT expression. One half μg of pRb wt or mts were cotrans-fected with 0.5 μg TGF-β2 reporter construct into 50 to 80% confluent CHO cells in 35 mm dishes. After incubation for 48 hr, CHO cells were harvested, lysed and subjected to the CAT assay as before. The CAT activities were calculated as before. The percentages of wild type are the ratio of CAT activities of mts to that of wt. pUC 18 was used to maintain the input DNA amount constant. **B. The effect of truncated pRb mutants on ATF-2's stimulation of CAT expression.** One half μg of wt or mts pRb were cotransfected with 0.5 μg of ATF-2 plasmid DNA and 0.5 μg of TGF-β2 construct. See A. for further details. **C. The effect of trun-cated pRb mutants on ATF-a's stimulation of CAT activity.** One half μg of pRb wt or mts were cotransfected with 0.5 μg of ATF-a plasmid DNA and 0.5 μg of TGF-β2 construct. See A. for further details. The percentages of wt were calculated, but since the pRb inhibited ATF-a's activation instead of enhancing it, all values of % of wt were set up as negative values. All three parts of this experiment have been repeated and each point is the average of at least five observations.

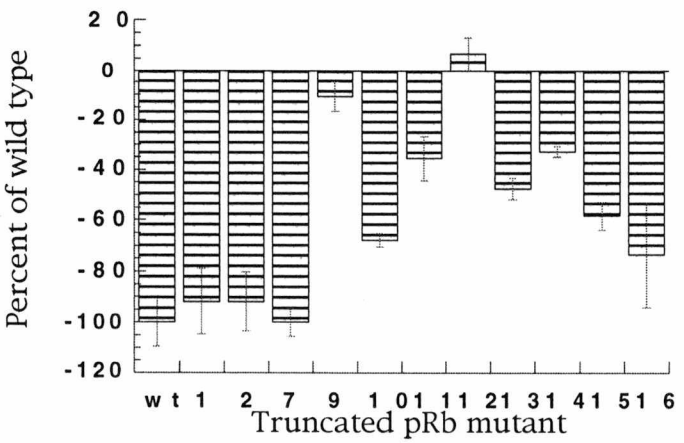
A. The effect of truncated pRb mutants on basal CAT expression



B. The effect of truncated pRb mutants on ATF-2's stimulation of CAT activity



C. The effect of truncated pRb mutants on ATF-a's stimulation of CAT activity



pRb alone

Transfected full length pRb caused a 4.2 ± 0.5 (n=22) fold stimulation of CAT activity over basal (the average CAT activities from CHO cells co-transfected with empty vector pSVE and TGF-β2 CAT is used as basal), which was similar to that (Park et al., 1994) found in CCL-64 cells (mink lung epithelial). When the same experiments were done with truncated pRb mutants, we found (Figure 11.A) that an intact A/B pocket was required for pRb's stimulation. Truncated mutants 9, 11 and 12, which have deletions in the A/B pocket, exhibited only 16, 44 and 9% of activity of wild type activity, respectively. The C pocket containing many phosphorylation sites is also important, since a significant decrease in activity (57, 55, 54% of wt) was found in C pocket-deleted mutants (mts 13, 14, 15).

Mutant 10 with the insert domain between A and B pocket deleted exhibited 67% of wt activity, which is consistent with the report that deletion of entire insert domain inactivates the protein-binding function of A/B pocket (Huang et al., 1990; Qian et al., 1992), but it also has been reported that replacement of the insert domain with a random amino acid sequence of identical length restores A/B pocket function (Huang et al., 1990; Qian et al., 1992). This implies that the insert domain functions as a "spacer" between the A and B domains, providing a physical separation necessary for the formation of the A/B pocket. Other mutants

tested containing intact A/B and C pockets (mts 1, 2, 7 and 16) were not significantly different from the wt pRb activity.

pRb with ATF-2

Overexpressing ATF-2 stimulated CAT activity 4.2 ± 0.4 (n=9) fold over the reporter vector alone (Table 2). Cotransfection of wt pRb with ATF-2 further stimulated CAT expression to 18.4 ± 1.2 (n=22) fold over basal, which was about the theoretical level for additivity (fold of ATF-2's activation multiplied by the fold increase caused by pRb alone).

Truncated pRb mutants exhibited stimulation of ATF-2's actions in CHO cells overexpressing ATF-2 (Figure 11 B) similar to those on endogenous ATF's, (i.e., overexpression of pRb alone) (Figure 11 A).

- An intact A/B pocket is required, since mutants 9, 11 and 12 showed the largest loss of activity (only 3, 29 and 2% of wt, respectively).
- The C pocket is also important, since mutants 13, 14 and 15 showed a significant loss of stimulation (28, 36 and 54 % of wt, respectively).
- Other mutants containing intact A/B and C pockets (mt 1, 2, 7 and 16) exhibited activities (79, 70, 94 and 97 %) roughly similar to wild type (100%).

pRb with ATF-a

As observed previously (Gong et al., 1995), co-expression of pRb did not stimulate CAT expression in the presence of ATF-a, but inhibited it (Figure 8 C). Cells cotransfected with wt pRb and ATF-a only exhibited a 1.9 ± 0.3 (n=22) fold stimulation over basal, which is lower than that seen with ATF-a alone (4.0 ± 0.3, n=12)

Interestingly enough, the truncated pRb mutants cotransfected with ATF-a showed a similar pattern of effects on ATF-a as with ATF-2, but in the opposite direction, dysfunctional mutants of pRb release the inhibitory effect of wt pRb (on ATF-a) instead of having a lower stimulatory effect of wt pRb (on ATF-2).

- An intact A/B pocket is required, since mts 9, 11 and 12 showed the largest loss of inhibitory activity. Mt 9 and 11 have only 11 and 36 % of wt activity, respectively while mt 12 completely lost its inhibitory activity (3.5 ± 0.2 for ATF-a with mt 12 vs. 3.4 ± 0.3 for ATF-a with the empty vector pSVE).
- The C pocket is also important, since deletion mutants 13, 14 and 15 showed a decrease in inhibitory activity (48, 33 and 58 % of wt, respectively).
- Other mutants containing intact A/B and C pockets (mt 1, 2, 7 and 16) exhibited inhibitory activities (92, 92, 100 and 74 %) very close to the wild type.

Although the transcriptional activities of ATF-a and ATF-2 are differentially modulated by co-expression of pRb, they appear

to share the same or similar interacting sites on pRb. Dr. Wicks tried to find evidence of the association of ATF-a with pRb under various conditions in vitro, but all the results have turned out to be negative. To date, there is no evidence that pRb binds to ATF-a directly, but pRb's stimulation on TGF-β2 has been shown to be exerted through the ATF regulatory element (Park et al., 1994; Gong et al., 1995); thus, the regulation could well be through a protein specifically binding to this site, for example, ATF-2 and/or ATF-a. On the other hand, adaptor proteins may mediate this interaction (e.g. hbrm or the cellular homolog of E1a etc.).

The basis for the different responses of ATF-2 and ATF-a to pRb's regulation is not clear. Since both ATF-2 and ATF-a can functionally interact with pRb, it is assumed that the interaction site should be in a conserved region between the two proteins. The non-conserved regions may play a role in determining the differential responses to pRb's actions.

Wang (Wang et al., 1994) has suggested that pRb functions as a match-maker: it possibly matches the C-pocket binding protein, tyrosine kinase, with its protein substrate. It is possible that the differential modulation of ATF-2 and ATF-a actions by pRb might be due to the different effects and/or different sites of phosphorylation by this nuclear tyrosine kinase (both ATF-a and ATF-2 contain 2 tyrosine residues in non-conserved regions). However, this hypothesis has two major questions to be answered: 1. Whether ATF-a and ATF-2 are (primary) substrates of the c-Abl tyrosine kinase? 2. Whether we can find (immunoprecipitate) a

pRb complex with c-Abl and ATF-a or ATF-2 associated at the same time in vivo?

Among other possible mechanisms are that pRb may affect the dimerization of ATF-a and ATF-2. This effect may due to changes in dimerization partner or lack of access of one or more partner. For example, both ATF-2 and ATF-a can form homodimers and heterodimers, and they share some partners from the Jun/Fos family but some of them, for example c-Fos, can associate with only one of them (ATF-a) to form an active complex. There is evidence that changes in the dimerization of ATF-a and ATF-2 can have an effect on DNA binding affinity or activation efficiency (Chatton et al., 1994). So the inhibition of ATF-a action by pRb could be explained hypothetically by suggesting that the interaction between pRb and ATF-a could make ATF-a inaccessible to c-Jun, a heterodimer partner of ATF-a which may be required for ATF-a's transcriptional activation. Furthermore, overexpressed ATF-a competes with cellular ATF-2 for interaction with pRb; thus, it inhibits the stimulation of pRb through cellular ATF-2 (i.e. the effect seen with pRb alone).

As stated earlier the effect of pRb can be exerted through direct or indirect interactions with transcription factors. A domain of pRb and TBP (TATA-binding protein of TFIID) has been shown to exhibit considerable homology. Transcription factors that bind pRb, such as PU1, c-Myc, E1A and IE2 protein, also bind TBP. It was suggested that one function of pRb may be to block the

communication between transcription factors (i.e. ATF-a and/or ATF-2) and TBP.

Table 3 shows the summary of the effects of truncated pRb mutants on the stimulation of TGF-β2 CAT expression. The t-test was used to determine whether the mean levels of CAT expression regulated by pRb mt differed significantly from that of pRb wt. If the computed t exceeds the tabular value (Tallarida and Murray, 1981) of a 99% confidence or more ($p \leq 0.01$), then the mean expression level with a pRb mt was taken to differ significantly from pRb wt. P values between 0.01 and 0.05 are considered to be of borderline significance.

Table 3. Summary of the effects of truncated pRb mutants on the stimulation of TGF-β2 CAT expression.

pRb Construct	Coding Region	Deletion Location	pRb		
			alone	with ATF-2	with ATF-a
wt	full-length	none	100	100	-100
mt1	D37-89	N-term Domain	93***	79**	-92***
mt2	D89-140	N-term Domain	92***	70**	-92***
mt7	D343-389	N-term Domain	91***	94***	-100***
mt9	D389-580	Pocket A	16*	3*	-11*
mt10	D580-614	Insert Domain	67*	53*	-68*
mt11	D614-662	Pocket B	44*	29*	-36*
mt12	D662-775	Pocket B	9*	2*	7*
mt13	D775-817	Pocket C	57*	28*	-48*
mt14	D817-839	Pocket C	55*	36*	-33*
mt15	D839-892	Pocket C	54*	54*	-58*
mt16	D892-926	C tail	90***	97***	-74**

Note: The above values are the percentage of the activity observed with pRb. The activities of wt pRb were set as 100% except that with ATF-a was set as -100% due to the inhibitory effects of wt pRb. * indicates a statistically significant difference ($P \leq 0.01$) between the value of mutant and that of wt; ** indicates a statistically borderline difference ($0.01 < P \leq 0.05$) between the value of mutant and that of wt; *** indicates that no significant difference ($P > 0.1$) exists between the value of mutant and that of wt. The quantity t is computed from the formula: $t = (X - Y)/ (Sx^2/Nx + Sy^2/Ny)^{1/2}$. X represents the mean level of pRb wt, Y represents the mean levels of pRb mts; Nx and Ny represent the number of test samples of pRb wt and mts, respectively; Sx and Sy represent the standard deviations of pRb wt and mts, respectively. The magnitudes of t were then compared with its distribution for a specified level of confidence and a number of degrees of freedom υ given by: $\upsilon = [Nx + Ny] -2$.

Level of expression of wt and truncated pRb mutants in transfected CHO cells.

One of the obvious possible explanations for the dysfunction of some of the pRb mutants is that those mutants might not be expressed properly in CHO cells. To test this, a hemagglutinin (HA) epitope-tagged form of pRb was used so that all of the deletion mutants could be analyzed by using a MAb directed against the HA epitope (12CA5). It also has the virtue of allowing one to distinguish the plasmid-encoded pRb and mutants from the normal pRb present in the cells.

Nuclear extracts were prepared from transfected CHO cells and the proteins separated on 10% SDS-polyacrylamide gels. After transfer to nitrocellulose, the membranes were probed with MAb 12CA5 (Figure 12 A). All of the deletion mutants produced an appropriate-sized protein (Table 4) at approximately the same level of expression as the wt (Figure 12 B) except for mutant 12, which was expressed about 23% as abundantly as the wild-type proteins in this blot. However, when we repeated this experiment three times to test different cell lysates from CHO cells transfected by pRb mt 12, the level of its expression was as high as 95%, 95% and 91% of the pRb wt protein. Therefore, we discarded the data (23%) of mt 12 expression level from that particular experiment, which may have been caused by technical mishandling, and took the average of the other three expression levels, which was 94% of the wt pRb. As expected, non-transfected CHO cells showed no

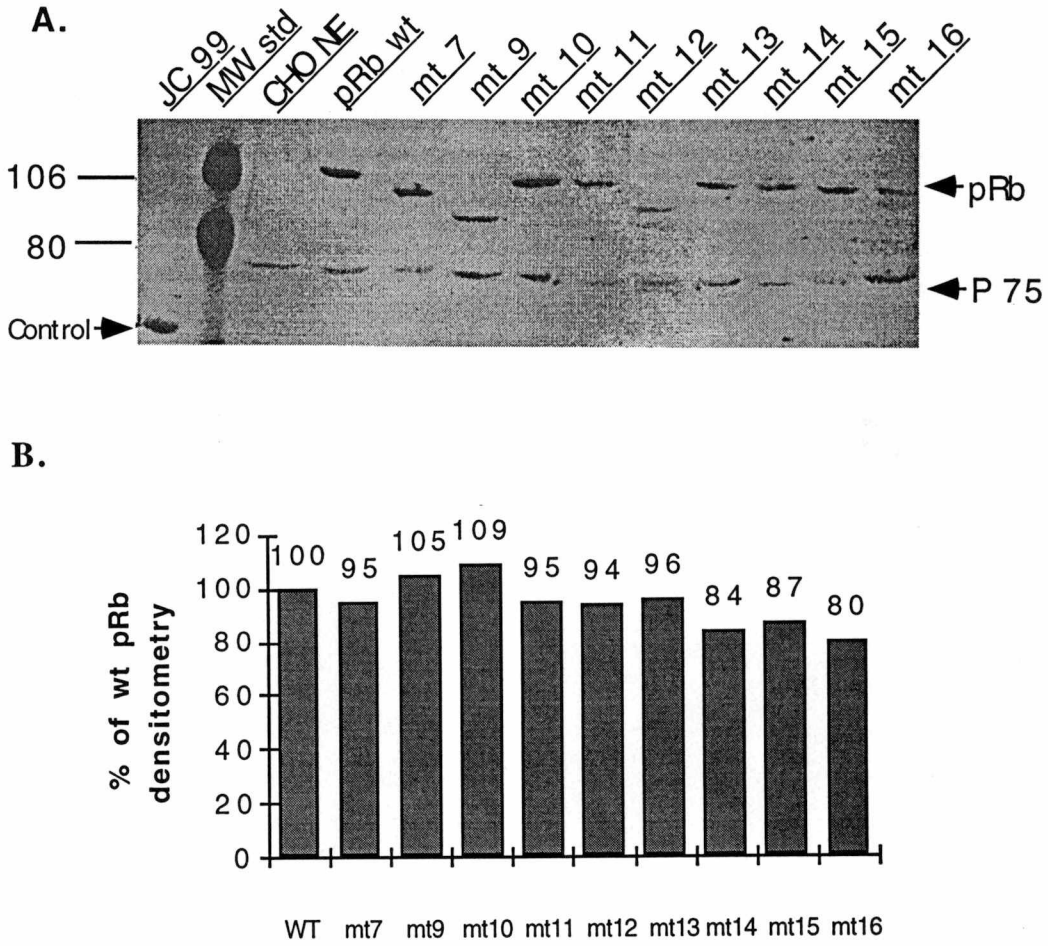


Figure 12. Expression of wild type and mutant pRb in CHO cells. **A)** Wt and mt pRb were detected by western blotting with anti-HA epitope MAb 12CA5. JC 99, a HA-tag containing protein, was used as a positive control. Numbers at the left indicate molecular weights (in kilodaltons) of the pre-stained marker proteins. The upper bands which react with the antibody are the expressed pRb wt or mts; and the lower band, referred as P75, is due to a cellular protein presumably containing an HA-like sequence in CHO cells. **B)** The densitometry of the bands due to pRb wt and mts is shown. The value for the wt pRb was set as 100%. The percentages shown above each bar are averages from different blotting experiments.

cross-reacting proteins in the region from 80 kD to 130 kD.

A lower molecular weight band (about 75 kD) was present in all CHO nuclear extract lanes including samples from untransfected cells, and its abundance was essentially constant. It appears likely that this unknown protein present in the nuclei of CHO cells contains a sequence closely related to that of the hemagglutinin epitope.

We also tested the expression of pRb wt and mt 12 when they were cotransfected with ATF-a / ATF-2. The results (Figure 13) show no apparent change in the expression of pRb wt and mt 12; therefore, ATF-a and ATF-2 appear to have no effect on the expression of foreign pRb proteins in CHO cells.

Thus, the effects of ATF-a and ATF-2 observed in the presence of pRb cannot be due to changes in the level of expression of pRb or its mutants (at least mt 12). It seems likely, therefore, that changes in the activity of pRb mutants are due to the absence of regions that are crucial for normal pRb functions. The following table shows the comparison of the expected and observed molecular weight of pRb full length and truncated mutants.

pRb also differentially regulates ATF-a and ATF-2's activation of TGF-β2 CAT expression in SKUT-1 cells

To further study the ability of pRb to differentially regulate the transcriptional actions of ATF-a and ATF-2, we conducted the same experiments in SKUT-1 cells. These cells were isolated

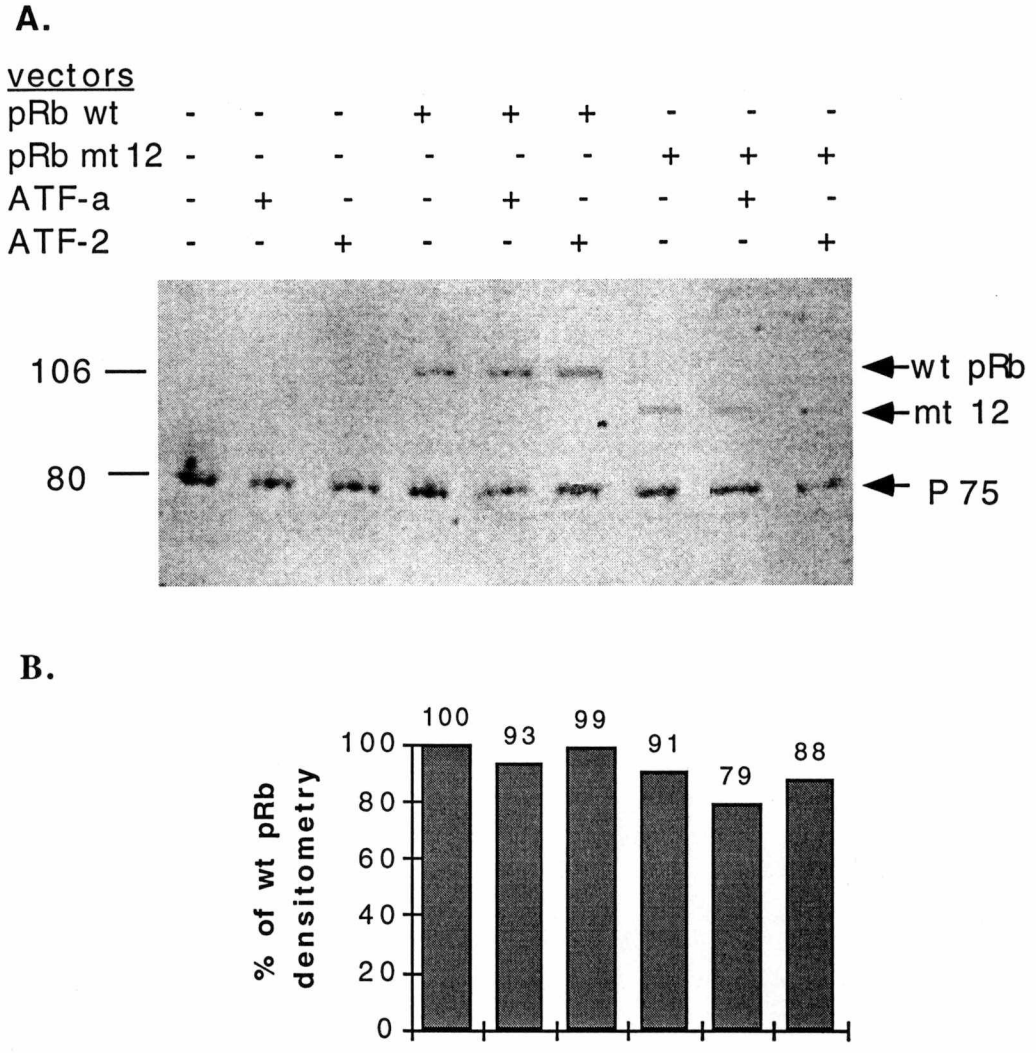


Figure 13. Cotransfection of ATF-a and ATF-2 has no effect on the expression of wild type and mutant pRb in CHO cells. A. C H O cells were transfected by pRb wt and mt 12 with or without ATF-a or ATF-2. Cell lysates were subjected SDS-PAGE, and probed by western blotting with anti-HA epitope MAb 12CA5. The upper bands of pRb samples are due to the introduced pRb wt or mt 12; and the lower bands, referred as P75, are due to a cellular protein containing an HA-like sequence. B. The densitometry of the bands represented pRb wt and mts. The value for the wt pRb was set as 100%. The percentages shown above each bars are averages from different blotting experiments.

Table 4. Comparison of the expected and observed molecular weight of pRb full length and truncated mutants from western blotting experiments.

CONSTRUCTS	*Expected MW (kD)*	*Measured MW (kD)*	*Difference (kD)*
pRb wt	117	117	0
mt 07	111	114	3
mt 09	92	93	1
mt 10	112	119	7
mt 11	111	112	1
mt 12	102	102	0
mt 13	111	112	1
mt 14	112	111	1
mt 15	110	111	1
mt 16	112	116	4

from human uterine mixed mesodermal tumor cells and no detectable pRb exists in this cell line (Lucas et al., 1995). The same conditions used for CHO cell transfection were chosen for transfecting SKUT-1 cells.

As shown in Figure 14, the same pattern of stimulation of CAT expression from TGF-β2 promoter was seen with SKUT-1 cells as with CHO cells (Figure 9 B). Although the basal activity of TGF-β2 CAT expressed in SKUT-1 cells (2.8±0.4 CPM/μg) was slightly lower than in CHO cells (4.0±0.6 CPM/μg), cotransfection of ATF-a or ATF-2 generated very similar stimulation of the TGF-β2 promoter. It is obvious that pRb is not necessary for ATF-a or ATF-2 to exert their effect on TGF-β2 promoter activity. Since there is no detectable endogenous source of pRb in SKUT-1 cells, that might account for the stronger stimulation seen with pRb alone in SKUT-1 cells (11.3 fold) than that in CHO cells (4.2 fold). Most impressively, pRb's differential modulation of ATF-2 and ATF-a actions in SKUT-1 cells is essentially identical to that seen in CHO cells. These results suggest that the same mechanism of differential regulation may be involved in both cell types, although the mechanism itself remains to be determined. Table 5 provides a comparison of the results from transfection experiments done in CHO cells and SKUT-1 cells.

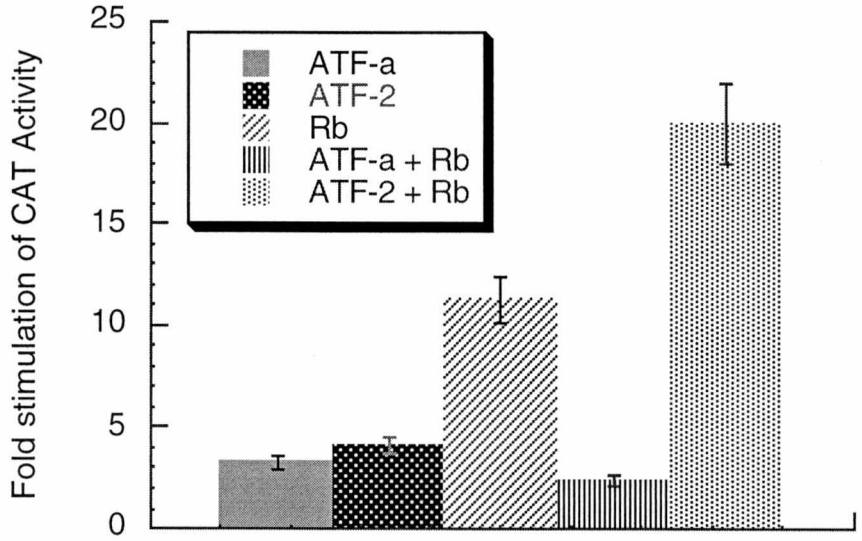


Figure 14. pRb differentially modulates the transcriptional activating effects of ATF-a and ATF-2 in SKUT-1 cells. One half μg of ATF-a/ATF-2/pRb plasmid DNA was cotransfected with 1.0 μg of TGF-β2 CAT reporter plasmid into 35 mm dishes of 50-80% confluent SKUT-1 cells. After transfection, SKUT-1 cells were incubated in the medium containing 10% fetal bovine serum for 48 hr, then SKUT-1 cells were harvested, lysed and subjected to CAT assay as before. Fold stimulations are the ratio of CAT activities of test samples to the average of basal CAT activities. pUC 18 was added to keep the total DNA input constant. This experiment was repeated and each point is the average of at least four samples, and error bars indicate the standard deviation of samples.

Table 5. Summary of pRb's differential regulation of ATF-a and ATF-2's activation of TGF-β2 CAT expression in CHO and SKUT-1 cells.

Expression Constructs	*Fold Increase in CAT Activity in CHO cells*	*Fold Increase in CAT Activity in SKUT-1 cells*
ATF-2	4.3 ± 0.3	4.1 ± 0.3
ATF-a	4.0 ± 0.3	3.3 ± 0.3
pRb	4.2 ± 0.5	11.3 ± 0.5
ATF-2 with pRb	18.4 ± 1.2	19.9 ± 1.1
ATF-a with pRb	1.9 ± 0.3	2.4 ± 0.3

Note: The pB_2-77 CAT reporter construct was cotransfected with each expression vector into 50 to 80% confluent CHO cells or SKUT-1 cells. The values shown represent the average CAT activity ± SD relative to that observed with cells transfected with reporter construct alone. The basal activity of reporter construct TGF-β2 CAT DNA is 4.0 ± 0.6 cpm/μg in transfected CHO cells, and 2.8 ± 0.4 cpm/μg in SKUT-1 cells.

CHAPTER IV
CONCLUSIONS AND SUMMARY

1. Optimal transient transfection conditions using lipofectamine as transfection inducer for the introduction of a foreign TGF-β2 CAT reporter construct in CHO cells have been selected.

2. The activation by co-transfected ATF-a, ATF-2 and the differential regulation mediated by pRb on the TGF-β2 promoter have been explored further in terms of varying input DNA concentrations and the time course for the expression of the regulated TGF-β2 CAT. Although the regulatory pattern was similar on days 1, 2 and 3, the greatest response was observed 48 hr after transient transfection.

3. The functional interaction sites for both ATF-2 and ATF-a on pRb have been probed by cotransfecting a series of truncated pRb mutants with ATF-2 or ATF-a. Our results demonstrate that ATF-2 and ATF-a share the same interaction sites on pRb in vivo: primarily in the so called A/B pocket domains, but the C pocket domain of pRb also appears to be involved.

4. By using an antibody against the HA tag located on the C-terminus of pRb, the levels of expression of all mutant forms of pRb have been tested and shown to be similar to wt pRb. Overexpression of ATF-2 and ATF-a did not effect the levels of expression of wt and mt pRb's.

5. We studied the differential modulation of the transcriptional activations generated by ATF-a and ATF-2 by pRb in CHO cells as well as in an alternative cell line, The human uterine mesodermal tumor cell line, SKUT-1. The same regulatory pattern as in CHO cells has been observed, although there were minor variations in the degree of stimulation/inhibition. These results also demonstrate that pRb is not required for the transcriptional actions of ATF-a and ATF-2 on the TGF-β2 promoter.

CHAPTER V
PERSPECTIVE AND FUTURE STUDIES

The differential regulation mediated by pRb on the TGF-β2 promoter has been explored, and ATF-a and ATF-2 were found to occupy the same interaction sites on the pRb molecule when they were differentially regulated. Since ATF-a and ATF-2 are very homologous in amino acid sequence and closely related in transcriptional function, it raises the question as to how these related proteins can be differentially regulated by pRb. To address this question, it is necessary to determine what the pRb's interaction sites on ATF-a and ATF-2 are. It is possible that ATF-a and ATF-2 use different motifs (non-conserved regions) to interact with pRb. Many proteins bind to pockets A and B of pRb, however, it has been suggested that different motifs were involved in binding to the A and B pockets of pRb, for example, T antigen of SV40, adenovirus E1A oncoprotein and other oncoproteins bind pRb via a conserved Leu-x-Cys-x-Glu motif (Kim et al., 1994); transcription factor E2F1, in contrast, uses a unique 18-amino acid motif to interact with pRb; and pp1α uses a different, large, C-terminal region to bind pRb (Lee et al., 1994). If pRb's regulation sites on ATF-a and ATF-2 are in the unconserved regions of these two, it may imply different binding regions of ATF-a and ATF-2 are involved and this could be the basis for differential regulation,

directly or by way of different bridge proteins involved in each case. To test this hypothesis, cotransfection of truncated mts of ATF-a and ATF-2 with pRb into the CHO and/or SKUT-1 cells could determine the binding sites for pRb, and help to further elucidate the mechanism of pRb's differential regulation of ATF-a and ATF-2.

The fact that ATF-2 and ATF-a activate TGF-β2 promoter in the similar pattern in CHO cells as in pRb-deficient SKUT-1 cells implies that pRb is not required for basal transcription activities of ATF-a and ATF-2. It is possible that ATF-a and ATF-2 competitively interact with pRb, and the interactions with pRb adjust their dimerization state, causing either changes between homodimer and heterodimer or changes in the heterodimeric partner. Since it has been suggested that changes in ATF-a and ATF-2 dimerization affect their DNA binding affinities and specificity (Chatton et al., 1994; Benbrook et al., 1990), this putative adjustment resulting from interaction with pRb may cause differential effects on ATF dimerization. For example, ATF-2 might form an active dimer, while ATF-a might form an inactive one (or cannot be dimerized) on the TGF-β2 promoter. To test the effects of interactions with pRb on the dimerization of ATF-a and ATF-2, the gel shift assay using a ^{32}P-labeled probe containing an ATF site can be performed to test transfected cell nuclear extracts. The comparison of the pattern of complexes formed ±pRb with cotransfected ATF-a or ATF-2 could suggest that changes in dimer species as well as in the DNA-binding affinities of different dimer species are taking place.

Another interesting question comes from the involvement of pRb's C-pocket in the interaction with ATF-a and ATF-2. Does it just mean that ATF-a and ATF-2 are large enough to occupy two distinct domains of pRb, or does it imply some other proteins' interactions with pRb are required for its regulation on ATF-2 and ATF-a? It is suggested that pRb may function as a match-maker, using the A/B pockets to bind one protein and the C pocket to another, thus bringing two proteins together (Wang et al., 1994). If this is true, pRb may bring the C pocket binding proteins (CBP) and ATF-a/ATF-2 together, and the CBP may cause the differential regulation due to the differential effects of this binding on ATF-a and ATF-2. To date the only known CBP is c-Abl nuclear tyrosine kinase. Since pRb cannot be phosphorylated by c-Abl, the binding to pRb may facilitate c-Abl's interaction with its potential substrates, A/B pockets binding proteins (Welch and Wang, 1995). To test this hypothesis, we are going to try to disrupt pRb's interaction with CBP by cotransfection of pRb with its C-pocket fragment, a dominant-negative inhibitor of pRb. It is expected that the disruption of the interaction between the putative CBP and ATF-a/ ATF-2 abolishes pRb's differential regulation. On the other hand, we can test if ATF-a and ATF-2 are reasonably good substrates of c-Abl tyrosine kinase by an in vitro phosphorylation assay, since both contain two tyrosine residues in their molecules, one is in the conserved region, the other in unconserved regions.

In the transfection experiments, we are assuming that foreign pRb in the transfected cells is unphosphorylated due to the

hypothesis that the overexpression of pRb in the cell may saturate the endogenous phosphorylation machinery (Qian et al., 1992). However, the cell-cycle dependent phosphorylation of pRb does affect its binding to transcription factors and its biological function--repressing cell cycle progression (Mancini et al., 1994); so it is worth determining the phosphorylation state of pRb in the transfected nuclear extracts by ^{32}P-labeled culture and immunoprecipitation. To test the effect of changing phosphory-lation state on pRb's function, we are going to leave serum out after transfection to examine the effect of pRb's differential regulation of ATF-a and ATF-2 in non-cycling cells. Since it has been suggested that pRb is a target of the cyclin D1/cdk4 pathway (Wang et al., 1994), the foreign pRb could be phosphorylated by activated cyclin D1/cdk4, and the phosphory-lation may affect pRb's differential regulation of ATF-2 and ATF-a.

Recently, ATF-2 has also been found to be a direct target of a signal transduction pathway, ending with phosphorylation by JNK (SAPK) of two closely spaced threonine residues. Since ATF-a has the same two crucial threonines and an identical amino acid sequence near these two threonines, it will be reasonable to study the phosphorylation of ATF-a by the same or similar kinases, and the functional modification in vivo by using factors known to stimulate the putative kinases. Therefore, the phosphorylation of ATF-a by the signal transduction pathway may provide clues as to the regulation of ATF-a's function.

Finally, what is the biological significance of pRb's differential regulation on two related transcription factors: ATF-a and ATF-2? To address this question, first we need to understand their individual biological functions. It has been suggested that pRb is important in repressing cell proliferation (Lee et al., 1992), and ATF-2 is required for skeletal and central nervous system development (Reimold et al., 1996); however, the biological function of ATF-a remains elusive. Thus, observations with mice or other animals carrying a germ line mutation in ATF-a will be extremely valuable to understand the biological function of ATF-a. The tissue distribution may provide some hints on the biological role of pRb's differential regulation on ATF-2 (Maekawa et al., 1989) and ATF-a (Pescini et al., 1994). Both ATF-a and ATF-2 are tissue-specifically distributed, and their distributions in human tissues are different: ATF-2 is relatively abundant in brain tissue, while ATF-a is expressed primarily in skeletal muscle tissue; and in the liver tissue, ATF-2 is expressed and ATF-a is not. Thus, pRb may interact with ATF-a and ATF-2 in a tissue-specific way: enhancing ATF-2's transcriptional activation on the CRE/ATF element mediated gene expression in some tissues and inhibiting that of ATF-a in the other tissues. Therefore, this differential regulation by pRb may imply a functional switch to determine the gene expression in different tissues, and help us to understand how stress/DNA damage may cause the cell either to exit the cell cycle or undergo apoptosis.

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APPENDIX

APPENDIX Expression and Regulation of pB_2-77 Construct in Transfected CHO Cells

Transfection Vectors	Ave. Activity (CPM/ug)	Standard Deviation	Ave. Fold Stimulation	Standard Deviation	n	percent fold with wt	t value (t-test)	P	Significance
pB2-77 alone	4.02	0.58	1.00	0.11	22				
pB2-77+Rbwt	16.63	1.71	4.18	0.51	22	100.0			
pB2-77+pSVE	3.90	0.67	0.97	0.17	9	0.0			
pB2-77+Rbmt01	16.63	1.56	3.95	0.25	5	92.8	1.45	>0.1	No
pB2-77+Rbmt02	16.53	1.47	3.93	0.22	5	92.2	1.70	>0.1	No
pB2-77+Rbmt07	15.87	0.92	3.90	0.23	6	91.2	1.96	.1>P>.05	
pB2-77+Rbmt09	5.93	0.45	1.48	0.15	6	15.9	21.47	<0.01	Yes
pB2-77+Rbmt10	12.62	0.17	3.11	0.05	6	66.7	9.60	<0.01	Yes
pB2-77+Rbmt11	9.77	1.49	2.38	0.26	7	43.9	12.27	<0.01	Yes
pB2-77+Rbmt12	5.15	0.16	1.26	0.04	5	9.2	26.38	<0.01	Yes
pB2-77+Rbmt13	11.58	0.80	2.79	0.10	5	56.7	11.69	<0.01	Yes
pB2-77+Rbmt14	11.05	0.39	2.73	0.12	6	54.7	12.18	<0.01	Yes
pB2-77+Rbmt15	11.00	1.24	2.70	0.30	6	54.0	8.95	<0.01	Yes
pB2-77+Rbmt16	15.64	0.83	3.85	0.21	6	89.7	2.43	.05>P>.01	

Transfection Vectors	Ave. Activity (CPM/ug)	Standard Deviation	Ave. Fold Stimulation	Standard Deviation	n	percent fold with wt	t value (t-test)	P	Significance
pB2-77+ATFa	15.50	1.24	4.05	0.26	12	N.A.			
pB2-77+ATFa+Rbwt	7.49	0.73	1.89	0.26	22	-100.0			
pB2-77+ATFa+pSVE	13.64	1.28	3.38	0.30	9	0.0			
pB2-77+ATFa+Rbmt01	8.47	1.08	2.01	0.39	4	-91.9	-0.59	>0.1	No
pB2-77+ATFa+Rbmt02	8.38	0.97	2.01	0.31	5	-91.9	-0.79	>0.1	No
pB2-77+ATFa+Rbmt07	7.68	0.43	1.89	0.10	6	-100.0	-0.06	>0.1	No
pB2-77+ATFa+Rbmt09	13.16	0.77	3.22	0.23	7	-10.7	-12.83	<0.01	Yes
pB2-77+ATFa+Rbmt10	9.60	0.26	2.37	0.06	6	-67.8	-7.83	<0.01	Yes
pB2-77+ATFa+Rbmt11	11.57	1.04	2.85	0.43	7	-35.6	-5.60	<0.01	Yes
pB2-77+ATFa+Rbmt12	14.15	0.92	3.48	0.23	6	*6.7*	-14.64	<0.01	Yes
pB2-77+ATFa+Rbmt13	10.99	0.47	2.67	0.26	5	-47.7	-6.04	<0.01	Yes
pB2-77+ATFa+Rbmt14	11.70	0.25	2.89	0.05	6	-32.9	-16.91	<0.01	Yes
pB2-77+ATFa+Rbmt15	10.22	0.55	2.51	0.14	6	-58.4	-7.88	<0.01	Yes
pB2-77+ATFa+Rbmt16	9.26	1.89	2.28	0.46	6	-73.8	-1.97	.1>P>.05	

Transfection Vectors	Ave. Activity (CPM/ug)	Standard Deviation	Ave. Fold Stimulation	Standard Deviation	n	percent fold with wt	t value (t-test)	P	Significance
pB2-77+ATF2	16.56	1.19	4.30	0.32	12	N.A.			
pB2-77+ATF2+Rbwt	73.42	4.67	18.44	1.24	22	100.0			
pB2-77+ATF2+pSVE	16.96	1.63	4.20	0.40	9	0.0			
pB2-77+ATF2+Rbmt01	64.80	7.19	15.44	1.79	5	78.9	3.56	<0.01	Yes
pB2-77+ATF2+Rbmt02	59.37	1.62	14.16	0.95	5	69.9	8.54	<0.01	Yes
pB2-77+ATF2+Rbmt07	71.29	7.91	17.59	2.03	6	94.0	0.98	>0.1	No
pB2-77+ATF2+Rbmt09	19.00	1.58	4.64	0.12	7	3.1	51.30	<0.01	Yes
pB2-77+ATF2+Rbmt10	47.49	3.40	11.71	0.74	6	52.7	16.76	<0.01	Yes
pB2-77+ATF2+Rbmt11	34.01	3.78	8.29	0.43	7	28.7	32.69	<0.01	Yes
pB2-77+ATF2+Rbmt12	18.22	0.77	4.48	0.19	6	2.0	50.57	<0.01	Yes
pB2-77+ATF2+Rbmt13	35.73	2.38	8.16	0.54	5	27.8	28.78	<0.01	Yes
pB2-77+ATF2+Rbmt14	38.05	1.76	9.38	0.39	6	36.4	29.28	<0.01	Yes
pB2-77+ATF2+Rbmt15	48.22	2.50	11.86	0.62	6	53.8	18.00	<0.01	Yes
pB2-77+ATF2+Rbmt16	73.14	8.45	17.99	2.08	6	96.8	0.51	>0.1	No

VITA

Huo Li was born in Sichuan, People's Republic of China on July 16, 1968. He received a Bachelor of Science degree with a major in Biochemistry from Sichuan University in July, 1989.

After graduation he was employed to work in the human plasma protein lab of the National Institute of Blood Transfusion, Chinese Academy of Medical Science as a research assistant for five years.

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