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I am submitting herewith a dissertation written by Huo Li entitled "Regulation of Transcription Factor ATF2 by the JNK/p38 Signal Transduction Pathway Is Facilitated by Interaction with Retinoblastoma Protein." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Philosophy of Doctor, with a major in Biochemistry and Cellular and Molecular Biology.

Wesley D. Wicks, Major Professor

We have read this thesis and
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Regulation of Transcription Factor ATF2 by the JNK/p38 Signal Transduction Pathway Is Facilitated by Interaction with Retinoblastoma Protein

A Dissertation Presented for the
Doctor of Philosophy
Degree

The University of Tennessee, Knoxville

Huo Li
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DEDICATION

This dissertation is dedicated to my wife

Mrs. (Bertie) Bing Ma Li

and my lovely seven-month old son

Jonathan Josh Li

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ABSTRACT

Two highly related transcription factors, ATF2 and ATFa, enhance the activity of the Transforming Growth Factor β2 (TGF-β2) promoter via a partial cAMP response element in transfected CHO cells. The retinoblastoma protein (Rb) also activates this promoter and enhances the stimulatory effects of ATF2 but causes near extinction of the effects of ATFa. Whereas Mitogen-activated protein Kinase Kinase 7 (MKK7) or cJun N-terminal protein Kinase (JNK) expression enhances the actions of both ATF's, MKK6 or p38 expression only augments the effects of ATF2. Immunoprecipitation of lysates from transfected cells with Rb antibodies brings down expressed ATF2 but not ATFa. Expressed JNK and p38 are also immunoprecipitated in the same complex. Similarly, ATF2 antibodies bring down expressed Rb, JNK and p38. Expression of Rb enhances the immunoprecipitation of both JNK and p38 by ATF2 antibodies. The results suggest that Rb is acting as a matchmaker by bridging either JNK or p38 with their common substrate ATF2 and, hence, facilitating its activation. This suggestion has been validated by direct measurements of the phosphorylation status of ATF2. cJun co-expression substantially enhances the

actions of ATF2, but has no significant effects on the actions of ATFa. These results were also confirmed by the inhibition of the actions of ATF2 but not those of ATFa by a dominant negative mutant of cJun. cFos has no significant effects on either ATF. Regulation of endogenous TGF-β2 gene expression in PC-3 cells is highly correlated to that of the transfected TGF-β2 promoter construct, indicating the physiological relevance of studies with the TGF-β2 promoter.

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LIST OF ABBREVIATIONS

ATF2	Activating Transcription Factor 2
ATFa	Activating Transcription Factor a
BSA	Bovine Serum Albumin
bZip	basic-Leucine-Zipper
CAT	Chloramphenicol Acetyltransferase
CBP	CREB binding protein
cdk	cyclin-dependent kinase
C/EBP	CAAT/enhancer-binding protein
CRE	Cyclic Adenosine 3', 5'-Monophosphate Response Element
CREB	CRE binding protein
CTD	C-terminal Domain
DMEM	Dulbecco's Modified Eagle's Medium
E1A	Adenovirus Early region 1A oncoprotein
ECL	Enhanced Chemiluminescence
EDTA	Disodium Ethylenediamine Tetraacetate
EGTA	Ethylene Glycol-bis(b-Aminoethylether)N,N,N',N',-Tetracetic

	Acid
ERK	extracellular signal regulated protein kinase
ESPG	EDTA-Sodium-Phosphate-Glucose
HA	Hemagglutinin
HB	Homogenization Buffer
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HRP	Horseradish Peroxidase
JNK	c-Jun N-terminal Kinase
JIP-1	JNK interacting protein-1
JSAP	JNK/SAPK-associated protein
MAPK	mitogen-activated protein kinase
MKK	mitogen-activated protein kinase kinase
MLK	mitogen-activated protein kinase kinase kinase
mt	mutant
NTD	N-terminal domain
NP-40	Nonidet P-40
PMSF	Phenylmethylsulfonyl Fluoride
PP1	Type I protein phosphatase
Rb	Retinoblastoma protein

SAPK	Stress-Activated Protein Kinase
TBP	TATA-Binding Protein
TGF-β2	Human Transforming Growth Factor β2
WT	wild type

CHAPTER I

INTRODUCTION

ATF2 and ATFa

1---Members of bZip family

The regulation of gene expression is a critical component in regulating cellular metabolism and maintaining the structural and functional differences that exist in cells during development. Regulation of transcription initiation, which is the best documented and may be the most common site of regulation of gene expression involves the interaction of RNA polymerase with its promoter. In eukaryotic systems, so-called transcription factors are the regulatory proteins which bind to specific sites in the promoter. A variety of structure motifs have been found to direct specific binding to sites located in target gene promoters. In the case of the transcription factors, we have been studying the DNA binding and dimerization sites are adjacent to each other and the entire region is referred to as the bZip domain. b is for the basic domain which binds DNA and Zip is

for theleucine zipper, a helical region involved in dimerization. There is a large super-family of bZip proteins, all of those members contain this common structural motif.

Activating transcription factors 2 (ATF2) and a (ATFa) are members of a subset of the family of bZip transcription factors (Meyer and Habener, 1993, review). Both ATF2 and ATFa were cloned from HeLa cell cDNA libraries (Gaire et al., 1990) generated by reverse transcription PCR.

Members of this bZip family typically have a basic region near their C-terminus, flanked by a leucine zipper motif. The basic region, rich in positively charged amino acids, 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) normally with 4 to 6 cycles, responsible for protein dimerization. Bzip proteins also usually have transactivation domains near the N terminus.

ATF2 and ATFa have a striking similarity to each other at the protein primary structure level (Figure 1, all figures and tables are in the appendix). Thus, they share significant identities in three regions [~90% over AA's 21-99 (ATF2); ~80% over AA's 354-426 including the bZip and ~70% over AA's 428-463] (Maekawa et al, 1989; Gaire et al, 1990; Meyer and Habener, 1993). Outside these regions there are isolated pockets of homology but the rest of the

two proteins are largely different. Both proteins are unique among the bZip family members in having a zinc finger at the N terminus.

Both ATF2 and ATFa act to form DNA binding homodimers and heterodimers (Hai et al., 1989; Benbrook et al., 1990). Such dimerization may result in ATF2 or ATFa being a constituent of factor complexes that have subtle differences in DNA binding specificity and regulatory potential (Meyer and Habener, 1993; Chatton et al., 1994).

ATF2-Jun heterodimers have been reported to act at a number of partial CRE/ATF sites (Karin and Hunter, 1995; Kaszubska et al, 1993; Liang et al, 1996; Newell et al, 1995). ATFa can interact with several members of the Jun/Fos subfamily (Benbrook and Jones, 1994; Chatton et al, 1994; Hai and Curran, 1991), but ATF2 only interacts with cJun. This suggests that some functional differences may exist between the two ATF's.

2---Promoter regulation

ATF2 and ATFa bind to cAMP response element (CRE) sites, full (TGACGTCA) or partial (CGTCA), which are closely related to so-called ATF sites (Benbrook and Jones, 1994; Hai and Curran, 1991; Meyer and Habener, 1993). These ATF/CRE sites are widely distributed in a variety of promoters (Meyer and Habener, 1993).

Despite their ability to bind to full and partial CRE sites, neither ATF2 nor ATFa has been found to mediate a response to cAMP (Gaire et al, 1990; Gong et al, 1995; Maekawa et al, 1989). However, there is a controversy regarding the cAMP effects on TGF-β2 expression (Kingsley-Kellesen et al., 1999). It has been suggested that the CRE binding protein (CREB) up-regulates TGF-β2 expression in F9 embryonal carcinoma cells. Since F9 cells are differentiating cells, cAMP's regulation in F9 cells may not be typical. We and others have found that ATF2 up-regulates the TGF-β2 in at least four different cell lines and no role for cAMP is evident (Kim et al., 1992; Gong et al., 1995). Indeed, evidence for a unique role for ATF2 in regulating transcription was provided by the fact that expression of ATF2 by itself caused significant stimulation of the activity of the TGF-β2 promoter while neither CREB or ATF1 were active (Kim et al., 1991; Kim et al., 1992; Gong et al., 1995).

ATF2 and/or ATFa have been found to enhance the activities of a variety of promoters through CRE/ATF sites including TGF-β2 [and probably β1] (Kim et al., 1991; Kim et al., 1992; Gong et al., 1995), E-selectin, Cyclins A and D1, TNFα, Fibronectin, FasL, PEPCK, DNA polymerase B, MHC enhancer A, Interferon γ, cJun, IL-2, Keratinocyte growth factor and Rb (Read et al., 1997; Shimizu et al., 1998; Joyce et al., 1999; Zhou et al., 1999; Hocevar et al., 1999; Fairs et al., 1998; Cheong et al., 1998; Kreidweiss et al., 1999;

Brockmann et al., 1999; Kim et al., 1998; Duyndam et al., 1999; Su et al., 1994). Mutation of the CRE/ATF sites in the promoters abolishes the effects of the ATF's in each case (Kim et al., 1992).

ATFa 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 ATFa protein is functionally masked by the C-terminal region of the protein (Chatton et al., 1994). Later, however, ATFa has been found to stimulate gene expression mediated by the ATF/CRE sites located in the TGF-β2 promoter in CHO cells (Gong et al., 1995) and in the E-selectin promoter in HeLa cells (Pescini et al., 1994). Mutation of these ATF-binding sites abolishes ATFa's transactivation in both cases (Pescini et al., 1994; Gong et al., 1995).

3---Phosphorylation

ATF2 and ATFa have been found to respond to environmental signals which are known to activate protein kinase cascade pathways including growth factors, cytokines and various forms of stress (Bocco et al., 1996; Gupta et al., 1995; Jiang et al., 1996; Raingeaud et al., 1995; Tournier et al., 1997; Raingeaud et al., 1996; Livingstone et al., 1995; van Dam et al., 1995; Tsai et

al., 1996). The activity of the N-terminal domain of ATF2 is also stimulated by UV irradiation and addition of serum. (Livingstone et al., 1995).

Consistent with the in vivo response to these stress signals, in vitro studies have suggested that ATF2 is the direct target of at least two signal transduction pathways (Karin and Hunter, 1995, review). ATF2 was found to be phosphorylated by the c-Jun N-terminal kinase (JNK) and protein kinase p38 (Gupta et al., 1995; Livingstone et al., 1995; van Dam et al., 1995) on two closely spaced threonine residues (positions 69 and 71) within the N-terminal activation domain. The replacement of these phosphorylation sites with alanine inhibited the transcriptional effects of ATF2 (Gupta et al., 1995; Livingstone et al., 1995; van Dam et al., 1995).

4---Physiological roles

The severe developmental defects observed in the ATF2 knockout mouse have implied its important roles in mediating growth and development. It has been shown that mice carrying a germ line mutation in ATF2 suffered widespread abnormalities, including development of the skeletal and central nervous system (Reimold et al., 1996). A more recent report using mouse null mutants of transcription factor ATF2 has shown that targeted mice died shortly

after birth and displayed symptoms of severe respiratory distress with their lungs filled with meconium (Maekawa et al., 1999)..

ATF's may also play important roles in virus infection and inflammation. There has been considerable interest in the role of ATF2 because it is a target of several viral proteins, including the adenoviral E1A, hepatitis B viral X and human T cell leukemia viral Tax proteins (Liu and Green, 1990; Chatton et al., 1993; Hagmeyer et al., 1995; Li and Green, 1996).

The expression of both ATF2 and ATFa is tissue-specific. The putative mRNA for ATF2 has been found in various human and monkey tissues and in many cell lines (Maekawa et al., 1989; Taketa et al., 1991), and it is 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 ATFa is found in human skeletal muscle, but it is 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 clearly different.

5---TGF-β2 regulation

In this study, we focused on the regulation of TGF-β2 promoter activity. 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-β's regulate cell differentiation, cell growth, 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 (Roberts and Sporn, 1990). Although TGF-β's 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). 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).

We have previously reported that both ATF2 and ATFa are able to stimulate the TGF-β2 promoter in CHO cells (Gong et al., 1995). As in CCL64 cells (Kim et al, 1992), Rb also was found to stimulate TGF-β2 promoter activity in CHO cells and it enhanced the effects of co-expressed ATF2 (Kim et al., 1992; Gong et al., 1995). Indeed, it was this effect which led to the discovery that ATF2 is able to regulate this promoter (Kim et al., 1995). In striking contrast, co-expression of Rb and ATFa led to near extinction of the stimulation of TGF-β2 promoter activity (Gong et al., 1995). This has provided

clear evidence for a functional difference between ATF2 and ATFa. The present study arose from a desire to uncover the molecular basis for this difference.

Retinoblastoma protein

1---Tumor suppressor

The retinoblastoma protein, Rb, was first identified as a suppressor of retinal cancer (for reviews, see Wang et al., 1994; Riley et al., 1994). It has been suggested that Rb inhibits tumorigenesis by inhibiting cell-cycle progression (Templeton et al., 1991; Pushkareva et al., 1995). Since then, Rb has been implicated in the regulation of many cellular processes, including cell-cycle arrest, differentiation and apoptosis (Pushkareva et al 1995; Qian et al, 1992; Wang, 1997). Mutations of the Rb gene have been found not only in retinoblastomas (Fung et al., 1987; Lee et al., 1987), but also in osteosarcomas, bladder carcinomas, small cell lung carcinomas, and a prostate carcinoma (Wang et al., 1994). Expression of wild type Rb, via retrovirus-mediated gene transfer, suppresses the tumorigenicity of several neoplastic cell lines that contain Rb mutations (Wang et al., 1994).

Fundamental functions of the tumor-suppressor protein Rb in vivo have been demonstrated in mice in which the Rb gene was inactivated in the germ

line (Lee et al., 1992 and 1994). In these animals, many Rb-deficient cells, most notably fetal neurons and hematopoietic precursors, fail to exit the cell cycle and continue to divide in aberrant locations. Reintroduction of functional Rb into Rb-deficient retinoblastoma cells resulted in reduced tumorigenicity in nude mice (Huang et al., 1988). The tumor-suppressing function of Rb was suggested to be tissue-specific by observations made in mice with heterozygous disruption of the Rb gene. These Rb +/- mice do not develop retinoblastomas, but have an unusually high incidence of pituitary tumors (Jacks et al., 1992).

In addition to the suppression of growth, Rb has also been suggested to play a role in cellular differentiation during development (Chen et al., 1996). Rb has been implicated in the regulation of apoptosis as well. For example, Rb 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).

2---Rb protein

The full length human Rb gene spans 4757 nucleotides. It has an open reading frame that encodes a protein of 928 amino acids (Riley et al., 1994, review). Rb contains several functionally distinct domains (Figure 2), which have been found to be important for its biological functions, including an N-

terminal domain, the so-called A and B pockets and a C-terminal domain (C pocket). Analysis of the domain structure of Rb by the use of synthetic deletion and naturally occurring mutations has revealed that A and B pockets are most important for the majority of its functions (Huang et al, 1990; Qian et al, 1992). The central A and B pocket domains, which are separated by a spacer region, have been found to be the binding sites for a large number of oncoproteins and transcription factors and to also regulate their activities (e.g., E2F, E1A, etc.)(Wang, 1997).

The C terminal region, referred to as the C pocket, has been found to contain several phosphorylation sites and is also the binding site for the nuclear c-Abl tyrosine kinase (Welch and Wang, 1995). The N-terminal domain of Rb also contains some phosphorylation sites (Inoue et al., 1995). Deletion studies indicate that amino terminal sequences are necessary for hyperphosphorylation of the full length molecule, which occurs during cell cycle progression (Qian et al., 1992; Durfee et al., 1994).

Rb has long been considered to be a negative regulator of gene expression based on its ability to sequester E2F family members away from target promoters (Helin et al., 1993; Wang, 1997; Brehm and Kouzarides, 1999), and more recently, to bind to histone deacetylases (Brehm and Kouzarides, 1999; Brehm et al., 1998; Magnaghi-Jaulin et al., 1998). E2F was

first defined as a transcriptional factor that activates the E2 promoter of adenovirus genes and it has since been found to activate a variety of G1/S-specific genes (Nevins, 1992, review). When E2F is bound by Rb, its transcriptional activity is blocked (Weintraub et al., 1995). The increase in Rb 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 Rb to dissociate from E2F, thus allowing it to activate S-phase genes (Weinberg, 1995, review).

However, increasing reports have appeared over the past few years showing that Rb is also able to positively regulate the expression of certain genes (Kim et al., 1992; Chen et al, 1996a; Chen et al,1996b; Park et al., 1994; Rohde et al., 1996; Wang, 1997). Thus, Rb can now be considered a versatile modulator of transcription the nature of whose actions depend at a minimum on the specific gene, the type of cell, its state of differentiation and the stage of the cell cycle.

The A and B pockets are critical for Rb's binding to transcription factors, such as E2F, C/EBP etc., as well as its tethering to nuclear structures and its growth suppressive effects in cell culture (Templeton et al., 1991). Table 1 shows a selected list of many of the oncoproteins and transcription factors that bind to the A and B pockets and regulate their activities (Adapted from Wang et

al., 1994, review). On the other hand, Rb-associated proteins use a variety of motifs to bind to Rb; for example, T antigen of SV40, adenovirus E1A oncoprotein and other oncoproteins bind Rb 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 Rb, while pp1a uses a different, large, C-terminal region to bind to Rb (Lee et al., 1994a).

3---Cell cycle related regulation

The ability of Rb to function as a tumor suppressor has been suggested to be due to its ability to regulate the expression of several key genes involved in growth control (Wagner and Green, 1991), including TGFβ1 and TGFβ2 (Kim et al, 1991; Kim et al, 1992). The biochemical mechanism by which Rb suppresses tumor formation is unclear; however, it has been suggested that Rb inhibits tumorigenesis by inhibiting cell-cycle progression (Templeton et al., 1991; Pushkareva et al., 1995). Introduction of functional Rb into certain Rb-deficient tumor cell lines results in G1 arrest (Templeton et al., 1991). This Rb-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).

During the G1 phase of the cell cycle, Rb is hypophosphorylated and is able to repress cell proliferation. At some point later in G1 in cycling cells, it

becomes hyperphosphorylated, by cyclin-dependent kinase 4 (cdk 4), and loses its growth suppressing function. Dephosphorylation of the Rb protein by type 1 protein phosphatases (pp1) occurs during mid-M phase, reactivating the protein prior to the next cell cycle (Ludlow et al., 1993).

The Rb gene product is a 110-kD nuclear phosphorylated protein that is phosphorylated on both serine and threonine residues (Wang et al., 1994). As stated above, these modifications were suggested to be important in regulating its interaction with oncoproteins and transcriptional factors. Hypophosphorylated Rb associates with the nuclear matrix during early G1, and is thought to bind peripheral matrix proteins lamin A and C (Mancini et al., 1994). Nonfunctional mutants of Rb, present in defective alleles of the Rb gene isolated from two spontaneously arising tumors, were found to be defective in phosphorylation, viral oncoprotein association and nuclear tethering (Templeton et al., 1991; Mancini et al., 1994).

Recently, other functional roles of Rb in growth repression have appeared. Rb has been suggested to regulate ribosomal RNA transcription mediated by pol I (Adnane et al., 1995; Nasmyth, 1996). Also, Rb 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 Rb might be a more general regulator of transcription needed for cell proliferation.

4---TGF-β2 connection

Rb inhibits the transcription of genes involved in growth control (Wagner and Green, 1991), but it was suggested that Rb activates transcription of the human TGF-β2 gene through ATF2 (Kim et al., 1992). Previous studies have indicated links between TGF-β2-mediated G1 growth arrest and the activity states of cyclins and cyclin-dependent kinases (cdk's) (Okamoto et al., 1994) and Rb (Koff et al., 1993). Overexpression of human cyclin D1 reduces the growth inhibition by TGF-β's. Treatment of cells with TGF-β's appears to prevent phosphorylation of Rb and retains it in the hypophosphorylated, growth-suppressive state.

JNK and p38 signal pathway

Actions of many signals in the cellular signal transduction network converge at one or more of the mitogen-activated protein kinase (MAPK) cascades, which activate transcription factors in the nucleus and other effectors throughout the cell (Wilhelm et al., 1995; Karin and Hunter, 1995; Waskiewicz and Cooper, 1997). Each MAPK is activated by its cognate MKK (MAPK Kinase) through a common mechanism involving phosphorylation of Thr and

Tyr residues in the regulatory triplet, Thr-X-Tyr (Raingeaud et al., 1996; Johnson et al., 1996; Fanger et al., 1997).

There are at least three major MAPK pathways: extracellular signal regulated protein kinase (ERK), JNK and p38; and seven MKK's (MKK1-7) have been identified in mammalian cells (Dérijard et al., 1995; Jiang et al., 1995; Karin and Hunter, 1995; Waskiewicz and Cooper, 1997; Fanger et al., 1997). ERKs, JNK, and p38 share 40-45% overall sequence identity. Generally speaking among MAPK pathways, the ERK pathway is often stimulated by growth and differentiation factors, whereas the JNK and p38 MAPK pathways are mainly activated by stress-related stimuli such as UV or gamma irradiation, ischemia and reperfusion, and inflammatory cytokines among others (Raingeaud et al., 1995; Johnson et al., 1995; Jiang et al., 1995; Karin and Hunter, 1995; Brunet and Pouysségur, 1996; Raingeaud et al., 1996; Tournier et al., 1996; Tournier et al., 1997; Waskiewicz and Cooper, 1997).

Stress-related stimuli do not alter expression levels of JNK's but rather activate existing JNK by phosphorylation on the two residues discussed above (Raingeaud et al., 1995; Fanger et al., 1997; Read et al., 1997). Two direct upstream kinases for JNK's are MKK4 (also known as SEK1, JNKK1), which also activates p38, and MKK7 (JNKK2), which has been shown to exclusively activate JNK(Raingeaud et al., 1996; Tournier et al., 1996; Tournier et al.,

1997). Activated JNK's phosphorylate a variety of transcription factors including ATF2, ATFa and c-Jun, leading to transcriptional activation (Gupta et al., 1995; Livingstone et al., 1995; Wilhelm et al., 1995; Bocco et al., 1996).

p38 MAPKs are activated by MKK3, MKK4, and MKK6 also through phosphorylation. Substrates for p38 include transcription factors such as ATF2, CHOP and ELK-1 (Jiang et al., 1996; Read et al., 1997; Cohen et al., 1997; Young et al., 1997; Brinkman et al., 1999).

Recent reports indicate that JNK interacts with the C pocket domain of Rb directly (Chauhan et al., 1999; Shim, et al., 2000). It has been suggested that JNK may phosphorylate Rb (Chauhan et al., 1999), and Rb inhibits JNK's phosphorylation of cJun in the in vitro kinase assay (Shim et al., 2000).

Research direction

Since ATFa shares a certain degree of sequence homology with ATF2 (Figure 1), especially in the zinc finger domain which has been suggested as the basis for transcriptional activation and interaction with regulatory proteins, it was of interest to test whether or not ATFa has functions similar to those of ATF2. We have been following up on earlier observations that ATF2 acts as the endogenous mediator of Rb's effects on the TGF-β2 promoter activity in CCL-

64 cells (Kim et al., 1992); and that Rb stimulates TGF-β2 promoter activity in CHO cells by itself. It also strongly enhances the effects of ATF2 while paradoxically reducing the stimulatory effects of the highly related ATFa (Gong et al., 1995).

Our approach has involved four aspects:

First, we have utilized truncation mutants of Rb to map the domains responsible for the three observed effects of Rb [stimulation of TGF-β2 promoter activity by itself (2-4 fold); enhancement of the effects of overexpressed ATF2 (3-5 fold) and inhibition of the effects of ATFa (80-90%)] (Li, 1996). These results show that despite differences in its effects on the two ATF's, the same regions of Rb are involved, namely the A and B pockets. However, significant inhibition of Rb action on the TGF-β2 promoter is observed with C pocket mutants. We also demonstrated that the differential effects of Rb in the human uterine mesodermal tumor cell line SKUT-1, which lacks endogenous Rb, are essentially the same as in CHO cells (Li, 1996).

The second aim was to compare effects of the immediate upstream signaling components responsible for phosphorylation and activation of the two ATF's. We used the co-expression of a series of protein kinases which are involved in the two major stress activated kinase signaling cascades (JNK and p38) to approach this aspect. Our results have shown firstly that overexpressed

MKK7 and JNK both enhance the effects of ATF2 and ATFa on TGF-β2 promoter activity but MKK6 and p38 only stimulate the actions of ATF2. These support the growing notion that JNK and p38 cascades possess overlapping but distinctive roles in regulating transcription, and have also revealed additional differential modulation of the actions of the ATF's.

The third aspect of our approach has been directed at examining the ability of Rb to interact with the two ATF's as well as the two upstream protein kinases found to enhance the effects of ATF2. Through co-immunoprecipitation experiments, we were able to show the association of Rb with JNK in transfected CHO cells. Furthermore, we demonstrated that p38 is associated with Rb as well. Based on the recent reports (Chauhan et al., 1999; Shim et al., 2000) and our results, the C pocket appears to be involved in direct interactions of Rb with JNK and p38. ATF2 was found to be able to associate with Rb apparently in the same complex as Rb bound to JNK or p38. Overexpression of Rb has been found to stimulate (3-4 fold) the interaction of ATF2 with JNK and p38 suggesting a model for the role of Rb in this complex as a match-maker to facilitate the interaction of protein kinases (JNK or p38) with their target (ATF2), thereby enhancing phosphorylation and activation of ATF2. Direct measurement of ATF2's phosphorylation status has confirmed that this is the case. In contrast, although Rb does not interact with ATFa under any

conditions, it reduces the latter's interaction with JNK which could account for the negative effects of Rb on ATFa's actions, at least in part.

Finally, a comparison of the effects of all of the regulatory agents used in studies of ectopic TGF-β2 promoter activity has been made with the chromosomal TGF-β2 promoter in PC3 cells using an ELISA assay for secreted TGF-β2. The results have shown that the pattern and even degree of effect of all of the combinations of expressed factors on the two promoters is indistinguishable. Thus, studies of the ectopic TGF-β2 promoter appear to accurately reflect, insofar as these studies have been carried, physiologically relevant regulatory events.

CHAPTER II

MATERIALS AND METHODS

Plasmids

Expression vectors for ATFa were kindly provided by Dr. C. Kedinger (CNRS/INSERM) (Chatton et al., 1993). pATF2 was a gift from Dr. M. Green (U. Mass) (Kim et al., 1992), and the CAT reporter construct pB2-77 was provided by Dr. S. Kim (NIH) (Kim et al., 1992). Constructs of full-length and truncated Rb expression vectors, the pRbWT3HA/SVE series, were provided by Dr. D. J. Templeton (Case Western Reserve) (Qian et al., 1992). The hemaggulutinin (HA) epitope (YPYDVPDYAK) is located at the carboxyl terminus of all of the Rb species used in this study. Expression plasmids for MKK6 and MMK7 were kindly provided by Dr. R. J. Davis (U. Mass) (Tournier et al., 1997; Raingeaud et al., 1996). dnJNKK (K116R), APF and JNK2 by Dr. G.L. Johnson (Natl. Jewish Hosp.) (Lin et al., 1995; Johnson et al., 1996), p38β by Dr. J. Han (The Scripps Res. Inst.) (Jiang et al., 1996), TAM 67

by Dr. M. Birrer (NIH), cJun by Dr. M. Karin (UCSD), and cFos by Dr. I. Verma (Salk Inst.). Plasmids were transformed into competent JM101 cells and prepared by the following procedures.

Plasmid preparation

Plasmids were prepared by a phenol/chloroform extraction protocol (Manufacturer's suggested protocol, 5 prime → 3 prime, Inc.). PZ 523 columns (5 prime → 3 prime, Inc.) were used to further purify the double-stranded plasmid DNA following the manufacturer's specifications.

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 (0.8 to 1.0%) were run at 100 volts for about 60 min in 0.5 X TBE (100 mM Tris-borate, 2 mM EDTA). After electrophoresis, the DNA

was stained in 1 μg/ml ethidium bromide for 30 min, and then visualized and photographed with UV light. A 100 bp standard ladder (Sigma) was included as a molecular weight standard in the agarose gels. Each plasmid prepared was tested by appropriate restriction enzyme digestion and subjected to agarose gel electrophoresis. The molecular weights of digested fragments were calculated and compared to the appropriate plasmid maps to validate the identity of the purified DNA.

Cell culture

Three cell lines, CHO, SKUT-1, and PC-3, were used in these experiments. The CHO cell line (ATCC) was cloned from Chinese hamster ovary, the SKUT-1 cell line (ATCC) was isolated from a human uterine mixed mesodermal tumor, and the PC-3 cell line (ATCC) was isolated from a prostate carcinoma. CHO and PC-3 cells were grown in Ham's F12 medium (Sigma) supplemented with 10% fetal bovine serum (Life Technologies). SKUT-1 cells were maintained in Dulbecco's modified Eagle's Medium (DMEM, Sigma) containing 10% fetal bovine serum. All cell lines were grown as monolayers in filter-sterilized medium on various sized plastic culture plates (Corning Glass Works) at 37 °C in a humidified CO_2 incubator.

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 NaCl, 0.01 M potassium phosphate, 1.2 g/liter of glucose, 1 mM EDTA, pH 7.5). The term "trypsin treatment" was 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 5 to 10 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 (Corning). Cocktails of plasmid DNA and Lipofectamine (Life Technologies) were prepared in serum-free medium (Opti-Mem I reduced serum medium, Life Technologies). For transfections done in 35 mm culture dishes, the following reagents were prepared in sterile tubes:

Solution A: For each transfection, 1-2 μg of DNA was diluted into 100 μl serum-free medium.

Solution B: For each transfection, 3 μl of lipofectamine 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 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 total amount of DNA transfected was kept constant by addition of pUC 18 plasmid DNA.

For transfections done in 100 mm cell culture dishes, 10 fold higher amounts of each reagent were used as above.

The cells were then incubated for 6 hr at 37 °C in a CO_2 incubator. Following this 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 48 to 54 hr after the start of transfection. Cell extracts were prepared by three freeze-thaw cycles, and clarified by centrifugation.

Lipofectamine was found to be the most efficient fusogenic agent from a variety that were examined. The conditions for transfection have been optimized through the following three steps. First, we examined the titration of basal CAT expression with pB2-77 vector alone, and the results indicated that 0.5 μg of plasmid yielded the highest CAT expression in 35 mm dishes. Second, we tested different amounts of each expression vector (including ATF2, ATFa, Rb, JNK, p38, MKK6 and MKK7), and found that between 0.1 and 0.5 μg of plasmid (specified in figure legends) was optimal for stimulating CAT expression. Third, we varied the post-transfection incubation time to monitor CAT activity in the cell extracts. The results suggested that 48 to 54 hr incubation gave the highest and most reproducible CAT activities.

A plasmid expressing the Green Fluorescent Protein (EGFP, from Clonetech) was used to monitor the transfection efficiency, which was found to be between 30 and 40% for CHO cells under the conditions used.

Protein assay

CHO cell extracts were subjected to the Bradford assay to determine the concentration of protein. Five μl of cell lysate was used for this assay. A standard curve was prepared with known concentrations of Bovine Serum

Albumin (BSA) and used to determine the total protein concentration of transfected cell extracts.

CAT activity assay

The CAT activity in 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 prior to incubation with 1.0 mM chloramphenicol (Sigma), 0.1 mM acetyl-CoA and 0.05 mCi [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} (NEN) was added and the samples were counted in a scintillation counter.

The assay was performed under conditions where radioactivity incorporated into chloramphenicol was linear with respect to both time and the amount of cell extract. Generally 100-200 cpm was observed with extracts from untransfected cells. This background radioactivity was subtracted from all samples from transfected cell extracts. 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. The relative CAT activity (fold stimulation) is defined as the ratio of specific CAT activity in extracts of cells

transfected with regulatory protein expression vectors to that in extracts of cells transfected with pB2-77 alone. The absolute CAT specific activities were highly reproducible from experiment to experiment [cpm/μg protein ± SEM: pB2-77 alone = 4.0 ± 0.1 (54); +ATF2 = 17.5 ± 0.4 (35); +ATFa = 16.4 ± 0.4 (27) ; + Rb = 16.4 ± 0.4 (31)] and no second reporter plasmid was used for normalization.

Preparation of nuclear extracts

Nuclear extracts were prepared from transfected cells by a modification of the procedure described by Borgmeyer et al. (1984). Cells were homogenized and lysed in 2 volumes of homogenization buffer (HB): 10 mM HEPES, pH 7.5/0.5 mM spermidine/0.15 mM spermine/5 mM EDTA/0.25 mM EGTA)/50 mM NaF/7 mM 2-mercaptoethanol/1 mM PMSF containing 0.5 M sucrose and 0.1% NP-40. The nuclei were sedimented by centrifugation at 12,000 g for 30 min. After two washes with HB containing 50mM NaCl, nuclei were extracted with HB containing 300mM NaCl and 10% glycerol for 1hr and clarified by centrifugation at 12,000 g.

SDS-PAGE and Immunoblotting

Ten µl of each nuclear extract (about 50 µg of protein was present in each loaded lane) or 15 µl of cell extract (about 30 µg of protein) was analyzed by sodium dodecyl sulfate (SDS)-polyacryamide gel electrophoresis (PAGE). Ten or twelve percent of 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 gels. After SDS-PAGE, the samples with a pre-stained molecular weight marker (BioRad) were electroblotted onto nitrocellulose membranes which were then blocked by incubation in 5% dry milk (Carnation) at room temperature for an hour to prevent non-specific binding.

The transfected CHO nuclear extract blots containing full length and truncated Rb mutants were probed with 10 µg/ml of monoclonal antibody 12CA5 (from Berkeley Antibody Company), which recognizes the carboxyl-terminal HA epitope (YPYDVPDYAK) present in all of the foreign Rb species in this study.

All other blots were probed by 1:2000 dilutions of appropriate polyclonal antibodies, including anti-ATF2, anti-Rb, anti-JNK, anti-p38 (all from Santa Cruz), anti-phospho-ATF2 (NEB), and anti-ATFa. Antibodies against ATFa

were prepared in rabbits from our animal facility with the His-tag protein purified by nickel chelate chromatography and SDS-PAGE electrophoresis. The band corresponding to ATFa was cut out of the gel and used for injection after emulsification with Freund's adjuvant following a standard immunization protocol.

Horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Fisher) were used as secondary antibodies as appropriate. For staining, H_2O_2 and 4-chloro-1-naphthol were used to visualize the presence of the respective proteins. For the more sensitive enhanced chemiluminescence (ECL) method, a protocol from Dr. Koontz's lab was used. After the blot was incubated with ECL reagents [Fluka; Sigma] for about 2 min, it was exposed to X-ray film, which was then subjected to development. The protein bands were scanned with a densitometer and the densities were used to calculate the relative amount of specific protein presented.

ELISA

An ELISA assay was used to measure the secretion of TGF-β2 from PC-3 cells. Serum-free medium was added to transfected cells and, after incubation for 48 hr, medium was collected and clarified by centrifugation at 6,000 g for 10

min. The medium was concentrated 20 fold using ultrafiltration (centricon 10, from Amicon). A series of dilutions of the resulting samples were added to microtiter plates. After the plates were coated with a solution of 1 mg/ml BSA, a 1:2000 dilution of anti-TGF-β2 antibody (Santa Cruz) was added. Horseradish peroxidase (HRP) linked Goat anti-rabbit IgG and its substrates (ortho-phenyl diamine/H_2O_2) were used to visualize TGF-β2. Absorbance was read by an ELISA plate reader at 490 nm.

Immunoprecipitation

For immunoprecipitation studies, 10 µl of the various antibodies to ATF2, ATFa, JNK, p38 and Rb were incubated with about 200µg of whole cell lysate in a volume of 200µl at 4 °C for 1hr. Fifteen µl of Protein A-Sepharose (Sigma) was then added and incubation continued another 60 min. The beads were washed 4 times with lysis buffer, boiled in 10 µl of SDS sample buffer and proteins separated on 10-12% polyacrylamide gels. After transfer to nitrocellulose, the proteins were probed with the appropriate primary antibodies and detected using ECL method.

CHAPTER IV

RESULTS

Differential modulation of ATFa and ATF2's activities by Rb

As shown in Figure 4 (adapted from Li, 1996), transfected full length Rb causes a 4.2 ± 0.5 (n = 22) fold stimulation of CAT activity over basal (the average CAT activity from CHO cells co-transfected with empty vector pSVE and TGF-β2 CAT is used as basal), which is similar to that (Park et al., 1994) found in CCL-64 cells (mink lung epithelial) and our previous results in CHO cells (Gong et al., 1995).

ATF2 expressed with the pSVE vector stimulates CAT expression 4.3 ± 0.3 (n=12) fold (Figure 4). Co-expression of WT Rb with ATF2 further enhances TGF-β2 promoter activity to 18.4 ± 1.2 (n=22) fold over basal.

As reported previously (Gong et al., 1995), co-expression of Rb with ATFa has the surprising effect of nearly extinguishing the stimulation of TGF-β2 promoter activity. In the present study, co-expression of wild type (WT) Rb

with ATFa leads to only a 1.9 ± 0.3 (n = 22) fold stimulation of CAT expression. This degree of stimulation is significantly lower (P value < 0.001. The P values used here and thereafter were obtained from the student t-test and are indicated as the probability of statistical significance for the mentioned comparison) than that seen with either factor alone [Rb = 4.2 fold; ATFa = 4.0 ± 0.3 (n=12)]. Furthermore, these effects all depend upon the integrity of the ATF site in this promoter (Gong et al., 1995). These differential effects were also observed in SKUT-1 cells (human uterine mixed mesodermal tumor cells) in a similar pattern (Table 2, adapted from Li, 1996).

In order to map the sites on Rb responsible for its effects alone and those observed with the two ATF's, we utilized a series of truncated mutants (Figure 5,) generated in the pSVE vector by D.J. Templeton (Qian et al., 1992). WT and mutant Rb's were overexpressed alone and in combination with the two ATF's. The results are expressed as a percentage of the effect seen with WT Rb.

Table 3 shows the summary of the effects of truncated Rb mutants on the stimulation of TGF-β2 CAT expression. An intact A/B pocket is required for Rb's stimulation. Since truncated mutants 9 and 12, which have deletions in the A/B pocket, exhibit only 16 and 9% of wild type activity, respectively. The C pocket is also important, since a significant decrease in activity (55% of WT) is found in C pocket-deleted mutant 14. Other mutants tested containing intact

A/B and C pockets (data not shown) are not significantly different from the WT Rb activity (Li, 1996).

As discussed previously, overexpressing ATF2 alone stimulates CAT activity 4.3 ± 0.3 (n= 12) over basal. Co-transfection of WT Rb with ATF2 further enhances CAT activity to 18.4 ± 1.2 (n=22) fold over basal (this value is set at 100% in Table 3, Column Rb with ATF2). Deletion mutants of Rb exhibit a pattern of stimulatory effects together with ATF2 that are virtually indistinguishable from those seen in the absence of ATF2 (Li, 1996). This result may not be surprising since the effects of Rb on this promoter appear to be mediated by endogenous ATF's, possibly ATF2 (Kim et al, 1992). Mutants 9 and 12 are the most severely affected with only 3 and 2% of WT stimulation. The C pocket is also important with mutant 14 exhibiting 36% of WT. Outside of the pocket domains, there was only modest inhibition (70-94% of WT, data not shown).

Co-transfection with WT Rb and ATFa leads to only a 1.9 ± 0.3 (n=22) fold enhancement of CAT activity (this value was set as -100% in Table 3, Column Rb with ATFa). Interestingly enough, the truncated Rb mutants exhibit a similar pattern of effects on ATFa as with ATF2 but in the opposite direction. Thus, dysfunctional mutants of Rb exhibit a diminished capacity to inhibit the effects of ATFa whereas the same mutants exhibited diminished capacity to

stimulate the actions of ATF2 (Table 3). Mutants 9 and 12 are once again the most severely affected (9 and 0% of WT). C pocket mutants 14 are moderately inhibited (33% of WT) while the other mutants outside the pocket domains are nearly normal (74-100%, data not shown).

Comparison of the effects of mutations in Rb indicates a close correspondence among the three effects (Rb alone and with the two ATF's, data not shown). In all cases, the effects of mutants outside of the A/B/C domains are marginally different at best from WT. In contrast, all of the A/B/C domain mutants exhibited effects significantly different from WT Rb.

To rule out the possibility that the dysfunction of the Rb mutants might be the result of reduced expression, we tested and found all of the mutant vectors generated an appropriately sized protein in CHO cells at approximately the same level of expression as the WT Rb (Li, 1996, data not shown).

Impact of perturbations in JNK/p38 kinase signaling cascades

MKK6 has been found to selectively activate the p38 kinase cascade while MKK7 has been found to selectively activate the JNK cascade (Raingeaud et al, 1996; Tournier et al, 1997). As an initial evaluation of the kinase cascade specificity involved in modulating the actions of the two ATF's

on TGF-β2 promoter activity, constitutively active forms of MKK6 and MKK7 have been expressed in CHO cells to see if they enhance the actions of either or both ATF's.

As shown in Figure 6, co-expression of either MKK6 or 7 strongly stimulates basal TGF-β2 promoter activity. Both MKK's enhance the actions of ATF2 significantly, in agreement with previous reports which used non-physiological promoter systems (Tournier et al., 1996; Raingeaud et al., 1996). In contrast, the action of ATFa was only augmented by MKK7 but to the same extent as with ATF2 (P value > 0.1 for differences between ATFa and ATF2). However, MKK6, whose effects have been shown to be specific for activation of p38 (Raingeaud et al., 1995; Raingeaud et al., 1996; Johnson et al., 1996), has no detectable effect on the actions of ATFa.

None of these effects is observed with a mutant TGF-β2 promoter (Gong et al, 1995), in which the CRE site is modified, that exhibits little or no response to the ATF's (data not shown).

The specificity of these effects has also been validated by the observation (Figure 6) that the stimulatory effects of MKK7 on basal and ATF-enhanced TGF-β2 promoter activity are all nearly abolished (~80% inhibition) by co-expression of dnJNKK (K116R), a dominant negative mutant of JNKK, shown

to be specific for the MKK7-JNK cascade (Lin et al., 1995). This mutant has little or no effect on the stimulatory actions of MKK6 (Figure 6).

If the actions of MKK7 accurately reflect activation of JNK, then co-expression of the latter protein should also enhance the actions of both ATF's. As shown in Figure 7, co-expression of JNK does, in fact, enhance the effects of both ATF's similarly and to virtually the same extent as MKK7 in Figure 6 (P value > 0.1). In addition, JNK also stimulates basal TGF-β2 promoter activity to about the same extent as MKK7 (P value > 0.1). These effects are all largely abolished (~80% inhibition) by co-expression of APF, a dominant negative mutant of JNK. [APF is a nonphosphorylatable mutant of JNK in which the phosphorylation site Thr-Pro-Tyr is changed to Ala-Pro-Phe (JNK-APF), and it has been observed to behave as a dominant competitive inhibitor of JNK (Johnson et al., 1996), presumably binds to Rb and ATF2 competitively with JNK.] Even the effects of ATF2 alone were substantially blocked (~50% inhibition) by APF.

As is true for JNK, the p38 pathway is activated by stress-related stimuli and leads to phosphorylation of ATF2. Thus it was of interest to see if p38 has a similar effect as JNK does on TGF-β2 expression. In accordance with the results obtained with MKK6, co-expressed p38 exerts stimulatory effects on both basal (5-6 fold) and ATF2-enhanced (12-13 fold) TGF-β2 promoter

activity (Figure 8). In contrast, p38 does not significantly enhance the actions of ATFa as was also the case with MKK6. The specific inhibitor of p38, SB 203580, used at a concentration which does not inhibit other protein kinases (Jiang et al., 1996; Cohen, 1997; Young et al., 1997; Brinkman et al., 1999), largely eliminates (~80% inhibition) the effects of p38 on both basal and ATF2-stimulated TGF-β2 promoter activity. These results strongly suggest that the actions of p38 are the result of expression of its protein kinase activity in the transfected CHO cells.

The specificity of APF and SB 203580 has been tested in transfected CHO cells. It has been found that APF inhibits the activities of MKK7 and JNK but not to those of MKK6 and p38, whereas, SB 203580 blocks the actions of MKK6 and p38 but not to those of MKK7 and JNK (data not shown). These results also imply that APF and SB 203580 are not likely to have effects on protein expression in general.

The congruence of the data with the co-expressed MKK's and JNK/p38 also are in agreement with the conclusion previously drawn (Gupta et al., 1995; Jiang et al., 1996; Raingeaud et al., 1995; Tournier et al., 1996; Raingeaud et al., 1996) that ATF2 is phosphorylated and activated by both JNK and p38 cascade pathways. On the other hand, ATFa appears to be regulated only by the JNK signaling pathway.

Consistent with the transfection data, we have observed in vitro phosphorylation of ATFa with purified JNK but purified p38 was unable to phosphorylate ATFa to any detectable extent (J. Blakemore and W.D. Wicks, unpublished results). Immunoblotting of cell extracts has confirmed the fact that the expression levels of the proteins encoded by the various plasmids transfected were not detectably affected by co-expression of the others (data shown in next section).

Protein expression levels in CHO cells.

To confirm that all proteins were properly expressed in the cells and that overexpression of one protein does not affect the expression of other proteins in the co-expression experiments, we systematically monitored the expression of all these proteins through western blots probed with specific antibodies. The expression levels of overexpressed proteins, including ATF2, ATFa, JNK, p38, MKK6, MKK7 and cJun, were examined.

1---Expression level of ATF2

Overexpression of ATF2 in transfected CHO cells was shown, as expected, to generate a single band (Figure 9, lane 2) on the western blot with

an estimated molecular weight of about 74 kD, close to that reported by others (72 kD by Meyer and Habener, 1993) and our results with purified ATF2 (data not shown). The expression of endogenous ATF2 was very low but detectable (Figure 9, lane 1). Co-expression of Rb (Figure 9, lane 3), JNK (lane 4), MKK6 (lane 5) and MKK7 (lane 6) did not have any observable effect on the expression level of ATF2.

2---Expression level of ATFa

The expression level of ATFa was examined using both antibodies made in our lab against ATFa by Dr. Wicks (see Materials and Methods for detail, Figure 10) and commercially obtained antibodies against ATF2 (Santa Cruz Tech, data not shown). Since ATF2 and ATFa are highly homologous, it was not surprising that polyclonal anti-ATF2 antibodies were able to cross-react with the expressed ATFa protein and vice versa. With both antibodies, overexpression of ATFa in transfected CHO cells was shown, as expected, to generate a single band on the western blots (Figure 10, lane 2). The calculated molecular weight was 70 kD reflecting the smaller size of ATFa relative to ATF2 (69 kD by Chatton et al., 1993). Endogenous ATFa was not detected (Figure 10, lane 1). Co-expression of Rb (Figure 10, lane 3), MKK7 (lane 4),

MKK 6 (lane 5) JNK (lane 6) and p38 (lane 7) did not have any observable effect on the expression level of overexpressed ATFa.

3---Expression level of JNK

Overexpression of JNK in transfected CHO cells was shown, as expected, to lead to a single band (Figure 11, lane 2) on the western blot probed with anti-JNK antibodies (Santa Cruz). The molecular weight of this band was calculated to be about 42 kD as expected (Gupta et al., 1995). Endogenous JNK is barely detectable (Figure 11, lanes 1) but it has the same mobility as overexpressed JNK (Figure 11, lane 2). Co-expression of MKK6 (Figure 11, lane 3), MKK7 (lane 4), ATF2 (lane 5) and Rb (lane 6) did not have a detectable effect on the expression of JNK.

4---Expression level of p38

Overexpression of p38 in transfected CHO cells generated a single band (Figure 12, lane 2) on the western blot probed with anti-p38 antibodies (Santa Cruz). The calculated molecular weight of this band was 38 kD as expected (Jiang et al., 1996). No endogenous p38 was detected in untransfected CHO cells (Figure 12, lanes 1). Extracts from cells co-expressing ATF2 (Figure 12, lane 3), ATFa (lane 4), MKK6 (lane 5), MKK7 (lane 6) and Rb (lane 7) all have

slightly higher level of p38 expression compared to p38 alone (lane 2). These results appear likely to be due to a loading error in lane 1 since additional experiments have shown that the expression level of p38 alone is not significantly different from the other co-expression combinations (data not shown).

5---Expression level of MKK6

Overexpression of MKK6 in transfected CHO cells led to a single band (Figure 13, lane 2) on the western blot probed with anti-MKK6 antibodies (Santa Cruz). The calculated molecular weight of this band was 44 kD compared to the expected 42 kD (Dickens et al., 1997). No endogenous MKK6 was detected in untransfected CHO cells (Figure 13, lanes 1). Co-expression of JNK (Figure 13, lane 3), p38 (lane 4), ATF2 (lane 5), ATFa (lane 6) and Rb (lane 7) did not have a significant effect on the expression level of MKK6.

6---Expression level of MKK7

Overexpression of MKK7 in transfected CHO cells generated a single band (Figure 14, lane 2). The calculated molecular weight of this band was 47 kD as expected (Raingeaud et al., 1996; Foltz et al., 1998). No endogenous MKK7 was detected in normal CHO cells (Figure 14, lanes 1). Co-expression of

JNK (Figure 14, lane 3), p38 (lane 4), ATF2 (lane 5), ATFa (lane 6) and Rb (lane 7) did not have a significant effect on the expression level of MKK7.

7---Summary

The expression level of cJun also has been examined (data not shown). We found that endogenous cJun expression was not detectable but over-expressed c-Jun in transfected CHO cells was reflected by a single band on the western blot using anti-cJun antibodies (Santa Cruz). Co-expression of ATF2, ATFa, JNK and MKK7 did not significantly affect the expression of cJun.

In summary, we examined the expression levels of seven different proteins in transfected CHO cells. The transfection procedure generates significantly higher amounts of each protein compared to their endogenous counterparts, which are not detectable except for endogenous JNK and ATF2. The expression level of each of the proteins examined was not significantly influenced by co-expression of the other proteins. Thus, the different effects on TGF-β2 promoter activity seen when two or more proteins are expressed in transfection experiments cannot be due to differences in their expression level.

Exploration of possible roles of cJun/cFos in the effects of ATF2/ATFa on TGF-β2 promoter activity

There are several potential explanations for the differential effects of Rb on the transcriptional activity of the two ATF's, including the possibility that heterodimerization with members of the cJun family may be involved. This seems reasonable given several reports that ATF2-cJun heterodimers are the active factor at some ATF sites (Karin, 1995; Kaszubska et al, 1995; Liang et al, 1996; Newell et al, 1995) as well as the fact that ATFa and ATF2 have different patterns of heterodimerization (Benbrook and Jones, 1994; Chatton et al, 1994; Hai and Curran, 1991; Kaszubska et al, 1995; Zhou et al, 1995). As an initial exploration of these possibilities, we observed the effects of co-expression cJun or cFos with the two ATF's on TGF-β2 promoter activity.

As shown in Figure 15, cJun does modestly enhance basal TGF-β2 promoter activity. More significantly, cJun co-expression substantially enhances the actions of ATF2. In contrast, cJun has no significant effect on the actions of ATFa. These results support the idea that ATF2 may be acting cooperatively with cJun but they also show that ATFa is acting independent of cJun.

Overexpression of cFos has no significant effect in all situations (Figure 15), even with cJun, underscoring the distinction of this ATF site from an AP-1 site. Combinations of either ATF with cJun and Rb yield similar results; i.e., cJun enhances the cooperation between ATF2 and Rb while it does not significantly perturb the inhibitory effects of Rb on ATFa (data not shown).

To further explore the effects of cJun, we co-transfected TAM 67, a dominant negative mutant of cJun with deletions in the transactivation domain (Brown et al, 1993; Xia et al, 1993). We felt that TAM 67 was likely to block interactions between Jun and the ATF's such as heterodimerization between ATF2 and cJun. TAM 67 was found to reduce basal CAT expression by 60-70% and this value was set at 1.0 for calculations of relative CAT activities ± ATF2/ATFa. Consistent with the effects of co-expression of cJun, TAM 67 causes severe inhibition of the effects of ATF2 at all vector input levels tested on TGF-β2 promoter activity (Figure 16). In marked contrast, TAM 67 has almost no ability to inhibit the actions of ATFa (Figure 17). The stimulation by Rb alone is reduced to a moderate extent by TAM 67 (data not shown).

These results are in agreement with those observed with cJun overexpression, i.e., that ATF2 is acting on the TGF-β2 promoter in cooperation with cJun, perhaps as a heterodimer as reported previously for other promoters. ATFa, on the other hand, appears to be acting independently of cJun.

Association of Rb with ATF2 but not ATFa

The simplest explanation for the ability of Rb to enhance the actions of ATF2 on TGF-β2 promoter activity would be that the two proteins interact which somehow facilitates the actions of ATF2. This possibility was first raised by Kim et al (Kim et al., 1992) using GST-Rb pull down ATF2 in crude extracts. In our studies, cell extracts from transfected CHO cells were subjected to immunoprecipitation with antibodies specific for Rb. Then the presence of ATF2 or ATFa in the immunoprecipitate was tested on western blots probed by anti-ATF2 or anti-ATFa antibodies.

Antibodies against Rb clearly pull down ATF2 from extracts of cells co-expressing the two proteins (Figure 18, top panel). The effect is dependent upon the integrity of the A/B pockets since the interaction is abolished with Rb mutants 9 (with a truncated A pocket) and mutant 12 (with a truncated B pocket). The association of Rb and ATF2 obviously fits Rb's up-regulation on ATF2's activity found in previous experiment since mutants 9 and 12, which fail to associate with ATF2, are also the most dysfunctional in modulating TGF-β2 promoter activity (Table 3).

In contrast, the C pocket mutant 14 is only slightly impaired (if at all) in its ability to interact with ATF2. In two experiments, the amount of ATF2 associated with mutant 14 is 65% and 95% of that associated with WT Rb (data not shown). Considering experimental variations, we conclude that mutant 14 has nearly the same ability to associate with ATF2 as WT Rb (P value > 0.1). Yet mutant 14 possesses only 36% of the ability of WT Rb to up-regulate ATF2's activity (P value < 0.001). This result suggests that the C-pocket of Rb may involve a different mechanism of modifying ATF2's activity from the A/B pockets. The ability of JNK or p38 to interact with the C pocket (discussed later) provides an alternative explanation for the role of the C pocket in regulating the activity of ATF2.

As can be seen in the Rb immunoblot (Figure 18, bottom panel), expression of both the WT and mutant Rb's are comparable and the appropriately sized proteins are generated. It also confirmed that the inability of mutants 9 and 12 to bring down ATF2 was not caused by inadequate expression of these mutant proteins. When Rb is not overexpressed, ATF2 is not present in the immunoprecipitate (data not shown) showing that the presence of ATF2 in the immunoprecipitate is dependent on the presence of overexpressed Rb.

These experiments have been repeated three times with the same results. We have also used ATF2 antibodies and they efficiently immunoprecipitate co-expressed Rb (see below).

In contrast to ATF2, ATFa does not appear to interact with Rb. As shown in the right side of the immunoblot in the top panel in Figure 19, both ATF's from cell extracts (not immunoprecipitates) react against the polyclonal antiserum used (lab-made). This is not surprising given the high degree of identity of these two proteins. ATFa exhibits a slightly higher mobility (69 kD) in this gel which reflects its 23 fewer amino acids relative to ATF2 (72 kD). Although the antiserum directed against ATFa reacts quite effectively with the co-expressed ATF2 (lane 4), no reaction is seen in the lane where ATFa should be if it interacted with Rb (lane 6).

The protein level of Rb present in the immunoprecipitate was monitored and found to be similar in each case (Figure 19, bottom panel) when Rb was co-transfected.

Thus, no evidence of association between Rb and ATFa has been found, even under the conditions where we observed the interaction between Rb and ATF2. These results may explain why Rb does not up-regulate ATFa's action as it does those of ATF2 because ATFa does not have the ability to interact with Rb. On the other hand, they do not enlighten us as to how Rb actually down-

regulates ATFa's activity. The latter question may partially be answered by the sequestration of JNK, which phosphorylates and up-regulates ATFa, to the C-pocket of Rb (discussed later).

Association of Rb with protein kinases JNK and p38

While these studies were in progress, a report appeared indicating that JNK interacts directly with the C pocket of Rb (Chauhan et al., 1999). We have been able to confirm this report as shown in Figure 20. Rb antibodies clearly are able to pull down JNK (Figure 20, top panel lanes 4, 7 and 8) as seen in immunoblots probed with anti-JNK antibodies. Co-expression of ATFa has no effect but ATF2 appears to enhance JNK immunoprecipitation slightly in this experiment (see below). The specificity of the immunoblot for JNK is shown by the use of co-expressed p38 which does not cross-react with the JNK antiserum (Figure 20, top panel, lanes 2, 3 and 6). Lane 1 contained cell extract from JNK-transfected cells included as a positive control.

The Rb protein level in the immunoprecipitate was monitored (Figure 20, bottom panel) and found to be comparable in all cells overexpressing Rb (lanes 3, 4, 5, 8 and 9). If either Rb or JNK was not overexpressed, JNK was not detected at all in the immunoprecipitate (lanes 6 and 7).

Based on the stimulatory effects of p38 on the actions of ATF2 and homologies between p38 and JNK, it seemed reasonable to expect that p38 also interacts with Rb. As shown in Figure 21, this proved to be the case (Figure 21, top panel, lanes 5, 7 and 8). Co-expression of either ATF has no detectable effect on p38 pull down in this experiment (top panel, lanes 7 and 8). The specificity of the immunoblot is once again validated by the lack of cross-reactivity of JNK with the p38 antiserum (top panel, lanes 2 and 4).

When either Rb or p38 was not overexpressed (top panel, lanes 3, 4 and 7), p38 was not detected at all. Lane 1 of the top panel contained cell extract from p38-transfected cells included as a positive control.

As in prior experiments, the Rb protein level in the immunoprecipitate was found to be similar in all cells overexpressing Rb (Figure 21, bottom panel, lanes 3, 4, 5, 7 and 8). Thus, we conclude that Rb associates with p38 in CHO cells and the presence of ATF2 or ATFa does not have any effect on the association between Rb and p38. To our knowledge, this observation has not been reported before.

Association of Rb with ATF2 and JNK or p38 in the same complex

Up to this point, the co-immunoprecipitation experiments have involved only two of the putative three members of the macromolecular complex formed with Rb, ATF2 and JNK/p38. It was important to monitor the levels of all three proteins in immunoprecipitates from extracts prepared in the same experiment.

For this purpose, the immunoprecipitates generated by anti-Rb antibodies were split into three aliquots. As can be seen in Figure 22, each sample was tested for the presence of ATF2 (top panel), JNK (middle panel) and Rb (bottom panel, as control). The results (lanes 8 and 9 in top and middle panels) show that both JNK and ATF2 are present in one Rb immunoprecipitate. Lane 8 (in all panels) was loaded with the same sample as lane 9 but with only half of the amount. Comparing lanes 7 and 9 of top panel, it can be concluded that ATF2's association with Rb is independent of the presence of JNK. Similarly, lane 6 and lane 9 of panel B indicate that JNK's association with Rb is also independent of the presence of ATF2. Lane 4 of the top panel and lane 3 of the middle panel are transfected cell extracts, which served as positive controls for ATF2 and JNK, respectively.

The protein level of Rb was monitored as before and found to be similar in all immunoprecipitates (Figure 22, bottom panel, lanes 5, 6, 7 and 9) except for lane 8 in which only half the amount of protein was loaded.

Similar results were also achieved when p38 was transfected and tested in place of JNK (Figure 23). From these results, we also conclude that Rb can interact with p38 and ATF2 in the same complex. Although, the interaction site of p38 on Rb remains to be determined, these results imply that p38, like JNK, is associated with the C pocket of Rb, especially since the interaction with ATF2 involves the A/B pockets.

Given the fact that ATF2 is a known substrate of JNK and p38 (Gupta et al., 1995; van Dam et al., 1995; Livingstone et al., 1995), the results to date suggest that Rb's role may be characterized as that of a match-maker facilitating the phosphorylation process by bringing JNK or p38 together with ATF2 (Figure 24).

Rb enhances the association of ATF2 with JNK or p38

Antibodies against ATF2 would be expected to immunoprecipitate both Rb and JNK/p38 based on the results obtained so far. This indeed proves to be the case as shown in Figures 25 to 28. Comparable amounts of Rb are brought

down when ATF2 is co-expressed ± JNK (Figure 25). On the other hand, the modest levels of JNK brought down by anti-ATF2 antibodies without co-expression of Rb are significantly enhanced by the latter's co-expression (compare lanes 5 and 7). In different experiments, the degree of stimulation by Rb co-expression of JNK immunoprecipitation by ATF2 antibodies is 3.3 ± 0.4 (n=4) fold.

As shown in Figure 26, the same results are seen with p38 brought down by anti-ATF2. In different experiments the degree of stimulation of p38 immunoprecipitation with ATF2 antibodies by co-expressed Rb is 3.8 ± 0.4 [n=3] fold. These results would not be predicted if Rb interacts independently with either ATF2 or JNK/p38.

As expected from the results with MKK7/JNK co-expression, ATFa antibodies are able to co-immunoprecipitate JNK (Figure 27). The amount of JNK brought down in this experiment compares roughly to that brought down by ATF2 antibodies (see Figure 25, top panel, lane 5). Co-expression of Rb, in contrast to its stimulatory effects on the association of ATF2 and JNK, reduces the amount of JNK in the immunoprecipitate by 31% ± 6% (n=2).

The fact that ATFa is not able to associate with Rb explains ATFa's inability to utilize the Rb mechanism to increase its activity. Furthermore, the presence of Rb in this experiment seems to trap JNK away from ATFa, which

may help to explain Rb's down-regulation of ATFa's activity. In agreement with the lack of effect of MKK6 and p38 on the actions of ATFa, no p38 could be detected in the anti-ATFa immunoprecipitates prepared with extracts from cells overexpressing ATFa and p38 ± Rb (data not shown).

In one experiment we have tested the ability of key Rb mutants to enhance JNK immunoprecipitation by ATF2 antibodies. Although the WT Rb exerted the same stimulation as in Figure 25 (Figure 28, top panel, lanes 6 and 7), both mutant 9 and 14 are unable to generate this effect (Figure 28, top panel, lanes 8 and 9). In addition, the interaction between ATF2 and JNK appears to be disrupted by co-expression of the Rb mutants (Figure 28, top panel, compare lane 6 with lanes 8 and 9). Once again, these results would not be predicted for independent interactions between Rb and ATF2, on the one hand, and Rb with JNK, on the other. The disruptive effect of C pocket mutant 14 is also consistent with the fact that C pocket is necessary for Rb's stimulatory effect on ATF2 (Figure 18, more details in Discussion).

Rb enhances the phosphorylation of ATF2 in CHO cells

Consistent with its suggested match-maker role, Rb enhances the phosphorylation of ATF2 as shown in Figure 29. Using phospho-specific anti-

ATF2 antibodies, we were able to measure the amount of phosphorylated ATF2 (Thr 69/71) in transfected CHO cells. Co-expression of Rb alone increases ATF2 phosphorylation by 4.8 fold (compare lane 1 and lane 4) presumably through endogenous JNK and/or p38. The effect of Rb is comparable to that of either co-transfected protein kinase JNK (6.1 fold, lane 2) or p38 (5.9 fold, lane 3). When Rb is co-expressed with JNK or p38, the phosphorylation of ATF2 is further increased to 13.3 and 13.0 fold, respectively. These results suggest that Rb increases ATF2's phosphorylation through the Thr 69 and 71 sites, which are known to be phosphorylated by JNK and p38 (Gupta et al., 1995, van Dam et al., 1995, Livingstone et al., 1995). Taken together, these results suggest that the ability of Rb to enhance the phosphorylation of ATF2 by JNK and p38 is very likely the consequence of Rb's ability to bind simultaneously to ATF2 and JNK/p38.

As controls, we find that APF inhibits JNK-mediated ATF2 phosphorylation by 90% (Figure 29, lane 7) and treatment of SB203580 inhibits p38's activity by 90% (lane 8). SB203580 has no significant effect on JNK's activity (6.1 fold to 5.6 fold, lanes 2 and 9), reflecting the specificity of SB203580 for p38.

Comparison of effects of regulatory proteins on ectopic TGF-β2 promoter activity with those on the endogenous TGF-β2 gene

A perennial question in work of this type is its relevance to the physiological situation in vivo. As one way of addressing this question, we have compared the effects of the various regulatory proteins used in these studies on CAT activity from the ectopic TGF-β2 promoter as above with those on endogenous TGF-β2 gene expression. For this purpose we have used the PC-3 cell line where the secretion of TGF-β2 protein has been found to reflect faithfully the effects of transcriptional regulators (Bang et al., 1992). An ELISA assay was used to measure TGF-β2 protein secreted into the culture medium and CAT activity was monitored in extracts of the attached cells which had been transfected with the pB2-77 CAT construct as described above.

Columns 1 and 2 of Figure 30 show that there is no significant difference in TGF-β2 secretion in the presence and absence of the pB2-77 plasmid (P value > 0.1). Thus, we set both the TGF-β2 secretion (about 0.05 Abs. 490 units per 40 μl of 20-fold-concentrated medium) and CAT activity levels (about 4.0 cpm per μg of protein in the cell extracts) as the basal level, when pB2-77 alone is the plasmid introduced into PC-3 cells. The values for both basal levels are

set arbitrarily as 1, and all other ELISA and CAT activities were compared to these basal levels and the ratios were used as the "fold stimulation".

In the presence of overexpressed ATFa (Figure 30, column 3), both the secretion of TGF-β2 and CAT activities increased to a similar extent (2.8 ± 0.4, 2.5 ± 0.6, n=8), and there is no significant difference between the values of the two assays (P value > 0.1). Thus, the introduced ATFa turns on the endogenous TGF-β2 gene and hence increases the level of secretion of TGF-β2, and also increases CAT expression from the ectopic TGF-β2 promoter presumably by the same mechanism.

The presence of overexpressed ATF2 (Figure30, lane 4) also increased the secretion of TGF-β2 and CAT activities to the same extent (3.1 ± 0.4, 2.7 ± 0.6, n=10). There was no significant difference (P value > 0.1) between these two actions of ATF2. Also, comparison of the effects of ATF2 and ATFa revealed no difference (Columns 3 and 4, P value > 0.1), which suggests that the cells have no preference in using either ATF over the other in regulating the TGF-β2 gene. We also noticed that ATF2 and ATFa's presumed transcriptional activation was somewhat smaller in PC-3 cells (2.7 ± 0.6) than in CHO cells (4.5 ± 0.5). Considering that great differences exist in the origins of these two cell lines, it may not be surprising that such a difference exists. It is probably

more significant that the same general pattern of up-regulation can be found in different cell lines, from CHO to SKUT-1 and to PC-3 cells.

We also examined the regulation of the two TGF-β2 promoter activities by overexpression of constitutively active protein kinases (Figure 31). Both protein kinases JNK (Figure 31, column 3) and p38 (column 4), and the kinase kinases MKK6 (column 5) and MKK7 (column 6) were found to up-regulate the activity of both TGF-β2 promoters. There were no significant differences between the secretion of TGF-β2 and CAT activities when various kinases were present (P value > 0.1).

Since the active forms of these protein kinases from two different kinase cascades are able to increase TGF-β2 promoter activities, both signal transduction pathways could be involved in the regulation of TGF-β2 gene expression in vivo. The pattern of regulation of TGF-β2 promoter by protein kinases and kinase kinases in PC-3 cells was comparable to that in CHO cells although the degree of stimulation was systematically smaller as discussed above.

We also tested the co-expression of the JNK kinases with ATF2 (Figure 32) and ATFa (Figure 33) in PC-3 cells. As expected, co-expression of ATF2 with JNK (Figure 33, column 3), p38(column 4), MKK7 (column 5) or MKK6 (column 6) further increased the secretion of TGF-β2 as well as the production

of CAT to the same extent (P value > 0.1), except for the case of MKK6 and ATF2, where the difference is of borderline significance (P = 0.02).

However, unlike ATF2, the combination of p38 (Figure 33, column 4) or MKK6 (column 6) with ATFa did not result in any more increase in either assay. On the other hand, as with ATF2, co-expression of ATFa with JNK (Figure 33, column 3) or MKK7 (column 5) further increased the secretion of TGF-β2 as well as the production of CAT. These results are consistent with our previous observations that ATF2 could be regulated by both MKK7/JNK and MKK6/p38 pathways, but ATFa is only responsive to MKK7/JNK.

Comparing the TGF-β2 secretion with CAT activities, we found that the combination of MKK7 and ATFa shows difference with borderline significance (P = 0.02). This discrepancy can be explained by one outlier in this combination (more discussion below).

The degree of increases seen with the various combinations of protein kinases and ATF's is not as high as the addition of their effects alone would predict. This might be explained by some upper-limitation (i.e. some key co-activator is present in limiting amounts) on TGF-β2 expression which has been reached. In any event, the increases were highly significant (P < 0.001) in all the combinations except MKK6/P38 + ATFa, which did not significantly differ from MKK6/p38 alone (P > 0.1).

To test the specificity of MKK7 and JNK's targeting to ATF2 or ATFa, APF, a dominant negative mutant of JNK, was included in the transfection experiment (Figure 34). APF was found to significantly reduce TGF-β2 expression in all combinations of MKK7/JNK + ATF2/ATFa (Figure 34, solid bars), compared to the absence of APF in these combinations (Figure 34, open bars). There were no differences in the effects of APF on TGF-β2 secretion and CAT production. Thus, we come to the conclusion that MKK7's up-regulation is exerted through activation of JNK, and inhibition of JNK's activity decreases the effects of both ATF2 and ATFa.

For the same purpose, SB203580, a specific inhibitor of p38, was used to treat transfected PC-3 cells (Figure 35). It was found that 10 μM SB203580 could significantly decrease the effects of the combination of ATF2 with p38 (Figures 35, columns 1)or MKK6 (column 3), yet it did not have a significant effect on the actions of ATFa (columns 2 and 4). These results suggest that MKK6's actions are being exerted through activation of p38. Again, we found no differences between TGF-β2 secretion and CAT production.

In summary, we have found that the secretion of TGF-β2 is highly correlated with the CAT activity induced in PC-3 cells. As shown in Figure 36, all tested samples (n=116) illustrate the high degree of correlation (R squared = 0.778) of changes in CAT activity and TGF-β2 secretion under all of the

conditions that we have employed. Each value was compared to that of pB2-77 alone which was set as the basal level. When we analyzed the data using the Rstudent test, one point (marked by the arrowhead in Figure 36) is an obvious outlier (R student = -9.9). If this outlier is legitimately excluded, R squared increases to 0.875 with 115 samples, which means that 87.5% of changes in TGF-β2 secretion can be predicted by changes in CAT activity.

Insofar as they go, these results suggest that the mechanisms involved in modulating the ectopic TGF-β2 promoter activity bear at least a reasonable relevance to those operating on the chromosomal TGF-β2 promoter.

The fact that MKK7 /JNK but not MKK6/p38 is able to enhance ATFa's actions is consistent with our inability to detect phosphorylation of ATFa by p38 in vitro (Blakemore and Wicks, unpublished results). In a broader context, various stimuli, especially stress and /or the inflammatory response could, by virtue of selective stimulation of appropriated kinase cascade, differentially activate these two ATF's leading to potentially differential actions on CRE-containing promoters. Given the presence of several isoforms of JNK and p38, however, the situation in any given cell type is likely to be more complex.

CHAPTER IV

DISCUSSION

Match-maker model

Our results have shown that Rb interacts with both the substrate ATF2 and the kinases JNK or p38 which phosphorylate it, and the joint binding of ATF2 and JNK/p38 to Rb facilitates phosphorylation/activation of ATF2. Thus, we propose a model in which Rb functions as a match-maker or so-called scaffold protein, bridging the protein kinases JNK or p38 with their substrate ATF2 (Figure 37).

One immediate question from the pull-down experiments is whether the three proteins (Rb, ATF2, and JNK/p38) are really in the same macromolecular complex, or associated with only one of other two in separate complexes. There are at least four arguments that support the presence of all three proteins in the same complex: 1] It would provide a simple mechanistic explanation for the ability of Rb to enhance the effects of ATF2; 2] The ability of Rb to enhance the

ability of ATF2 antibodies to bring down JNK and p38 indicates interaction among the three proteins; 3] The fact that expression of Rb mutants 9 and 14 strongly reduces the immunoprecipitation of JNK by ATF2 antibodies is hard to explain any other way (i.e., it sequesters JNK away from ATF2 which cannot bind to the mutant Rb); and 4] The increase in phosphorylation of ATF2 seen with Rb co-expression make it the only logical explanation.

The bridge function was first proposed by Wang et al. (1994), suggesting that the tyrosine kinase c-Abl could interact with the C pocket of Rb, while the A/B pockets of Rb presumably interact with a hypothetical transcription factor which can be phosphorylated by c-Abl. They showed that the integrity of both the A/B and C pockets is necessary for the full function of Rb, but they have not yet pinpointed a candidate protein (i.e. substrate for c-Abl) which could fit in the model.

Recently, rapid progress in studying cellular signaling pathways has led to the identification of scaffold proteins, i.e., JNK interacting protein-1 (JIP-1, Whitmarsh et al., 1998) and JNK/SAPK-associated protein 1 (JSAP1, Ito et al., 1999). The scaffold proteins interact with multiple components of the JNK signaling pathway, including the mixed-lineage group of MAP kinase kinase kinases (MLK), MKK7 and JNK, and selectively enhance JNK's activation by the MLK signaling pathway (Whitmarsh et al., 1998; Ito et al., 1999). It has

been suggested that scaffold proteins can mediate the activation of individual MAP kinase signaling pathway, increasing its specificity and efficiency through formation of multienzyme complexes in the cytoplasm (Whitmarsh and Davis, 1998). As pointed out by Schaeffer and Weber (1999), this scaffold-like effect of bridging proteins which are known to interact in solution (Dickens et al., 1997), may also serve to enhance the "regulatory flexibility" of such systems.

Do these scaffold proteins have counterparts in the nucleus to mediate downstream MAP kinase pathways? With what protein(s) does the proposed match-maker Rb actually connect? In this study, we confirmed recent reports (Chauhan et al., 1999; Shim et al., 2000) that JNK associates with Rb. We further discovered that the p38 kinase co-immunoprecipitates with Rb as well, presumably through the same mechanism as with JNK (discussed below). As discussed above, our co-immunoprecipitation results have suggested that ATF2 associates with the A/B pockets of Rb, and the evidences indicates that Rb interacts with JNK/p38 and ATF2 in the same macromolecular complex. Thus, we believe that Rb is a nuclear scaffold protein mediating the activation of ATF2 by either the JNK or p38 pathway.

The interaction of JNK with Rb has been shown to be direct and to involve the C pocket (Chauhan et al., 1999; Shim et al., 2000). The results we obtained with the different Rb mutants support this conclusion. Consistent with

the match-maker model, mutant 14, with a deletion in the C pocket which has little effect on ATF2 binding, exhibits significantly reduced stimulation of basal or ATF2-regulated TGF-β2 promoter activity. More importantly, mutant 14 shows a loss of the ability to enhance JNK immunoprecipitation by ATF2 antibodies despite its ability to bind ATF2 normally. This is most easily explained by the loss of JNK binding to the Rb mutant.

Similarly, mutant 9 with a deletion in A/B pockets shows an almost total loss of stimulatory effects on TGF-β2 promoter activity, most likely due to the loss of ATF2 binding. However, it should still be able to bind JNK since the C pocket is intact. The immunoprecipitation results show that overexpression of mutant 9 disrupts the association of JNK with ATF2, presumably due to loss of the scaffolding function of Rb.

The match-maker model suggests that Rb increases ATF2 activity by facilitating its interaction with JNK/p38, and hence increasing its phosphorylation/activation. Indeed, both JNK and p38 have been found to be able to phosphorylate ATF2 in vitro and in vivo (Gupta et al., 1995; Livingstone et al., 1995; van Dam et al., 1997). Our cotransfection results with MKK6, MKK7, JNK and p38 show substantial enhancement of the activity of ATF2 on the TGF-β2 promoter, which suggests that this process is activated by both JNK's and p38, in agreement with the results of others (Raingeaud et al, 1996;

Tournier et al, 1997). Thus, phosphorylation of ATF2 could account for the stimulation we have obtained.

It has been suggested that phosphorylation of Thr 69 and 71 is essential for ATF2 to exert its full effects (Gupta et al., 1995; Tournier et al., 1997; Raingeaud et al., 1996; Livingstone et al., 1995; Young et al., 1997) and the stimulation of ATF2's actions by Rb is significantly reduced when the Ala 69,71 double mutant protein is used (Gupta et al., 1995). These observations support our model that Rb increases the ATF2's activity through phosphorylation, although it was hard to understand this possibility when Gupta et al. (1995) observed it because Rb does not have any known kinase activity.

In addition to the phosphorylation mechanism, the possibility exists that formation of the triple complex with Rb, ATF2 and JNK/p38 increases the efficiency of subsequent interaction of ATF2 with co-activators like CBP/p300 (Kingsley-Kellesen et al., 1999; Kawasawi et al., 1998). It is also possible that both mechanisms operate in vivo but, whatever the case, it is clear that Rb is facilitating the interaction of other proteins.

In summary, the match-maker model provides a connection between MAP kinase signaling pathways and the tumor suppressor function of Rb. Since both are involved in the regulation of important functions associated with cell growth, differentiation and cell cycling, it is satisfying to observe that they

cross-react with each other, and regulate common downstream targets, like transcription factors such as ATF2 and C/EBP (see below), in a highly efficient, specific and controllable manner.

MAP kinase signaling pathway

The present results with ATF2 and the two kinases add to the list of systems in which both the JNK and p38 signaling pathways converge on a single transcription factor (Gupta et al., 1995; Jiang et al., 1996; Raingeaud et al., 1995; Tournier et al., 1997; Raingeaud et al., 1996; Cohen, 1997; Schaeffer et al., 1999; Yang et al., 1998). The two pathways can be regulated separately (Lin et al., 1995; Johnson et al., 1996; Cohen, 1997) but presumably ATF2 would play an important role under conditions which activated either pathway.

ATFa, on the other hand, appears to be selectively activated by the JNK pathway. One obvious question arises as to how the two kinases are able to distinguish between the two ATF's given the fact that they are highly homologous, especially around the site phosphorylated by both JNK and p38 (Meyer et al., 1993; Gupta et al., 1995; Tournier et al., 1997; Raingeaud et al., 1996; Livingstone et al., 1995; Cohen, 1997). Interestingly, p38 has been found to interact with different regions of substrates that are phosphorylated in

common with JNK and/or ERK (Schaeffer and Webber, 1999; Yang et al., 1998). Since JNK has been found to dock with the 20-30 amino acids just upstream of the phosphorylation site in ATF2 (Gupta et al., 1995; Livingstone et al., 1995), a region that is ~95% identical with ATFa and to which JNK2 has been found to bind (Bocco et al., 1996), p38 is likely to bind to a region that is not homologous in the two proteins, several of which can be found in several locations between the phosphorylation site and the basic DNA binding domain (Meyer and Habener 1993). Indeed, binding of p38 to ATF2 is quite weak relative to JNK when the N-terminal fragment (1-109) of ATF2 is used (Raingeaud et al., 1995).

Our results have confirmed not only the report by Chauhan et al (1999) but also extended it to include p38. Based on the fact that the common substrate ATF2 is bound at the A/B pockets of Rb and that JNK and p38 recognize several proteins in common (Jiang et al., 1996; Raingeaud et al., 1995; Raingeaud et al., 1996; Cohen, 1997; Schaeffer et al., 1999; Yang et al., 1998), it seems reasonable to suggest that p38 also binds to the C pocket. C/EBP, another bZip family member, has been found to bind to the A/B pockets of Rb and this interaction enhances adipocyte differentiation (Chen et al., 1996). Recently, it has been found that p38 is also able to regulate the ability of C/EBP to bring about adipocyte differentiation (Wang et al., 1996; Engelman et al.,

1999; Nebreda and Porras, 2000). Based on our results, it is tempting to predict that the role of Rb in that system will be to form a triple complex with C/EBP and p38.

It is intriguing to point out that the JNK/p38-ATF2 signaling systems are employed not only in the elaboration of TGF-β2 and TNFα (Kim et al., 1992; Gong et al., 1995; Tsai et al., 1996) but also in the responses generated by their subsequent binding to receptors in similar or different cells (Brinkman et al., 1999; Hanafusa et al., 1999). If similar cells are involved, then an auto-stimulatory cycle would result. If different cells are involved, then other outcomes could take place depending upon the state of differentiation of the target cells. These effects could represent an important way to provide sustained signaling to a variety of cells involved in such processes as differentiation, inflammation and wound healing (Meyer and Habener, 1993; Raingeaud et al., 1995; Cohen, 1997; Brinkman et al., 1999; Schaeffer and Weber, 1999; Nebreda and Prorras, 2000; Hanafusa et al., 1999).

Association of ATF2 with Rb

One question from the co-immunoprecipitation results is whether the interaction between ATF2 and Rb is direct. Although the co-

immunoprecipitation results imply that Rb interacts with ATF2, they do not eliminate the possibility of indirect interaction through a mediator protein. Our results and those of Kim et al (1992) can be easily explained by the fact that retention of ATF2 by Rb is obtained only in the context of a crude nuclear extract from CHO or HeLa cells and a bridging protein could account for the observed interaction. Indeed, hBrm, a human SWI2/SNF2 homologue, has been found to bridge between the glucocorticoid receptor and Rb (Singh et al, 1995) in mediating the latter's enhancing effects on those of adrenal steroids (e.g. hbrm).

Although it is possible that an additional protein[s] actually binds to the A/B pocket domains and it interacts with ATF2, no extra stained bands are seen in gels from the immunoprecipitates that might support the existence of such a bridge protein. Dr. Wicks tried for several months to obtain evidence for direct interaction between Rb and ATF2 in vitro (data not shown). His approach was to use a GST-Rb fusion protein and bacterially expressed purified N-terminal His-tagged ATF's. Neither ATF2 or ATFa could be retained on immobilized GST-Rb using GSH agarose beads. Similarly, negative results were obtained using nickel sepharose beads to bind the His-tagged ATF's after incubation with Rb in vitro. These results imply that the binding between ATF2 and Rb may not be direct, although the experiments did not use the natural proteins, i.e., the His

tag on the ATF2 molecule may interfere with the potential binding site. This seems especially possible given that the N-terminal domain of ATF2, where the His tag is located, contains the phosphorylation and transactivation sites (Meyer and Habener, 1993).

Another fact consistent with possible indirect binding is that none of the known Rb binding motifs can be found in either ATF. Although many proteins bind to the A/B pockets of Rb, it has been suggested that different motifs are involved. For example, the T antigen of SV40, adenovirus E1A oncoprotein and other oncoproteins bind Rb 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 Rb; and pp1a uses a different, large, C-terminal region to bind Rb (Lee et al., 1994).

On the other hand, Rb has been reported to bind to both C/EBP β and NF-IL6, two closely related bZip family members, although the former interaction was reported to be unstable when a target DNA oligonucleotide was present (Chen et al 1996a; Chen et al, 1996b). The Rb binding site on C/EBP β was not determined but NF-IL6 has sites in both the NTD and CTD capable of binding to Rb. Both C/EBP β and NF-IL6 contain a Rb binding motif in their NTD which is homologous to that found in E2F (Chen et al 1996a; Chen et al; 1996b;). The motif involved is not present in either ATF, however. Rb has also

been shown to bind to c-Myc and to upregulate its transcriptional activity (Adnane and Robbins, 1995). The NTD of Myc is responsible for binding to Rb, the NTD of ATF2 is required for its interaction with Rb in HeLa nuclear extracts (Kim et al, 1992) and the NTD's of C/EBP β and NF-IL6 have also been implicated in binding to Rb. However, there is little homology between ATF2 and these other factors in their NTD's . Indeed, the common feature shared by c-Myc, C/EBP β, NF-IL6 and the ATF's is their bZip domain which is in the CTD. From these results it is difficult to identify a consensus domain which could account for the actions of Rb on bZip protein functions. It is clear, however, that all of these transcription factors depend on the integrity of the A and B pockets of Rb for their functional interactions. This is true for many Rb binding proteins. It does not suggest, on the one hand, that all bZip proteins interact directly with Rb or rule out, on the other hand, that bridge proteins may be involved in interactions of some of them with Rb.

Differences between ATFa and ATF2

So far, based on the present study and earlier report (Gong et al., 1995), three functional differences in the actions of ATFa and ATF2 have been recognized: 1] Rb inhibits ATFa's activity while it enhances that of ATF2; 2]

MKK7/JNK but not MKK6/p38 are able to enhance the effects of ATFa while both sets augment the actions of ATF2; and 3] No significant effects of any of the cJun/cFos constructs has been observed on the actions of ATFa but cJun enhances the effects of ATF2.

First, since ATFa and ATF2 are very homologous in terms of amino acid sequence and closely related in their transcriptional functions, it raises the question as to how these related proteins can be differentially regulated by Rb. The lack of interaction of ATFa and Rb is striking and can certainly account for the fact that Rb does not enhance the actions of ATFa. It is harder to explain the inhibitory effects of the combination of Rb and ATFa. Given the fact that Rb reduces the amount of JNK in the anti-ATFa immunoprecipitate, it is conceivable that Rb acts by sequestering JNK largely away from ATFa. This suggestion is supported by a recent report that Rb has a inhibitory effect on JNK through its C pocket presumably by sequestering JNK away from cJun (Shim et al., 2000). However, the impact of mutations in the A/B pocket of Rb in reducing the actions of ATFa is difficult to understand. It suggests the requirement for a protein to be bound to the A/B pockets which is important for the actions of ATFa. The putative A/B pocket protein could be a co-activator or hetero-dimeric partner of ATFa. At present, we simply do not have enough information to fully explain the negative effects of Rb on ATFa's actions.

Second, both ATF's activities are enhanced by co-transfection of MKK7/JNK. These results are consistent with the reported interaction of ATFa with JNK in vivo (Bocco et al, 1996) and in vitro (Gupta et al, 1995). ATFa and ATF2 are readily phosphorylated in our hands by JNK and the cAMP dependent protein kinase (data not shown). Thus, our observed lack of phosphorylation/activation of ATFa by p38 is not likely to be an artifact. As discussed above, p38 may use docking sites which are different between ATF2 and ATFa. In a broader context, various stimuli, especially stress and/or the inflammatory response could, by virtue of selective stimulation of the appropriate kinase cascade, differentially activate these two ATF's leading to potentially differential actions on CRE-containing promoters. Given the presence of several isoforms of JNK and p38 (Fanger et al, 1997; Gupta et al, 1996; Jiang et al, 1996), however, the situation in any given cell type is likely to be more complex.

Third, our results indicate that cJun increases ATF2's action but not that of ATFa, while cFos does not have any significant effect on either ATF. Consistent with these results, a very clear pattern was obtained with the inhibitory effects of the dominant negative cJun mutant TAM on ATF2's actions supporting a role for ATF2-Jun heterodimers acting on the TGF-β2 promoter. These results are not surprising, considering the previous observations of

differences in heterodimerization between ATFa and ATF2 (Hai and Curran, 1991; Benbrook and Jones, 1994; Chatton et al 1994), and are consistent with the fact that ATF2-cJun heterodimers activate several promoters bearing partial CRE's (Kaszubska et al, 1995; Liang et al, 1996; Newell et al, 1995; Karin, 1995).

These results argue strongly against any role for Jun or Fos in the actions of ATFa, thus presumably removing one potential explanation for the differential effects of Rb on these two ATF's; i.e., formation of ATFa-Fos heterodimers which appear to be inactive as transcription factors (Chatton et al, 1994). Since cycling cells have little or no Fos (Karin, 1995; Lallemand et al, 1997), it is unlikely that dimerization with it could be responsible for the negative effects of Rb on ATFa in any case.

Taking the absence of endogenous ATFa into account, ATFa may not play any important role in regulating TGF-β2 in the cells we used for this study. The overall roles of ATFa and ATF2 are still evolving and their involvement in global transcriptional regulation is uncertain. However, the ATF2 knockout mouse exhibited many more severe abnormalities than was seen with the CREB knockout (Reimold et al, 1996; Maekawa et al., 1999). Many major systems were affected raising the suggestion that ATF2 is also important in regulating

development. Whether or not ATFa plays a global role anything like that of its related factor and how Rb inhibits its actions both remain to be determined.

The results with PC-3 cells suggest that the mechanisms involved in regulating ectopic TGF-β2 promoter activity must bear significant similarity to those operating on the chromosomal TGF-β2 promoter. In no instance did we observe a significant divergence in the effects of any combination of expressed proteins between the two assays and 26 different combinations were tested, and the overall correlation is very high with a R squared of 0.875 (n = 115, excluding one outlier). Thus, we have evidence to believe that our study is physiologically relevant.

Perspective and future studies

As with many other studies, this study raised more interesting questions than it answered. Some of them are listed here. Hopefully, these may serve as an incentive for potential future projects to uncover more information about the molecular mechanism involved in the regulation of gene expression by the Rb-driven complex.

First, what is the phosphorylation status of Rb and how does its phosphorylation affect its putative scaffold protein function? It has been known

that the phosphorylation of Rb is cell-cycle dependent and is mediated primarily by the cyclin D1/cdk4 pathway (Mancini et al., 1994; Wang et al., 1994). Recently, it has been suggested that JNK binds to the C pocket domain of Rb and subsequently phosphorylates it (Chauhan et al., 1999; Shim et al., 2000). It is also been suggested that p38 can reverse Rb's inhibitory function on E2F1 (Wang et al., 1999). Taking together, these results suggest that Rb's function may be regulated by the MAP kinase signaling pathway through phosphorylation. We suspect that phosphorylation of the C pocket domain by JNK/p38 may well serve as a switch to determine the protein binding activity of the A/B pockets, and hence to regulate the function and/or formation of this macromolecular complex.

Second, do ATF2, Rb and JNK/p38 co-localize in the nucleus? Since Rb, ATF2 and JNK/p38 exist in the same complex as suggested by the pull-down experiments, we predict that these proteins should be localized together at least to some detectable extent. Rb has been found to be a nuclear protein and bind to nuclear matrix (Mancini et al., 1994; Wang et al., 1994); ATF2 and activated JNK/p38 have been found inside the nuclear membrane (Gupta et al., 1995); thus, it is clear that the putative macromolecular complex is likely to be in the nucleus. The co-localization experiments would provide further evidence for the

existence of this macromolecular complex and provide more physiological relevance.

Third, does interaction of Rb and ATF2 involve direct binding? As we discussed above, this issue has not been solved by the current study. Different approaches, i.e., using pure proteins or using an antibody immobilization method, may be helpful to detect possible direct binding. Or, in an effort to identify potential mediating protein(s), one may purify the active component(s) from cell extracts monitored with an assay that detects the association of Rb and ATF2. These studies, together with truncated mutation approach, may also help to find the Rb's interaction site on ATF2. We predict that it may be located in the middle region of the ATF2 molecule for two reasons: 1] This region is where ATF2 and ATFa differ the most; 2] The NTD of ATF2 is involved in the phosphorylation and activation function while the CTD binds to DNA promoter elements and the dimeric partner. It has not been observed that interaction with Rb interferes with any of these interactions so far. Thus, it seems likely that those regions may not mediate the interaction with Rb.

Fourth, what is the mechanism by which phosphorylation of ATF2 increases TGF-β2 promoter activity? Our results suggest that phosphorylation of ATF2 is facilitated by interaction with Rb and causes increase in ATF2 activity. The mechanism involved in the latter process remains to be answered.

It has been suggested that phosphorylation increases the ability of ATF2 to bind to CRE-containing promoters (Gupta et al., 1995). It is also possible that phosphorylation may affect the ability of ATF2 to bind to the Cys/His-rich region of its coactivator CBP/p300 via bZip domain (Sano et al., 1998). CBP binds to multiple components of the basal transcriptional machinery, including TFIIB, and facilitates the activities of ATF2 (Kwok et al., 1994). Additionally, it has been found recently that ATF2 has intrinsic histone acetyltransferase activity which is modulated by phosphorylation (Kawasaki et al., 2000). It will be very interesting to examine which of the three mechanisms, among others, is involved in regulation of TGF-β2 promoter activity in transfected CHO cells and how the putative Rb complex connects to the activation mechanism, i.e., whether or not the match-maker model may extend to include CBP/p300.

Fifth, what is the specificity of the macromolecular complex? We tested JNK2 and p38β for their interaction with Rb and roles as regulators in this study. There are more isoforms for each MAP kinase, and clear differences in function have been reported (Dérijard et al., 1995; Jiang et al., 1995; Karin and Hunter, 1995; Waskiewicz and Cooper, 1997; Fanger et al., 1997). It is important to examine whether other isoforms of JNK and p38 can interact with Rb and up-regulate ATF2's actions. These experiments can potentially help to reveal the specificity of the macromolecular complex. It is likely that different

kinases have different affinities (if any) to interact with Rb, and hence their ability to modify downstream targets could be selectively regulated by Rb.

Finally, in addition to TGF-β2 gene, what other downstream gene expression targets are there for the putative Rb-driven macromolecular complex? As we discussed for the case of C/EBP above, the cross-reaction between MAP kinases JNK/p38 and Rb very likely regulates other transcription factors, and, hence, can influence expression of a variety of genes. This suggestion is based upon the facts that numerous transcription factors have been found to associate with Rb (Wang et al., 1994), and JNK and p38 regulate many different targets as well (Karin and Hunter, 1995; Waskiewicz and Cooper, 1997). In the unlikely scenario that only ATF2 can be regulated by this mechanism, even activated ATF2 may enhance the expression of many different genes including TGF-β2 (see Introduction for the long list). Thus, it would be very interesting to study the global regulatory effects of the combinations of Rb ± JNK/p38 ± ATF2, to determine which genes are influenced by this complex and the mechanism involved. Recent development of DNA micro-array techniques may provide great leverage in exploration of this aspect.

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APPENDIX

Tables and Figures

Table 1. List of selected Rb-associated proteins.

Protein	Function	Interaction sites in Rb	Consequence of interacting with Rb
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
ATF2	Transcription factor	A/B	Enhances activity
c-Abl	Tyrosine kinase	C	Inhibits tyrosine kinase
D1, D2, D3	Cyclins	Large A/B	Targets Rb for phosphorylation?
cdk2, cdk4	Ser/Thr kinase	?	Rb phosphorylation?
pp1a	Phosphatase	Large A/B	Rb de-phosphorylation?

Table 2. Summary of Rb's differential regulation of ATFa and ATF2'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
ATF2	4.3 ± 0.3 (n=12)	4.1 ± 0.3 (n=4)
ATFa	4.0 ± 0.3 (n=12)	3.3 ± 0.3 (n=4)
Rb	4.2 ± 0.5 (n=22)	11.3 ± 0.5 (n=4)
ATF2 with Rb	18.4 ± 1.2 (n=22)	19.9 ± 1.1 (n=4)
ATFa with Rb	1.9 ± 0.3 (n=22)	2.4 ± 0.3 (n=4)

Note: Adapted from Li, 1996. The pB2-77 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 ± 0.3 cpm/μg in transfected CHO cells, and 2.8 ± 0.4 cpm/μg in SKUT-1 cells.

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

Rb Construct	Deletion Location	% activity of WT Rb		
		alone	with ATF2	with ATFa
WT	none	100	100	-100
mt9	Pocket A	16*	3*	-11*
mt12	Pocket B	9*	2*	7*
mt14	Pocket C	55*	36*	-33*

Note: Adapted from Li (1996). The above values are the percentage of the activity observed with Rb. The activities of wt Rb were set as 100% except that with ATF-a was set as -100% due to the inhibitory effects of wt Rb. * indicates a statistically significant difference ($P < 0.001$) 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 Rb wt, Y represents the mean levels of Rb mutants; Nx and Ny represent the number of test samples of Rb WT and mutants, respectively; Sx and Sy represent the standard deviations of Rb WT and mutants, 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: $\upsilon = [Nx + Ny] -2$.

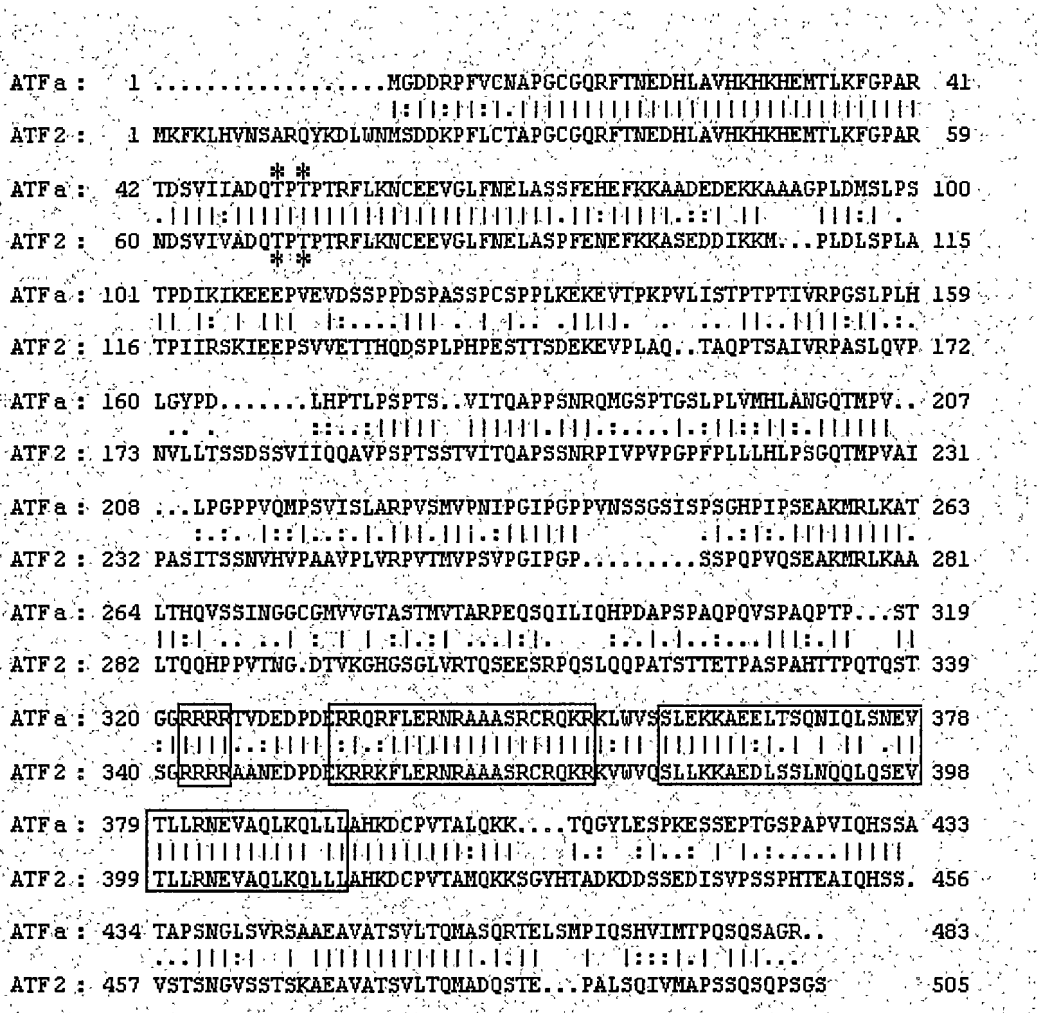

Figure 1. Amino acid sequence and homology comparison between ATFa and ATF2. The boxed areas correspond to the basic region (ATFa: 322-325 and 334-354; ATF2: 342-347 and 356-376) and Leucine Zipper domain (ATFa: 360-391; ATF2: 380-411). Asterisk symbols indicate the phosphoralation sites on ATF2 (Thr 69 and 71) and ATFa (Thr 51 and 53).

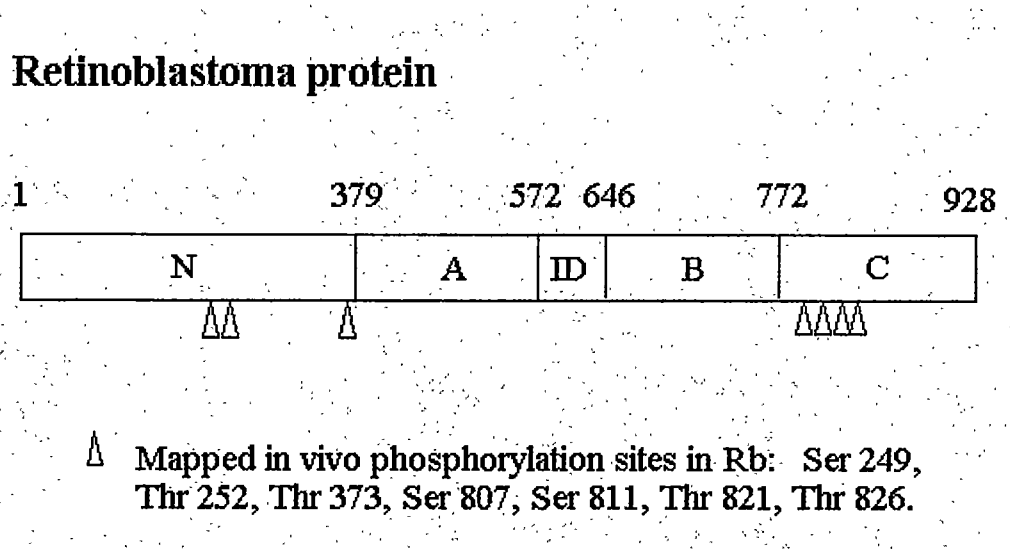

Figure 2. Domain structure and phosphorylation sites of Rb. The human Rb protein contains 928 amino acids that are organized into several domains: the N terminal region (amino acids 1-378), domain A (A, amino acids 379-572), the insert domain (ID, amino acids 573-645), domain B (B, amino acids 646-772) and the C terminal region (C, amino acids 773-928). There are 7 Ser/Thr sites in Rb (indicated by arrow heads), which have been shown to be phosphorylated by the cyclin-dependent protein kinases in vivo.

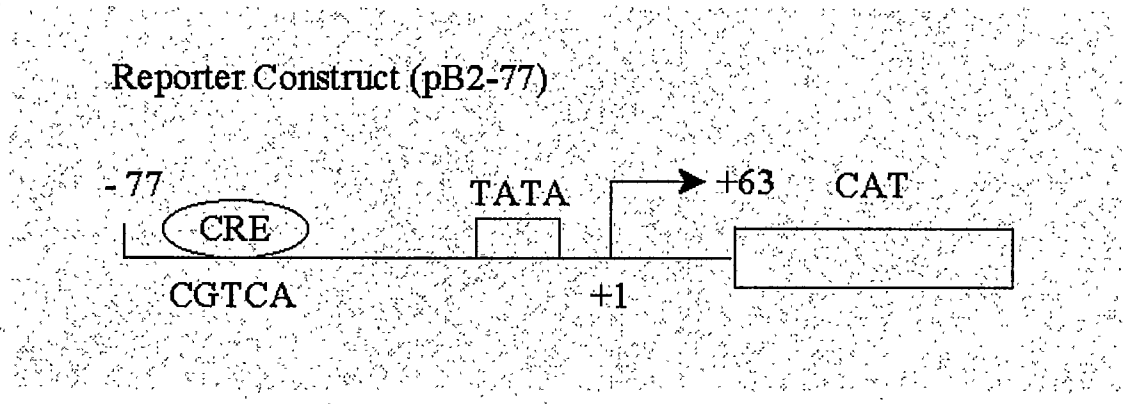

Figure 3. Schematic structure of pB2-77 construct. As indicated in the figure, the construct has a partial CRE site (CGTCA) and TATA box from the TGF-β2 promoter (-77 to 63), which is linked to the downstream cDNA coding for the CAT protein.

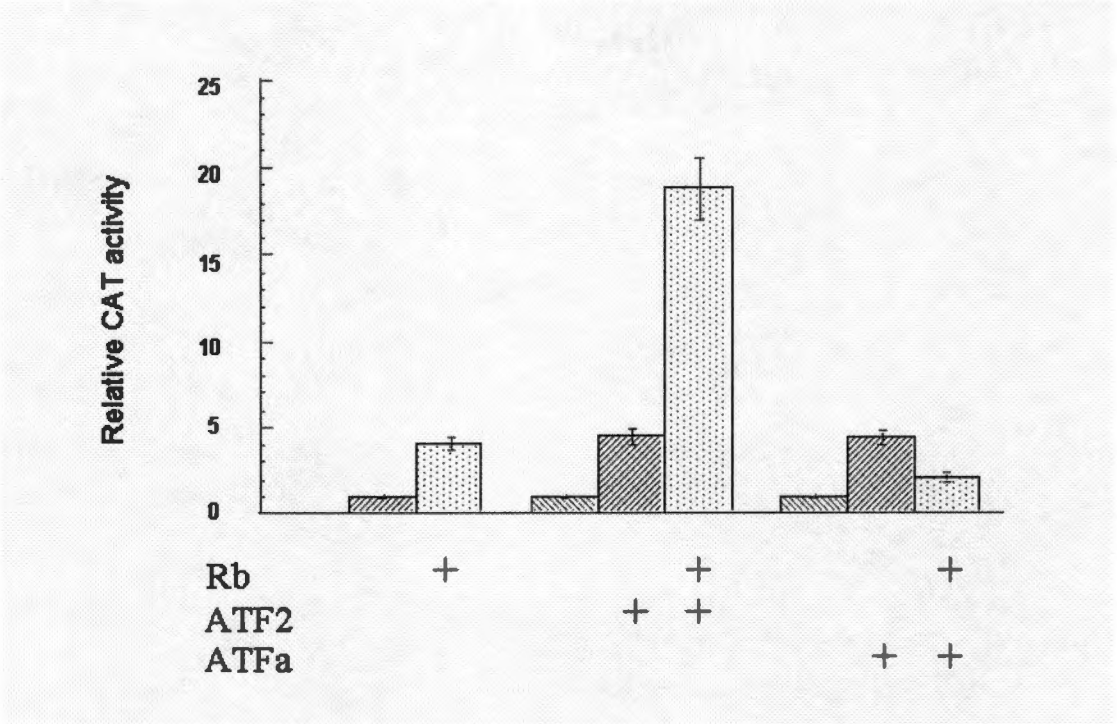

Figure 4. Rb differentially modulates the transcriptional activating effects of ATFa and ATF2 in CHO cells. Adapted from Li, 1996. One half μg of each ATFa, ATF2, or Rb plasmid DNA (as indicated in the figure) was co-transfected with 0.5 μg of TGF-β2 CAT reporter plasmid DNA into 35 mm dishes of 50-80% confluent CHO cells. After transfection, CHO cells were incubated in the medium containing 10% fetal bovine serum for about 54 hr, then CHO cells were harvested, lysed and subjected to CAT assay as described in Methods and Materials. 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 twelve samples in no less than six independent experiments, and error bars indicate the standard deviation of samples. In this and subsequent figures relative CAT activity is defined as the ratio of CAT activity to that observed with cells transfected with reporter construct alone. The basal activity of reporter construct TGF-β2 CAT DNA is 4.0 ± 0.3 cpm/μg in transfected CHO cells.

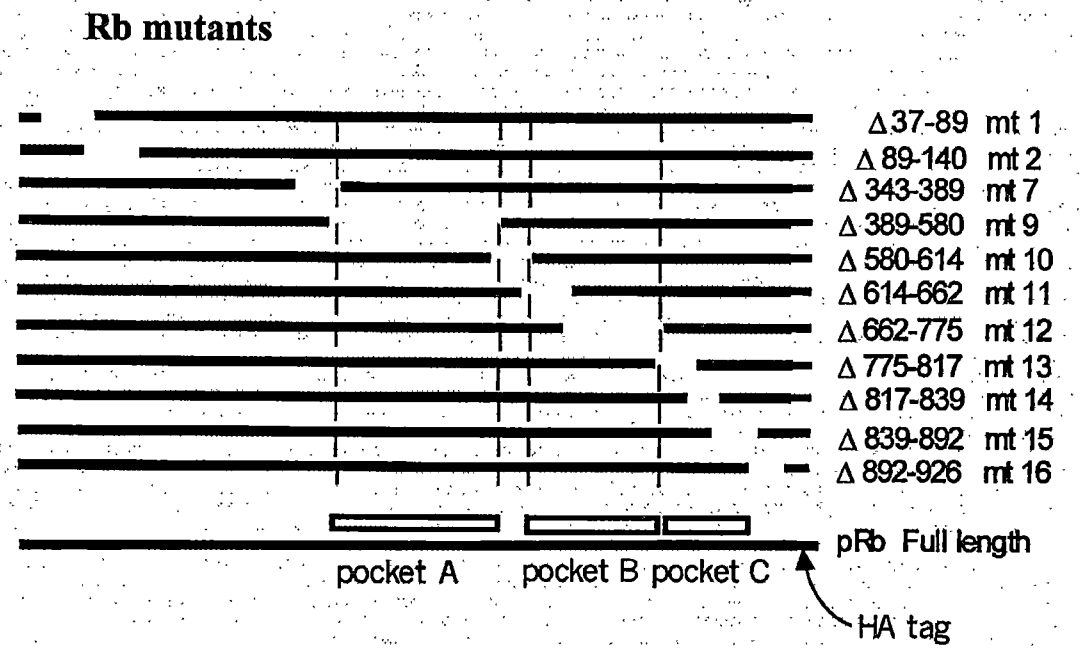

Figure 5. Schematic representation of deletion mutants of Rb expression vectors used in this study. The coding region of Rb is represented by the line at the bottom, locations of pockets A, B and C are represented by the bottom boxes, and the synthetic epitope tag is represented by the shaded box at 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.

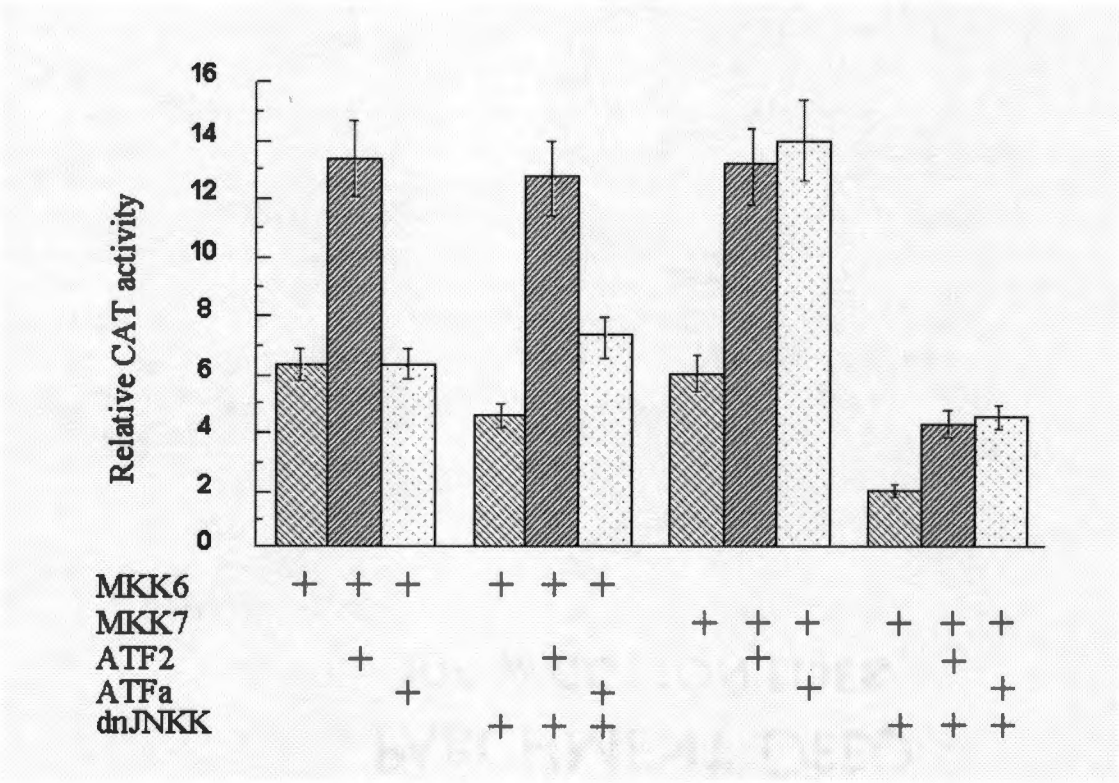

Figure 6. Effects of a dominant negative JNKK mutant [K116R] on the ability of MKK6 and MKK7 to enhance TGF-β2-CAT activity in CHO cells ± ATFa and ATF2. CHO cells were transfected under conditions similar to those in Figure 4 with the following DNA constructs: 0.5 μg of TGF-β2–CAT reporter plasmid and, where indicated, 0.5 μg of ATF2 , ATFa or dnJNKK[K116R] expression plasmids ± 0.2 μg of MKK6 or 0.1 μg of MKK7 expression plasmids. The amounts of MKK6 and MKK7 plasmid DNA were optimized in titration experiments [data not shown]. Cells were harvested for assays 54 hr after transfection was initiated as described in Materials and Methods. Each value represents the mean fold increase in CAT activity relative to that in cells transfected with reporter construct alone. This experiment was repeated and each column is the average of at least four samples in no less than two independent experiments, and error bars indicate the standard deviation of samples.

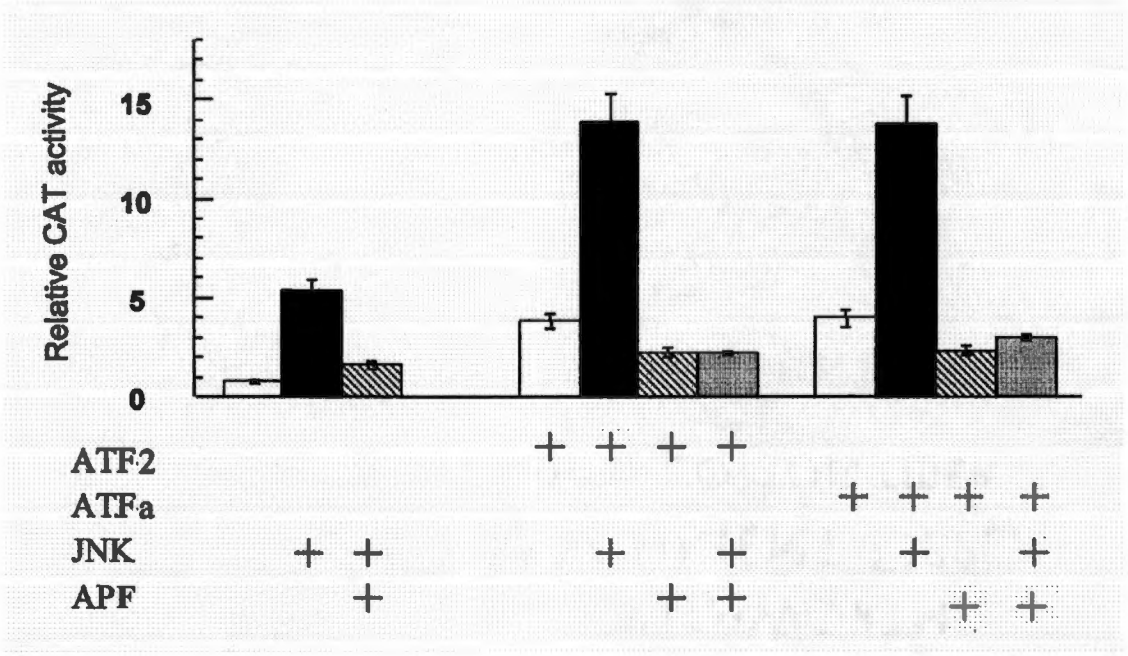

Figure 7. Effects of a dominant negative JNK mutant [APF] on the ability of JNK to enhance TGF-β2-CAT activity in CHO cells ± ATFa and ATF2. CHO cells were transfected under conditions similar to those in Figure 4 with the following DNA constructs: 0.5 μg of TGF-β2–CAT reporter plasmid and, where indicated, 0.5 μg of ATF2 , ATFa or APF expression plasmids ± 0.2 μg of JNK expression plasmids. The amount of JNK plasmid DNA were optimized in titration experiments [data not shown]. Cells were harvested for assays 54hr after transfection was initiated as described in Materials and Methods. Each value represents the mean fold increase in CAT activity relative to that in cells transfected with reporter construct alone. This experiment was repeated and each column is the average of at least four samples in no less than two independent experiments, and error bars indicate the standard deviation of samples.

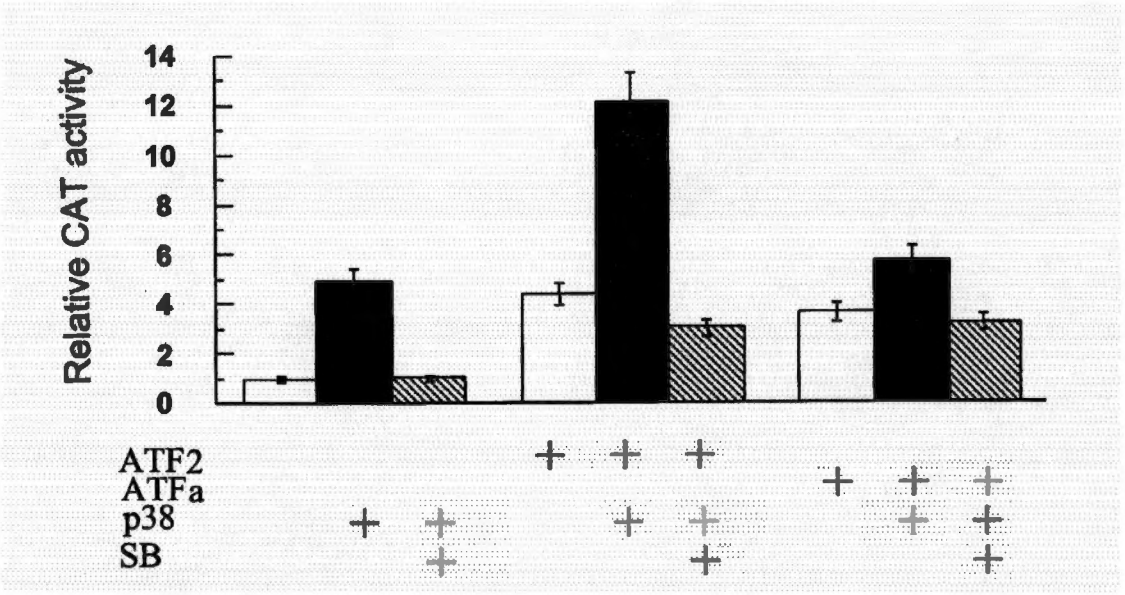

Figure 8. Effects of SB203580 on the ability of JNK to enhance TGF-β2-CAT activity in CHO cells ± ATFa and ATF2. CHO cells were transfected under conditions similar to those in Figure 4 with the following DNA constructs: 0.5μg of TGF-β2–CAT reporter plasmid and, where indicated, 0.5 μg of ATF2 , ATFa, ± 0.2 μg of p38 expression plasmids. The amount of p38 plasmid DNA used was optimized in titration experiments [data not shown]. 10 μM of SB 203580 (SB) was added 6 hr after transfection. Cells were harvested for assays 54hr after transfection was initiated as described in Materials and Methods. Each value represents the mean fold increase in CAT activity relative to that in cells transfected with reporter construct alone. This experiment was repeated and each column is the average of at least four samples in no less than two independent experiments, and error bars indicate the standard deviation of samples.

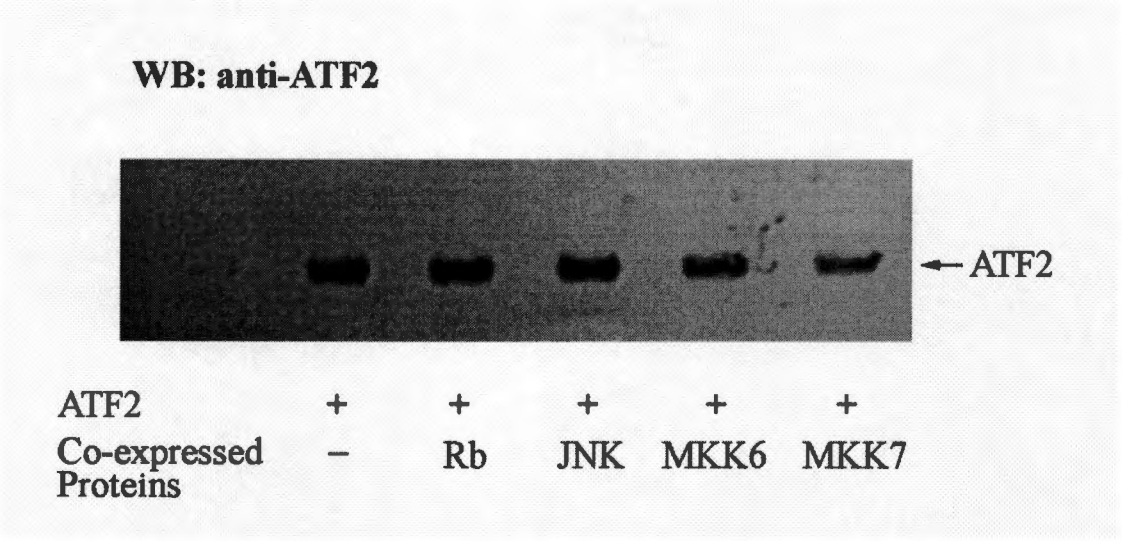

Figure 9. Protein expression level of ATF2 in transfected CHO cells. CHO cells were transfected by ATF2 or with other indicated expression vectors: Rb, JNK, MKK6, or MKK7. Fifteen µl of cell lysates were subjected to SDS-PAGE, and probed by western blotting with anti-ATF2 antibodies (see Materials and Methods for details). The first lane was loaded with cell extract from non-transfected cells, to show the expression level of endogenous ATF2. The molecular weights of the bands were determined by the standard curve using included pre-stained molecular markers (bands not shown), and were approximately as expected for ATF2. This experiment has been repeated with similar results.

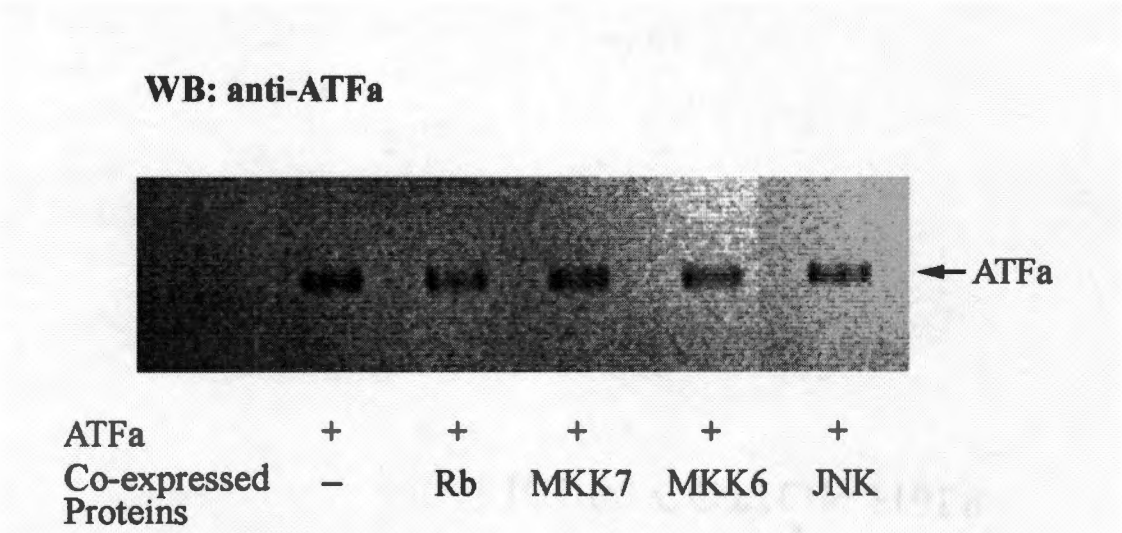

Figure 10. Protein expression level of ATFa in transfected CHO cells. CHO cells were transfected by ATFa or with other indicated expression vectors: Rb, MKK7, MKK6, or JNK. Fifteen μl of cell lysates were subjected to SDS-PAGE, and probed by western blotting with anti-ATFa antibodies (see Figure 9 for other details). The molecular weights of the bands were approximately as expected for ATFa. This experiment has been repeated with similar results.

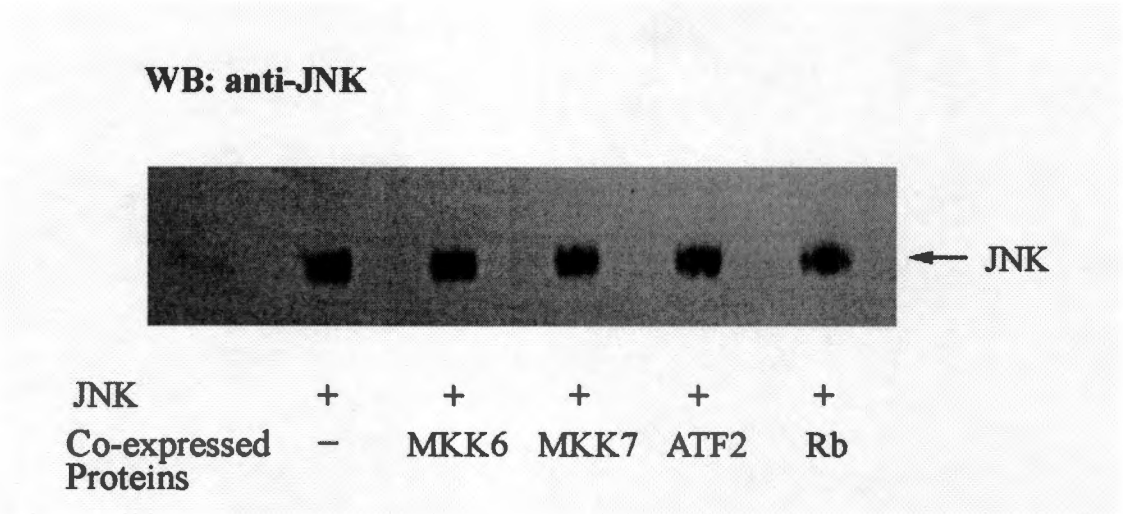

Figure 11. Protein expression level of JNK in transfected CHO cells. CHO cells were transfected by JNK or with other indicated expression vectors: MKK6, MKK7, ATF2 or Rb. Fifteen μl of cell lysates were subjected to SDS-PAGE, and probed by western blotting with anti-JNK antibodies (see Figure 9 for other details). The molecular weights of the bands were approximately as expected for JNK. This experiment has been repeated with similar results.

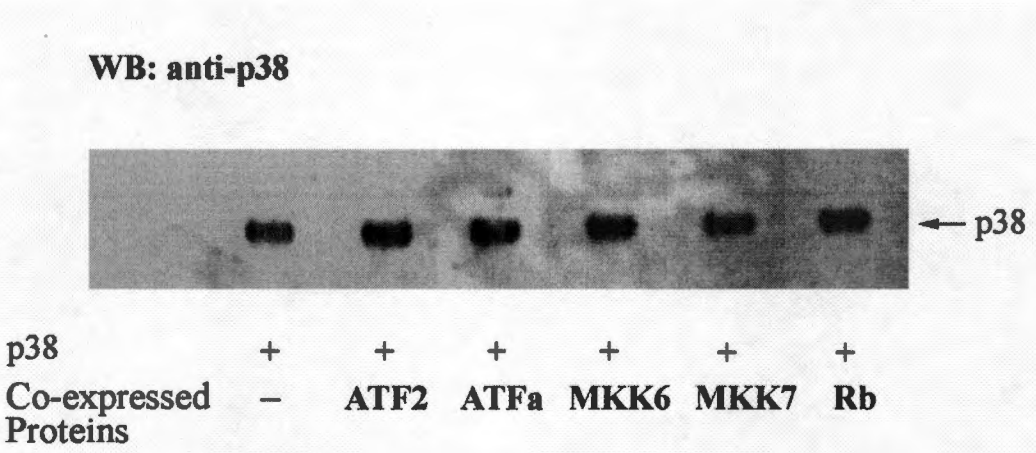

Figure 12. Protein expression level of p38 in transfected CHO cells. CHO cells were transfected by p38 or with other indicated expression vectors: ATF2, ATFa, MKK6, MKK7, or Rb. Fifteen μl of cell lysates were subjected to SDS-PAGE, and probed by western blotting with anti-p38 antibodies (see Figure 9 for other details). The molecular weights of the bands were approximately as expected for p38. This experiment has been repeated with similar results.

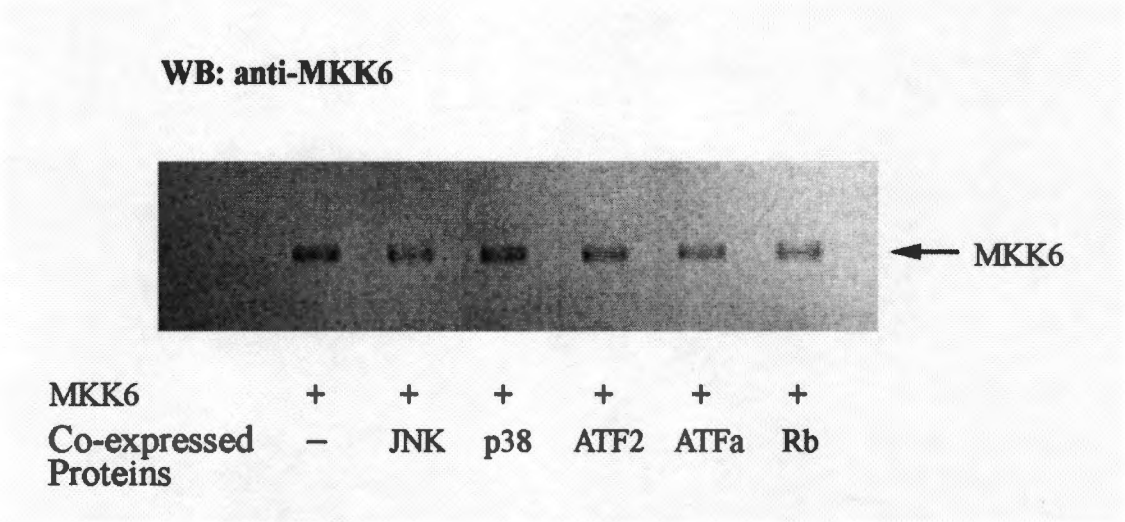

Figure 13. Protein expression level of MKK6 in transfected CHO cells. CHO cells were transfected by MKK6 or with other indicated expression vectors: JNK, p38, ATF2, ATFa, or Rb. Fifteen μl of cell lysates were subjected to SDS-PAGE, and probed by western blotting with anti-MKK6 antibodies (see Figure 9 for other details). The molecular weights of the bands were approximately as expected for MKK6. This experiment has been repeated with similar results.

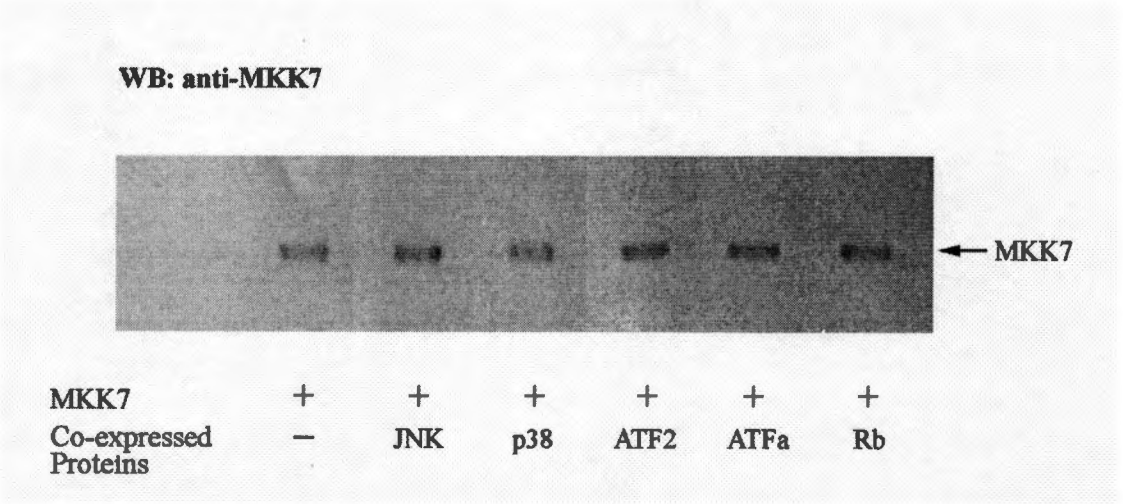

Figure 14. Protein expression level of MKK7 in transfected CHO cells. CHO cells were transfected by MKK7 or with other indicated expression vectors: JNK, p38, ATF2, ATFa or Rb. Fifteen μl of cell lysates were subjected to SDS-PAGE, and probed by western blotting with anti-MKK7 antibodies (see Figure 9 for other details). The molecular weights of the bands were approximately as that of MKK7. This experiment has been repeated, and observed similar results.

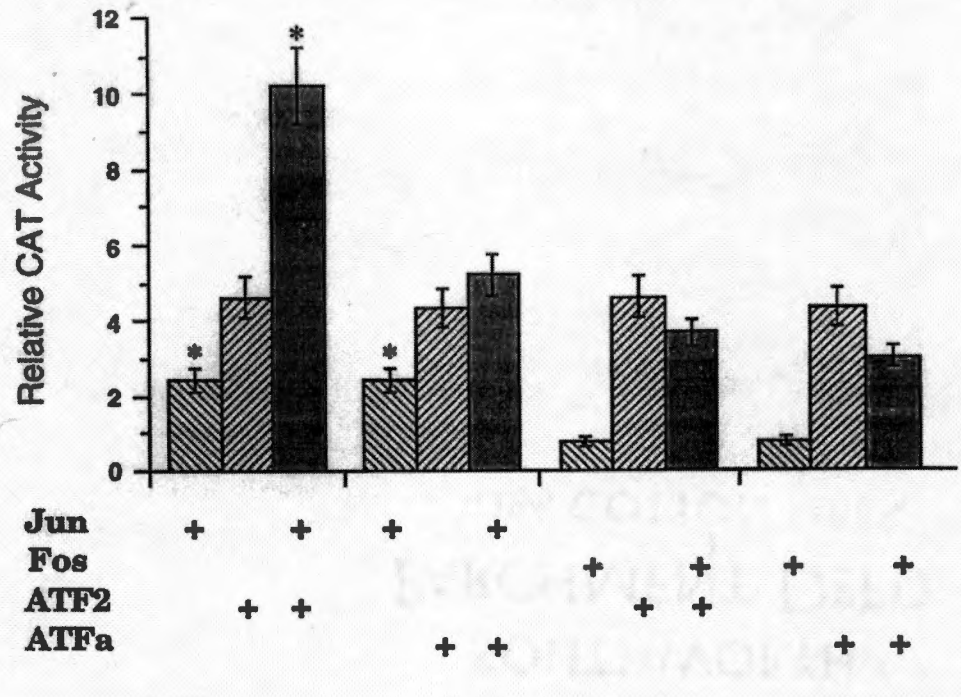

Figure 15. Effects of cJun and cFos co-expressed with ATFa and ATF2 on TGF-β2–CAT activity in CHO cells. CHO cells were transfected with 0.5 μg of TGF-β2–CAT DNA and 0.5 μg of ATFa, ATF2, cJun or cFos expression plasmid DNA's under conditions similar to those described in the legend to Figure 1. Cells were harvested 54 hr later for assays as described in Materials and Methods. Each value is the mean fold increase in CAT activity relative to that in cells transfected with reporter construct alone ± SD of 4-8 observations. Asterisk symbols indicate a statistically significant effect of cJun/cFos (i.e., $P < 0.001$).

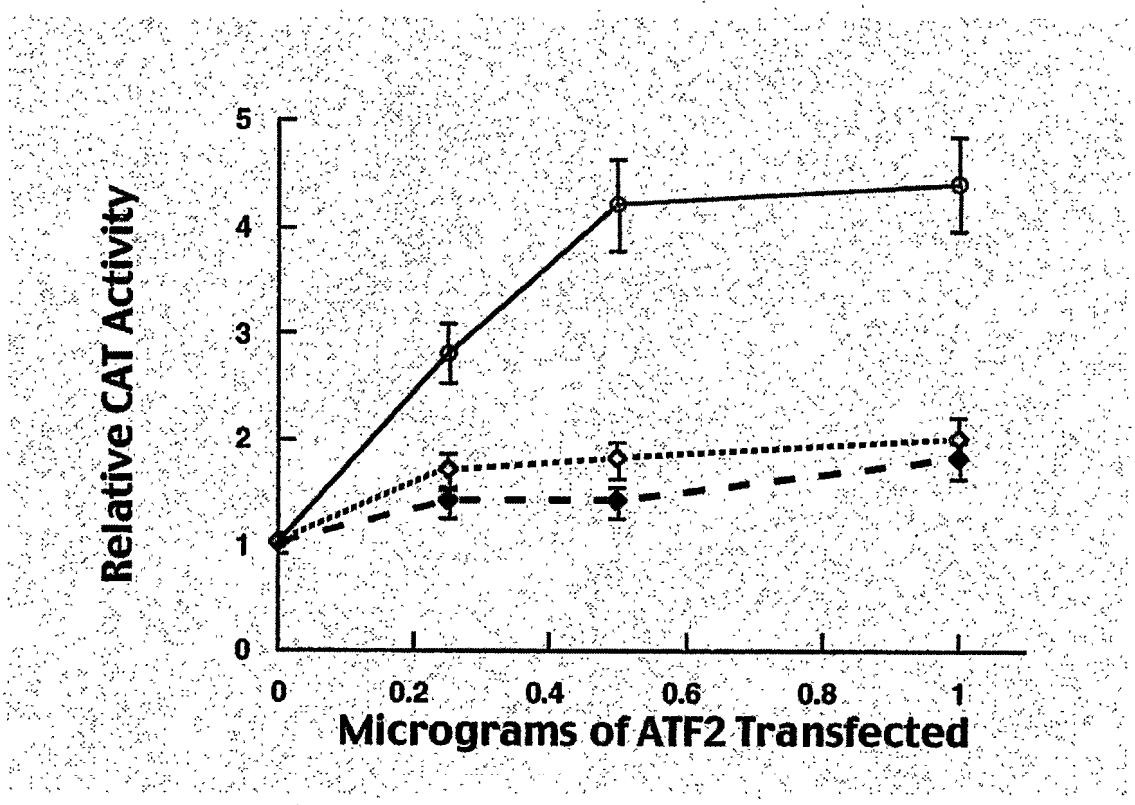

Figure 16. Titration of ATF2 with different amounts of TAM67. Varying amounts of ATF2 plasmid DNA's were co-transfected ± the indicated amounts of TAM 67 plasmid DNA and 0.5 μg of the TGF–β2–CAT reporter construct into CHO cells under conditions similar to those described in the legend to Figure 4. After incubation for 54 hr, cells were harvested for assays as described in Materials and Methods. Since TAM 67 significantly reduced basal TGF–β2–CAT expression (not shown), these values have been normalized to 1.0 for calculation of relative CAT activities [i.e., specific activity of test sample/ specific activity of samples from cells transfected with TGF–β2–CAT DNA alone ± TAM 67]. Each point is the average of at least 6 observations with SD's indicated by the bars. Symbols in the figure indicates the following.
Open circle: no TAM 67 co-transfected.
Open diamond: 0.5 μg TAM 67 DNA co-transfected.
Filled diamond: 1.0 μg TAM 67 DNA co-transfected.
See the legend to Figure 4 for further details.

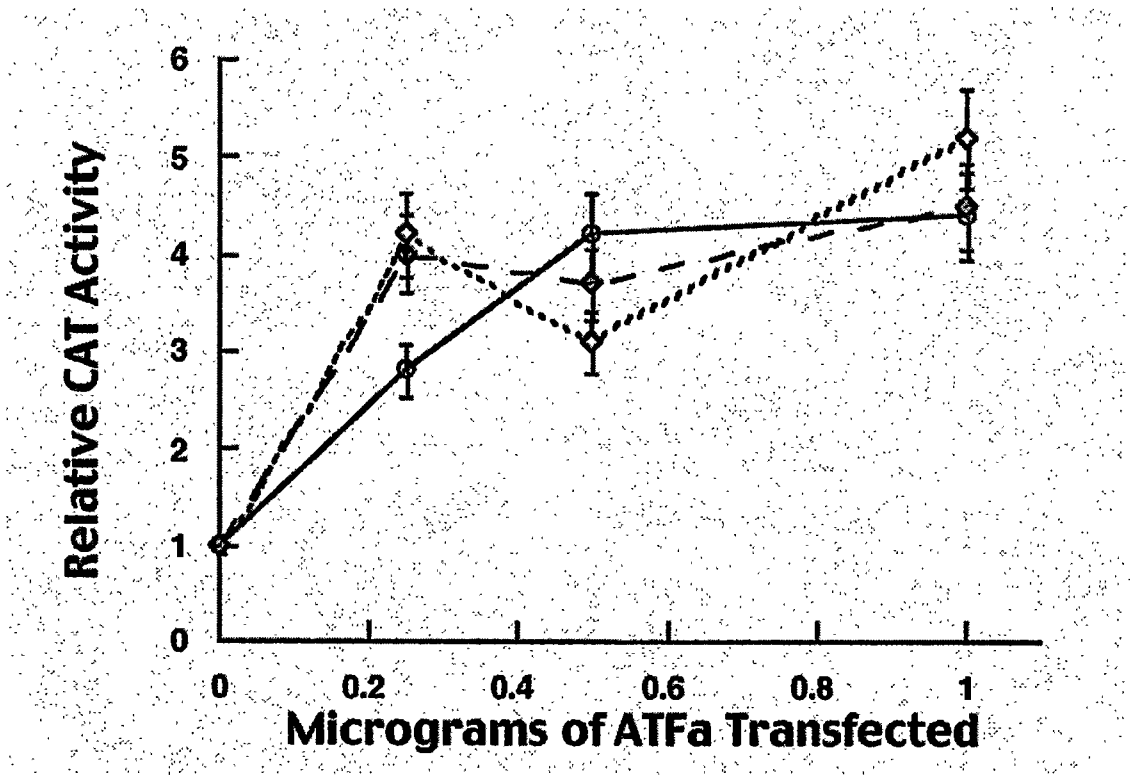

Figure 17. Titration of ATFa with different amounts of TAM67. Varying amounts of ATFa plasmid DNA's were co-transfected ± the indicated amounts of TAM 67 plasmid DNA and 0.5 μg of the TGF–β2–CAT reporter construct into CHO cells under conditions similar to those described in the legend to Figure 4. Each point is the average of at least 6 observations with SD's indicated by the bars. Symbols in the figure are the same as in Figure 16. See the legend to Figure 4 for further details.

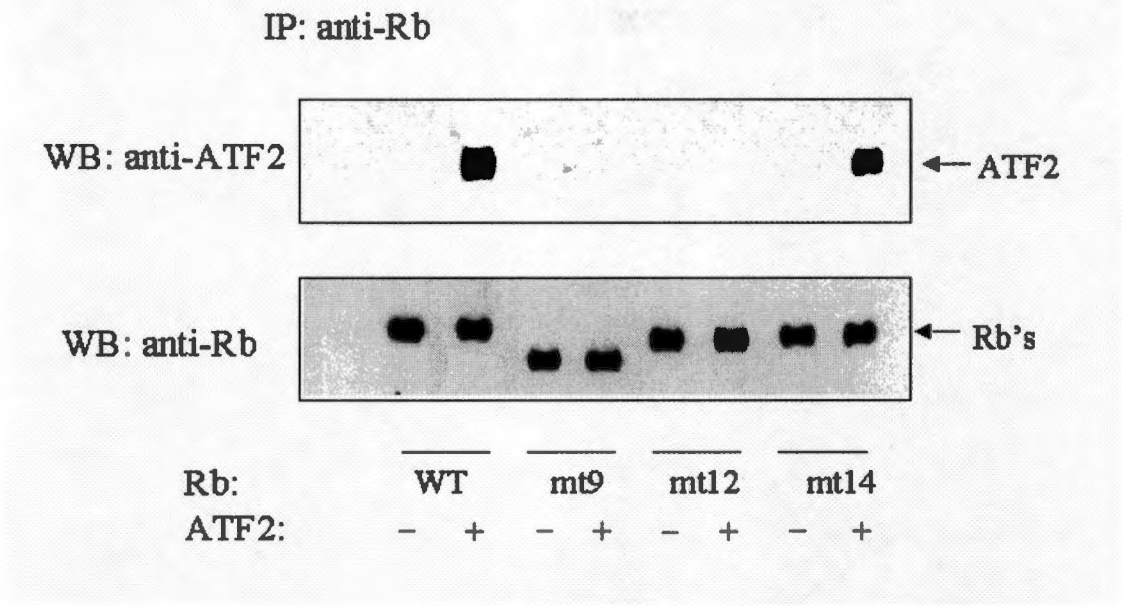

Figure 18. Co-immunoprecipitation of Rb and ATF2 in over-expressed CHO cell extract. Extracts (about 200 μg of proteins) from CHO cells transfected with the indicated expression vectors (Rb WT or mutants ± ATF2) at the bottom were subjected to immunoprecipitation by 10 μl antibodies against Rb followed by western blotting of immunoprecipitates with the antibodies against ATF2 or Rb (see Materials and Methods for details) as indicated on the left side. The molecular weights of the bands were estimated and found as expected for each protein as marked on the right side. This experiment has been repeated with similar results.

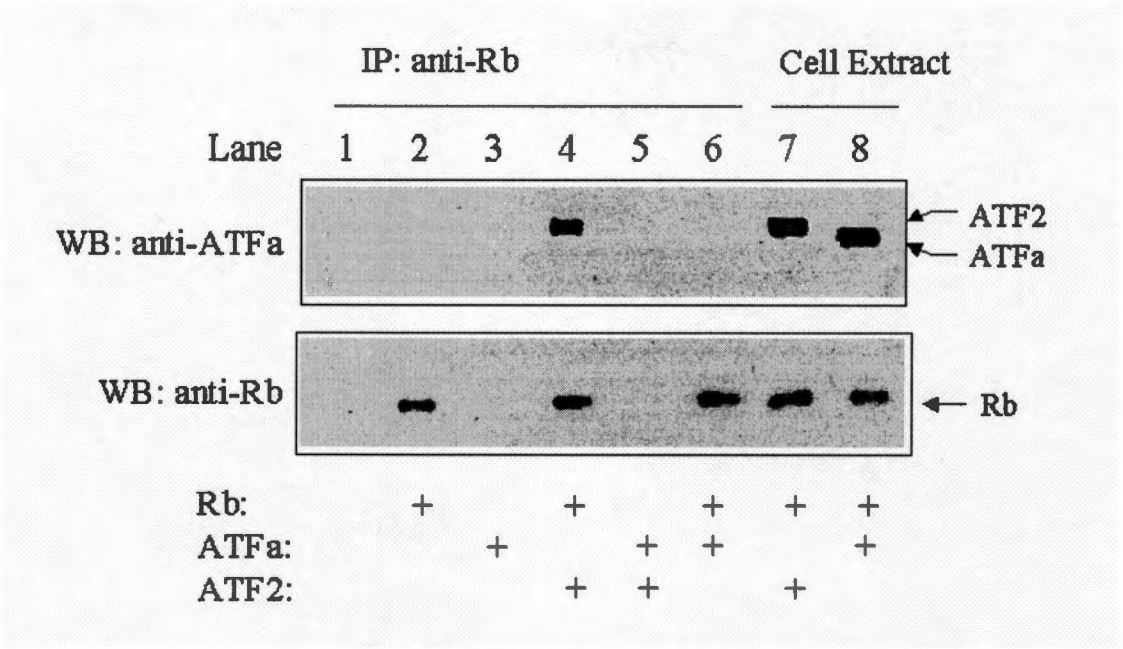

Figure 19. Co-immunoprecipitation of Rb and ATFa in over-expressed CHO cell extract. Extracts (about 200 μg of proteins) from CHO cells transfected with the indicated expression vectors (Rb/ATF2/ATFa) at the bottom were subjected to immunoprecipitation by 10 μl antibodies against Rb followed by western blotting of immunoprecipitates with the antibodies against ATFa or Rb (see Materials and Methods for details) as indicated on the left side. The molecular weights of the bands were estimated and found as expected for each protein as marked on the right side. This experiment has been repeated with similar results.

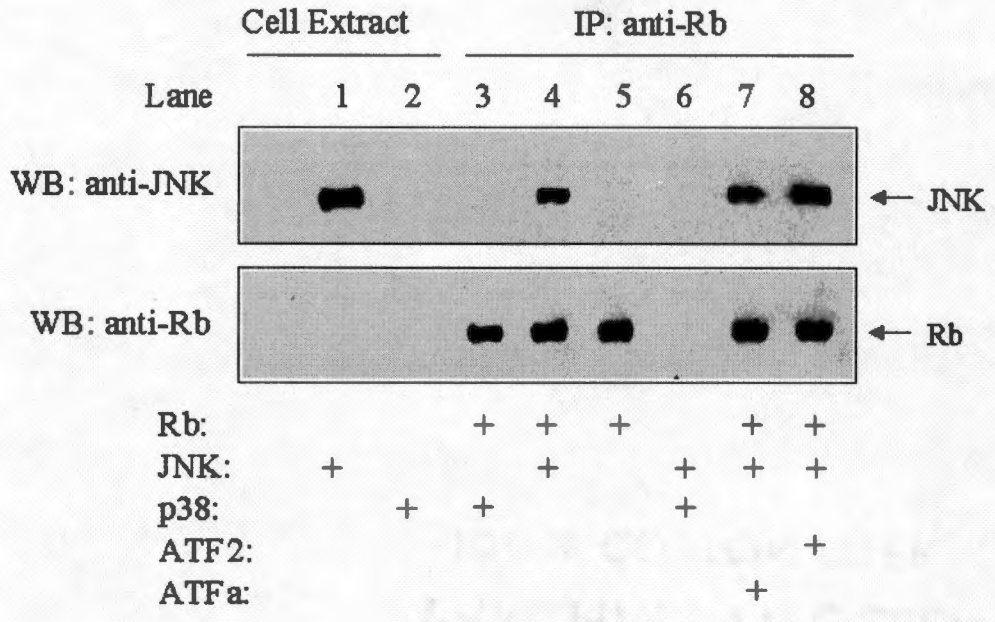

Figure 20. Co-immunoprecipitation of Rb and JNK in over-expressed CHO cell extract. Extracts (about 200 μg of proteins) from CHO cells transfected with the indicated expression vectors (Rb/JNK/p38/ATF2/ATFa) at the bottom were subjected to immunoprecipitation by 10 μl antibodies against Rb followed by western blotting of immunoprecipitates with the antibodies against JNK or Rb (see Materials and Methods for details) as indicated on the left side. The molecular weights of the bands were estimated and found as expected for each protein as marked on the right side. This experiment has been repeated with similar results.

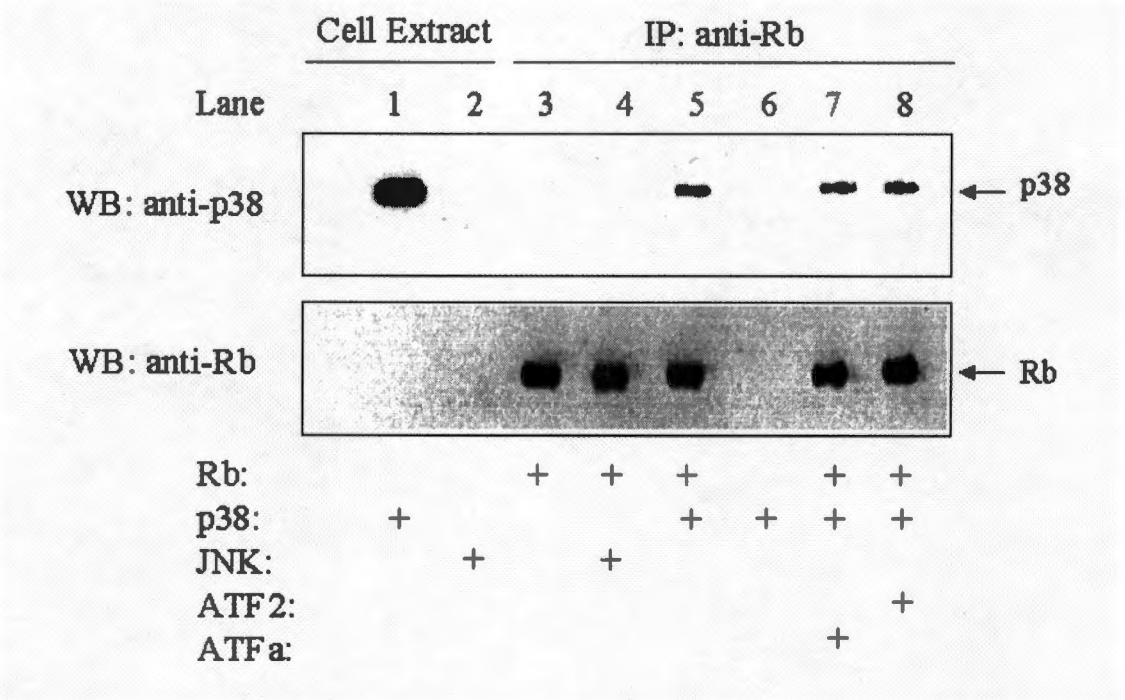

Figure 21. Co-immunoprecipitation of Rb and p38 in over-expressed CHO cell extract. Extracts (about 200 μg of proteins) from CHO cells transfected with the indicated expression vectors (Rb/p38/JNK/ATF2/ATFa) at the bottom were subjected to immunoprecipitation by 10 μl antibodies against Rb followed by western blotting of immunoprecipitates with the antibodies against p38 or Rb (see Materials and Methods for details) as indicated on the left side. The molecular weights of the bands were estimated and found as expected for each protein as marked on the right side. This experiment has been repeated with similar results.

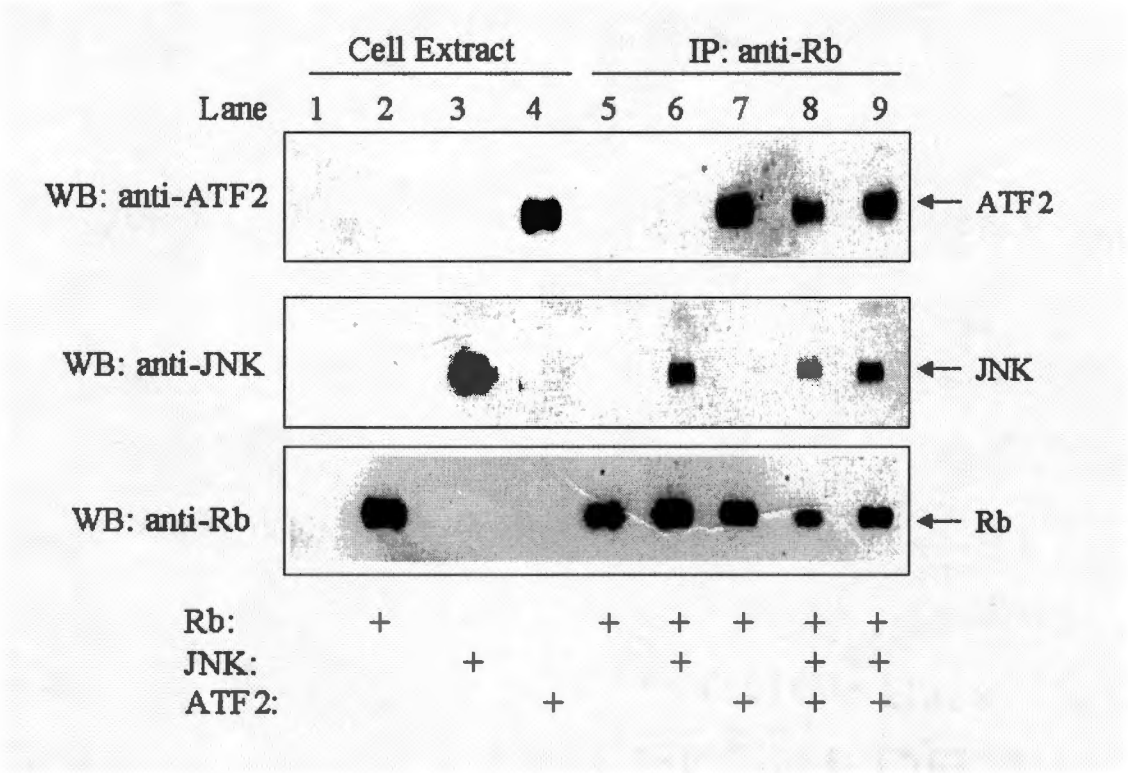

Figure 22. Co-immunoprecipitation of Rb with ATF2 and JNK simultaneously. Extracts (about 200 μg of proteins) from CHO cells transfected with the indicated expression vectors (Rb/JNK/ATF2) at the bottom were subjected to immunoprecipitation by 10 μl antibodies against Rb followed by western blotting of immunoprecipitates with the antibodies against ATF2, JNK or Rb (see Materials and Methods for details) as indicated on the left side. The molecular weights of the bands were estimated and found as expected for each protein as marked on the right side. Lane 8 in all panels was loaded with the same sample as lane 9 but with only half of the amount. This experiment has been repeated with similar results.

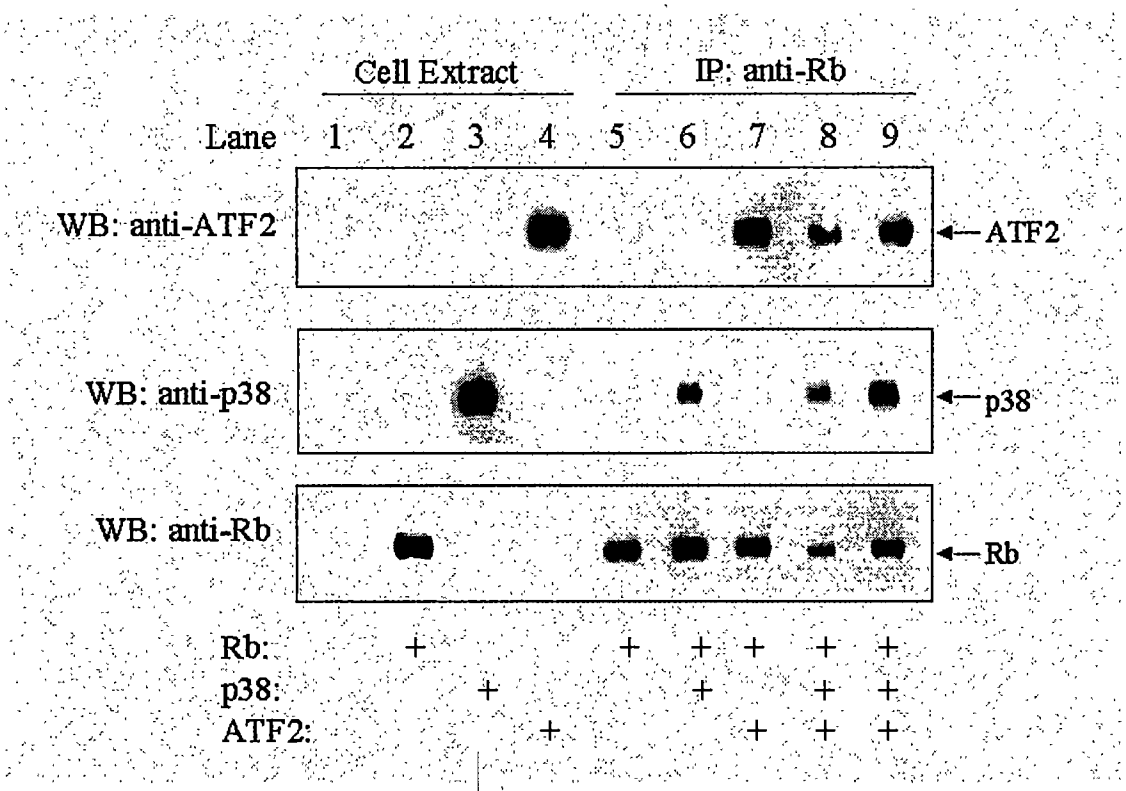

Figure 23. Co-immunoprecipitation of Rb with ATF2 and p38 simultaneously. Extracts (about 200 μg of proteins) from CHO cells transfected with the indicated expression vectors (Rb/p38/ATF2) at the bottom were subjected to immunoprecipitation by 10 μl antibodies against Rb followed by western blotting of immunoprecipitates with the antibodies against ATF2, JNK or Rb (see Materials and Methods for details) as indicated on the left side. The molecular weights of the bands were estimated and found as expected for each protein as marked on the right side. Lane 8 in all panels was loaded with the same sample as lane 9 but with only half of the amount. This experiment has been repeated with similar results.

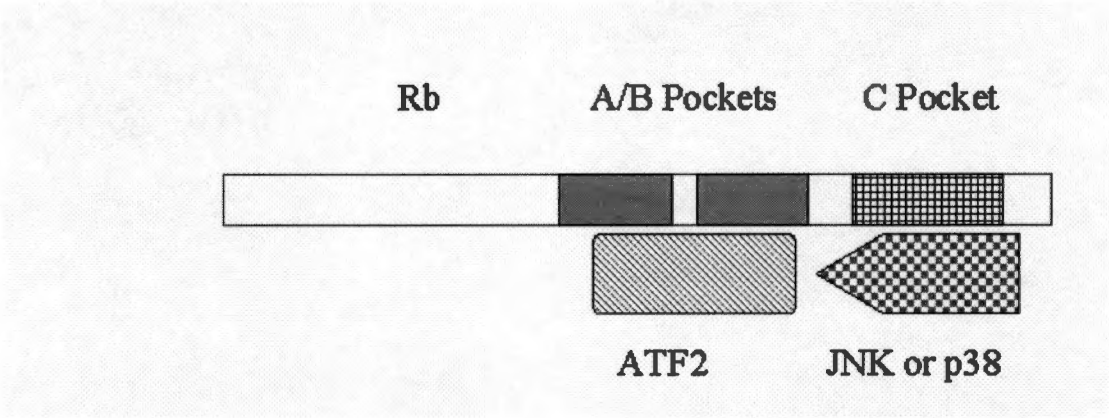

Figure 24. Matchmaker hypothesis for Rb's function. Schematic linear structure of Rb with suggested matchmaker function. Rb binds to JNK or p38 using its C-pocket, and interacts (directly or indirectly) with ATF2 using its A/B pockets. These interactions increase the accessibility of ATF2 to JNK or p38, and may potentially increase ATF2's phosphorylation/activation.

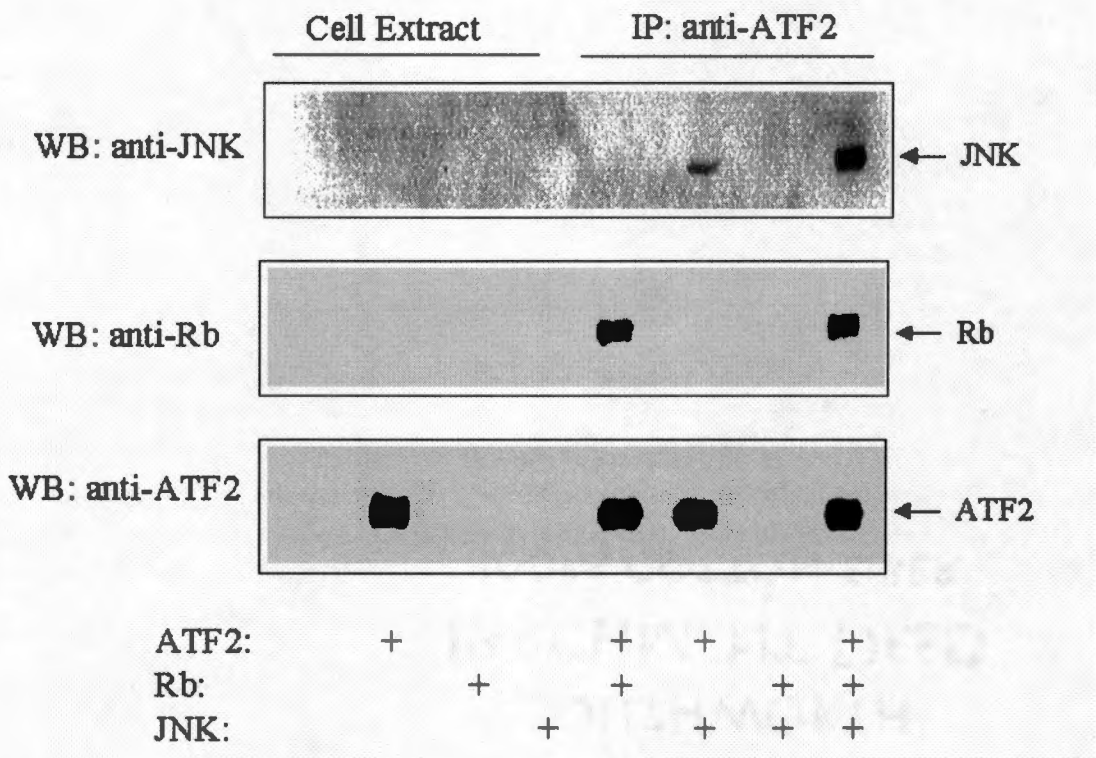

Figure 25. Rb enhances the association of ATF2 with JNK. Extracts (about 200 μg of proteins) from CHO cells transfected with the indicated expression vectors (ATF2/Rb/JNK) at the bottom were subjected to immunoprecipitation by 10 μl antibodies against ATF2 followed by western blotting of immunoprecipitates with the antibodies against JNK, Rb, or ATF2 as indicated on the left side. The molecular weights of the bands were estimated and found as expected for each protein as marked on the right side. This experiment has been repeated with similar results. See Materials and Methods for additional details.

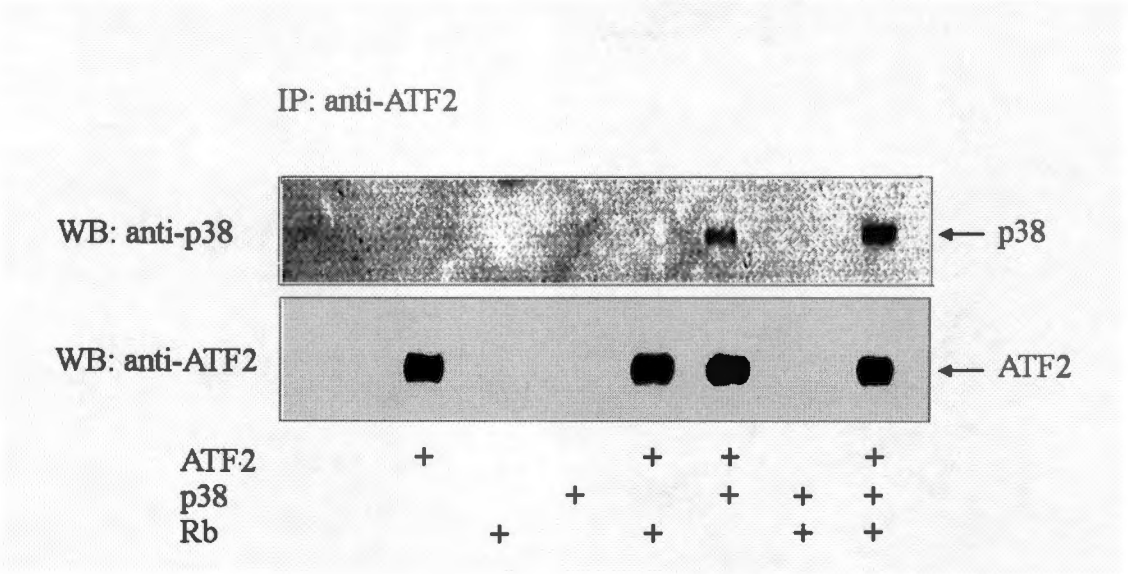

Figure 26. Rb enhances the association of ATF2 with p38. Extracts (about 200 μg of proteins) from CHO cells transfected with the indicated expression vectors (ATF2/p38/Rb) at the bottom were subjected to immunoprecipitation by 10 μl antibodies against ATF2 followed by western blotting of immunoprecipitates with the antibodies against p38 or ATF2 as indicated on the left side. The molecular weights of the bands were estimated and found as expected for each protein as marked on the right side. This experiment has been repeated with similar results. See Materials and Methods for additional details.

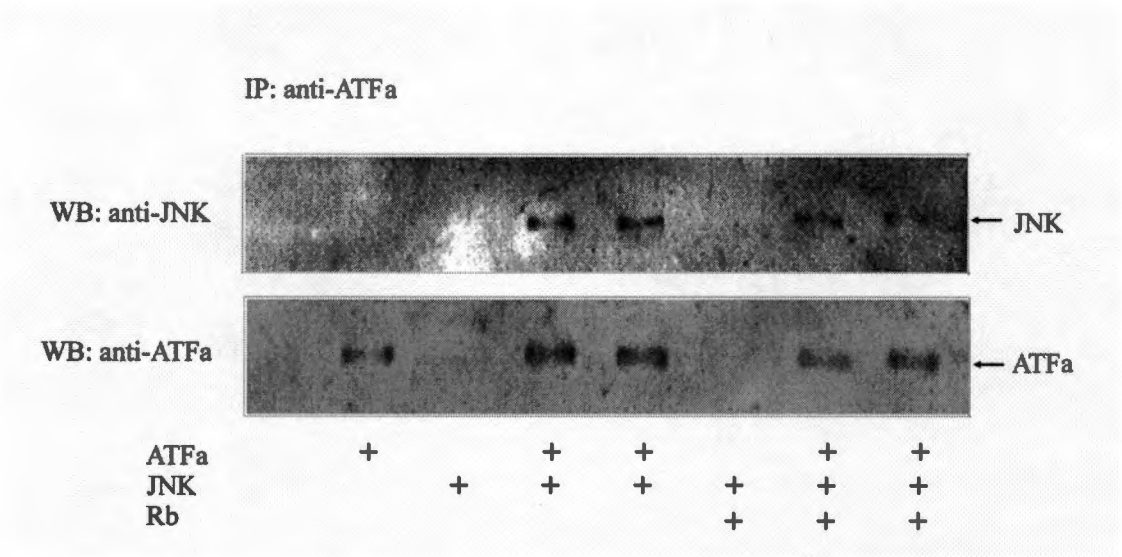

Figure 27. Effects of Rb on the co-immunoprecipitation of ATFa with JNK. Extracts (about 200 μg of proteins) from CHO cells transfected with the indicated expression vectors (ATFa/Rb/JNK) at the bottom were subjected to immunoprecipitation by 10 μl antibodies against ATFa followed by western blotting of immunoprecipitates with the antibodies against JNK or ATFa as indicated on the left side. The molecular weights of the bands were estimated and found as expected for each protein as marked on the right side. This experiment has been repeated with similar results. See Materials and Methods for additional details.

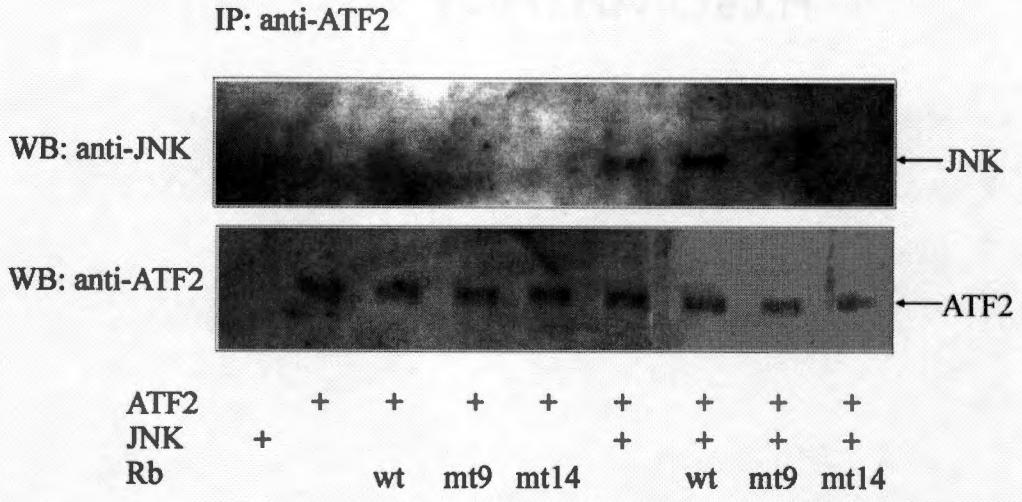

Figure 28. Effects of Rb mutants on the co-immunoprecipitation of ATF2 with JNK. Extracts (about 200 μg of proteins) from CHO cells transfected with the expression vectors (Rb WT/mutant/ATF2/JNK) indicated at the bottom were subjected to immunoprecipitation by 10 μl antibodies against ATF2 followed by western blotting of immunoprecipitates with the antibodies against JNK or ATF2 as indicated on the left side. The molecular weights of the bands were estimated and found as expected for each protein as marked on the right side. This experiment has been repeated with similar results. See Materials and Methods for additional details.

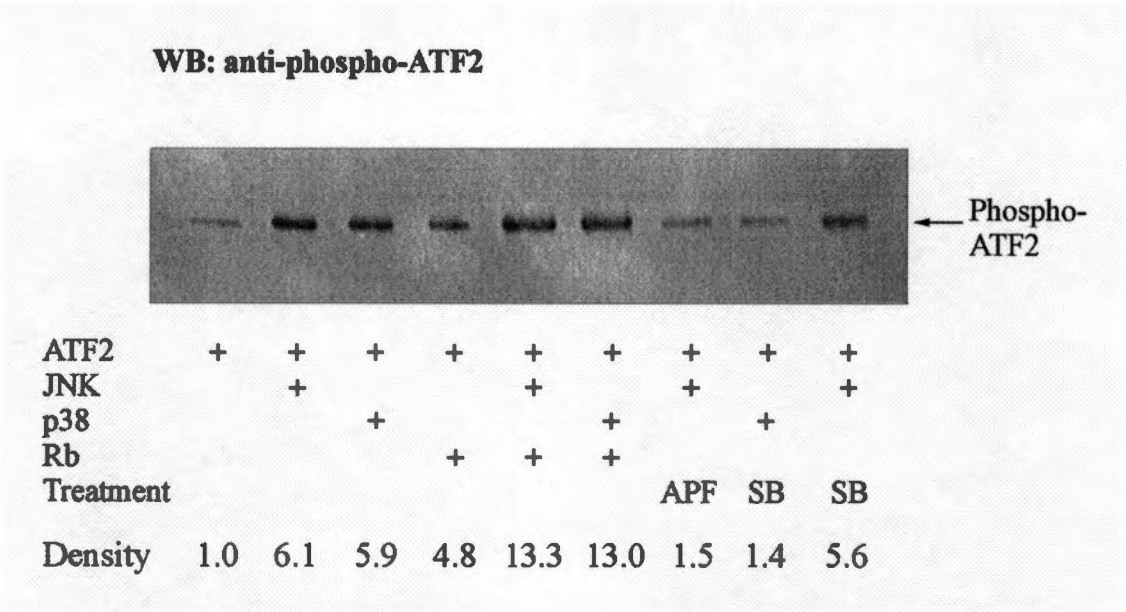

Figure 29. Phosphorylation status of ATF2 in transfected CHO cells. CHO cells were transfected with ATF2 alone or with other indicated expression vectors: JNK, p38, Rb or APF. Fifteen µl of cell lysates were subjected to SDS-PAGE, and probed by western blotting with anti-phospho-ATF2 (Thr 69/71) antibodies (NEB). The molecular weight of the band is as expected for phospho-ATF2 (i.e. 75 kD). See Figure 9 for other details. APF and SB 203580 (SB) were used as described in Figures 7 and 8.

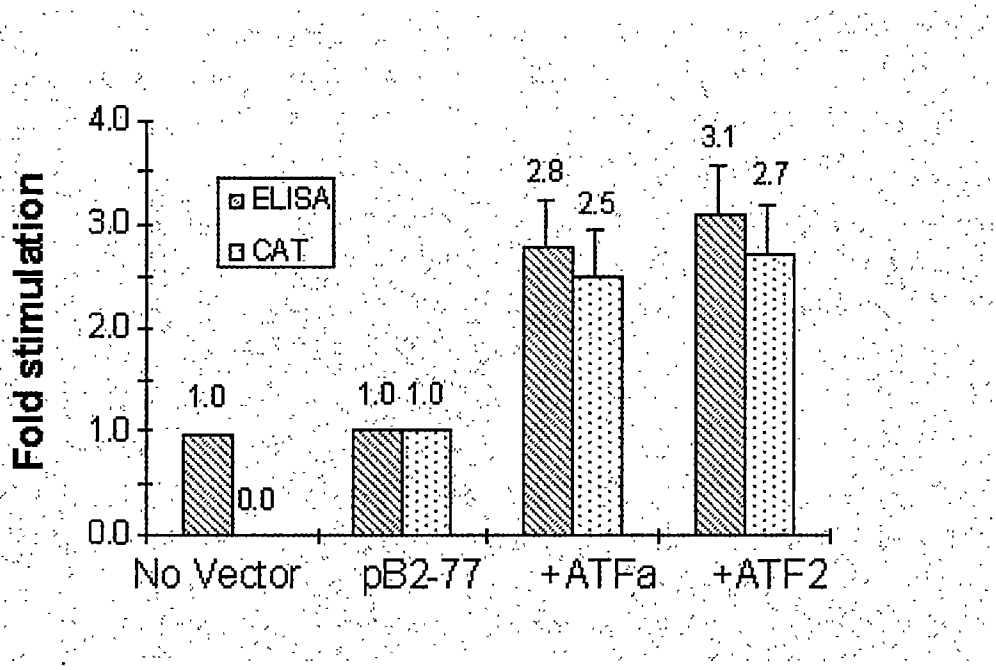

Figure 30. Comparison of endogenous TGF-β2 gene expression with ectopic TGF-β2-CAT promoter regulation. PC-3 cells were transfected with 0.5 μg pB2-77 plasmid DNA, without or with 0.5 μg ATFa or ATF2 DNA as indicated in the figure. Forty-eight hours post-transfection, the culture medium was collected, concentrated 20 fold, and subjected to an ELISA assay using antibody against TGF-β2; the cells were harvested and lysed, and subjected to the CAT assay. The values for both basal levels (transfected with pB2-77 alone) are set arbitrarily as 1, and all other ELISA and CAT activities are compared to these basal levels and the ratios were used as their "fold stimulation". The bars on the left represent the fold stimulation from the ELISA assay (endogenous TGF-β2 activity), while the bars on the right represent that from the CAT assay (ectopic TGF-β2 promoter).

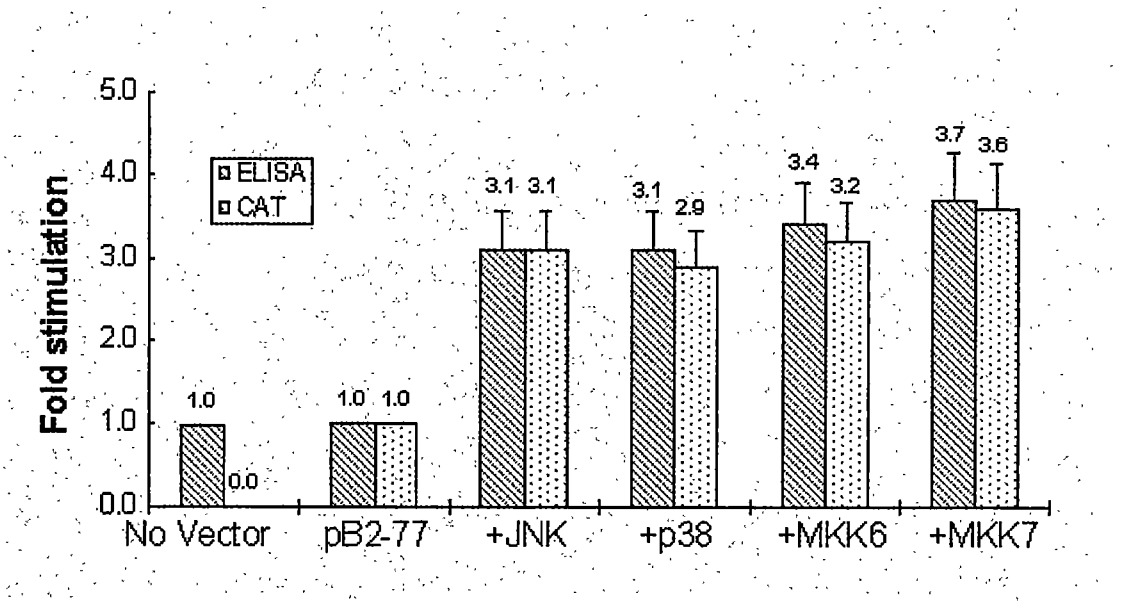

Figure 31. Comparison of the regulation of endogenous TGF-β2 gene expression and ectopic TGF-β2-CAT activity by various protein kinases in the absence of overexpressed ATF's. PC-3 cells were transfected with 0.5 μg pB2-77 plasmid DNA alone, or with 0.2 μg JNK, p38, MKK6 or MKK7 expression vector DNA as indicated in the figure. See Figure 30 for other details.

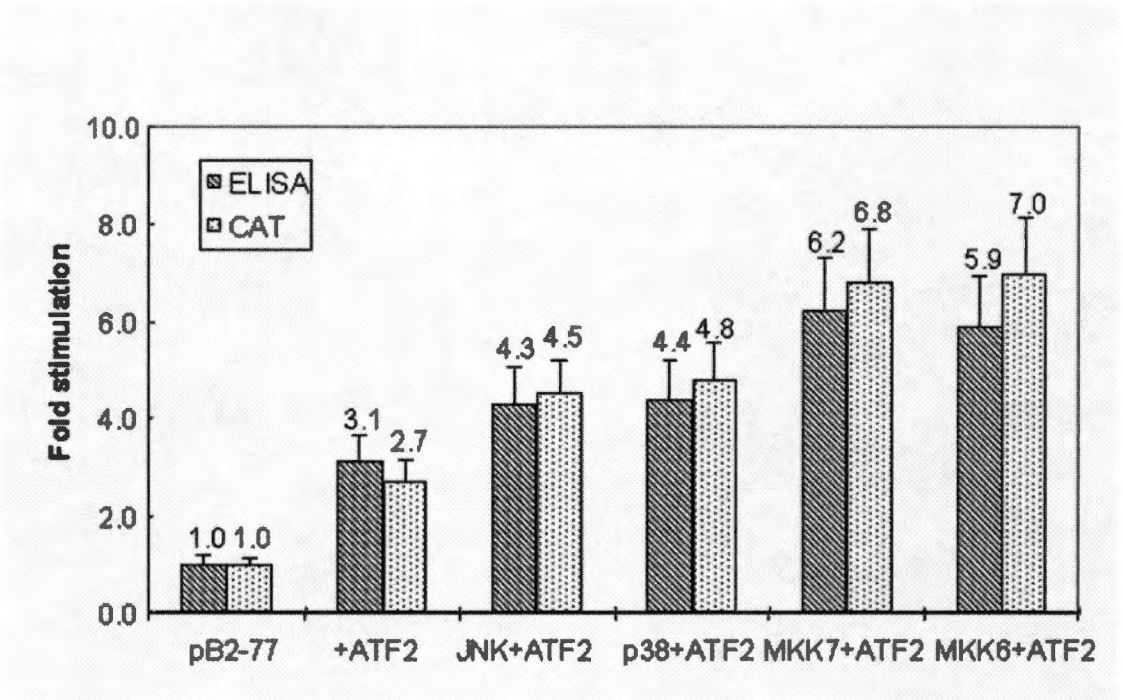

Figure 32. Comparison of the ability of co-transfected protein kinases to augment the effects of ATF2 on TGF-β2 promoter activity. PC-3 cells were transfected by 0.5 μg pB2-77 plasmid DNA alone, or with 0.5 μg ATF2 with or without 0.2 μg JNK, p38, MKK6 or MKK7 expression vector DNA as indicated in the figure. See Figure 30 for other details.

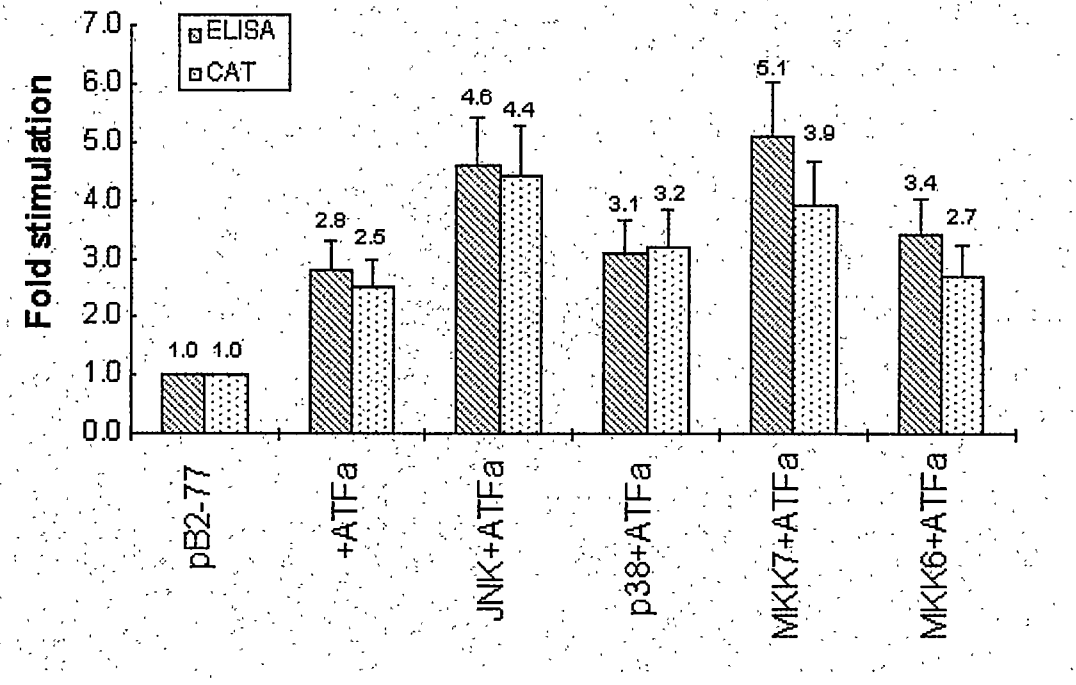

Figure 33. Comparison of the ability of co-transfected protein kinases to augment the effects of ATFa on TGF-β2 promoter activity. PC-3 cells were transfected with 0.5 μg pB2-77 plasmid DNA alone, or with 0.5 μg ATFa with or without 0.2 μg JNK, p38, MKK6 or MKK7 expression vector DNA as indicated in the figure. See Figure 30 for other details.

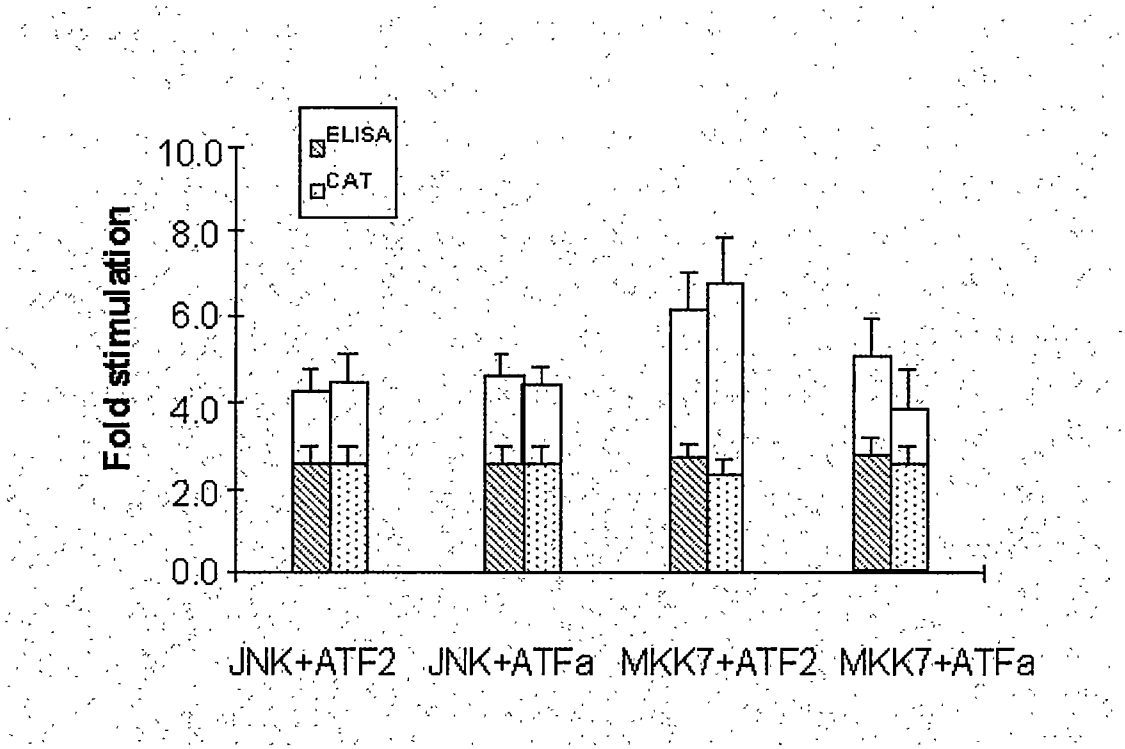

Figure 34. Inhibition of the effects of MKK7 and JNK on endogenous and ectopic TGF-β2 promoter activity by APF. PC-3 cells were transfected with 0.5 μg pB2-77 reporter plasmid and 0.5 μg ATF2 or ATFa, with or without 0.2 μg JNK, MKK7 expression vector DNA in the presence (filled bars) or absence (open bars) of APF expression vector. See Figure 30 for other details.

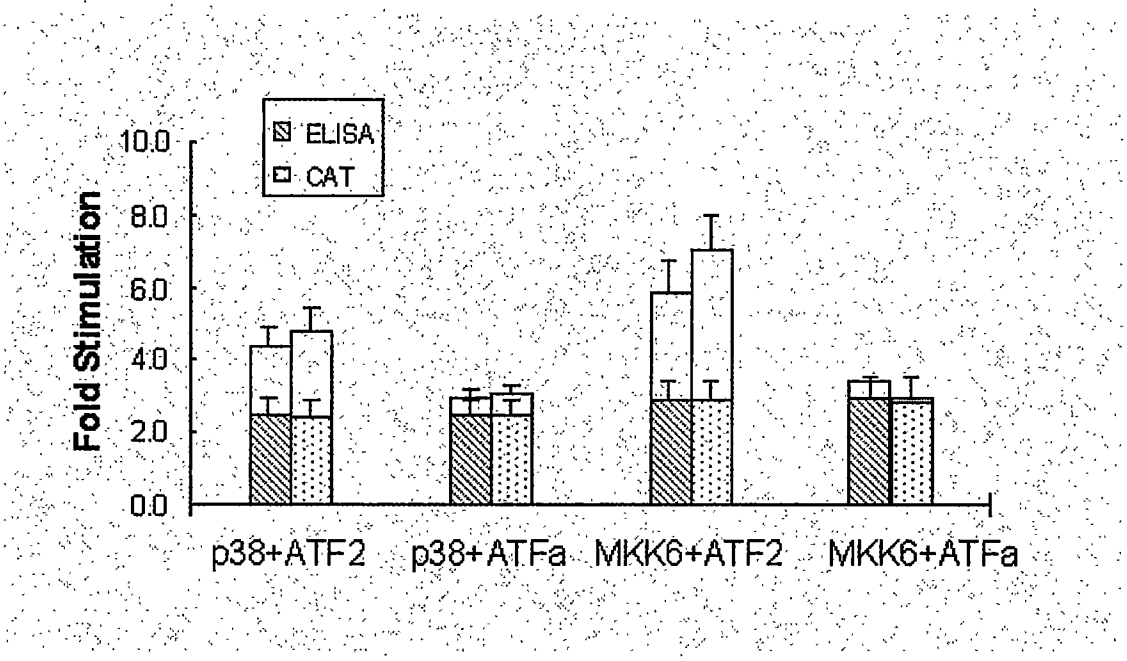

Figure 35. Inhibition of the effects of MKK6 and p38 on endogenous and ectopic TGF-β2 promoter activity by SB203580. PC-3 cells were transfected with 0.5 μg pB2-77 reporter plasmid and 0.5 μg ATF2 or ATFa, with or without 0.2 μg p38 or MKK6 expression vector DNA. SB203580, a specific inhibitor of p38 was included in the medium at a final concentration of 10 μM (filled bars). Open bars represent the result in the absence of SB203580. See Figure 30 for other details.

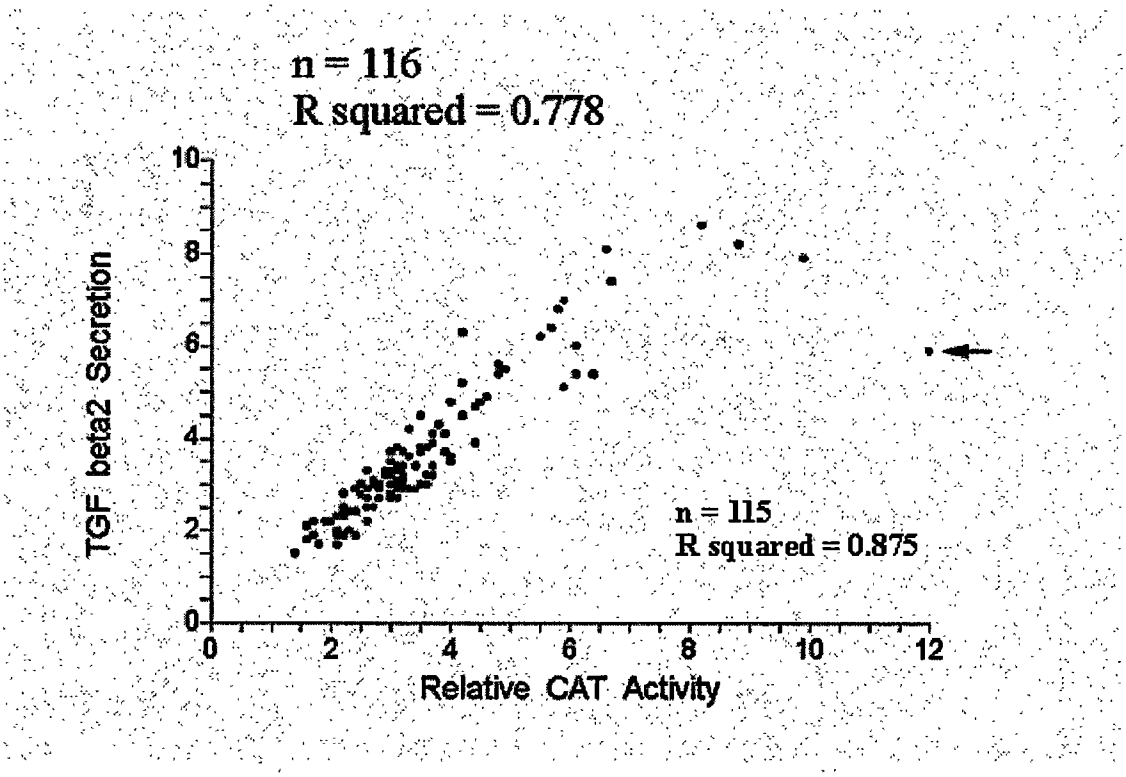

Figure 36. Correlation plot of relative CAT activity with TGF-β2 secretion. PC-3 cells were transfected with 0.5 μg pB2-77 reporter plasmid and various combinations of expression plasmids shown in Figures 30 to 35. Each point represents an individual experiment, with TGF-β2 secretion vs. CAT activity. The value was compared to that of pB2-77 alone. There are total of 116 samples with a R^2 of 0.778 using simple linear regression, which means 77.8% of variation in TGF-β2 secretion can be explained by changes in CAT activity. The arrow indicates the only outlier in the samples determined by R student analysis (R student = -9.9). If this outlier is excluded, R^2 increases to 0.875 with 115 samples, which means 87.5% of variation in TGF-β2 secretion can be explained by changes in CAT activity.

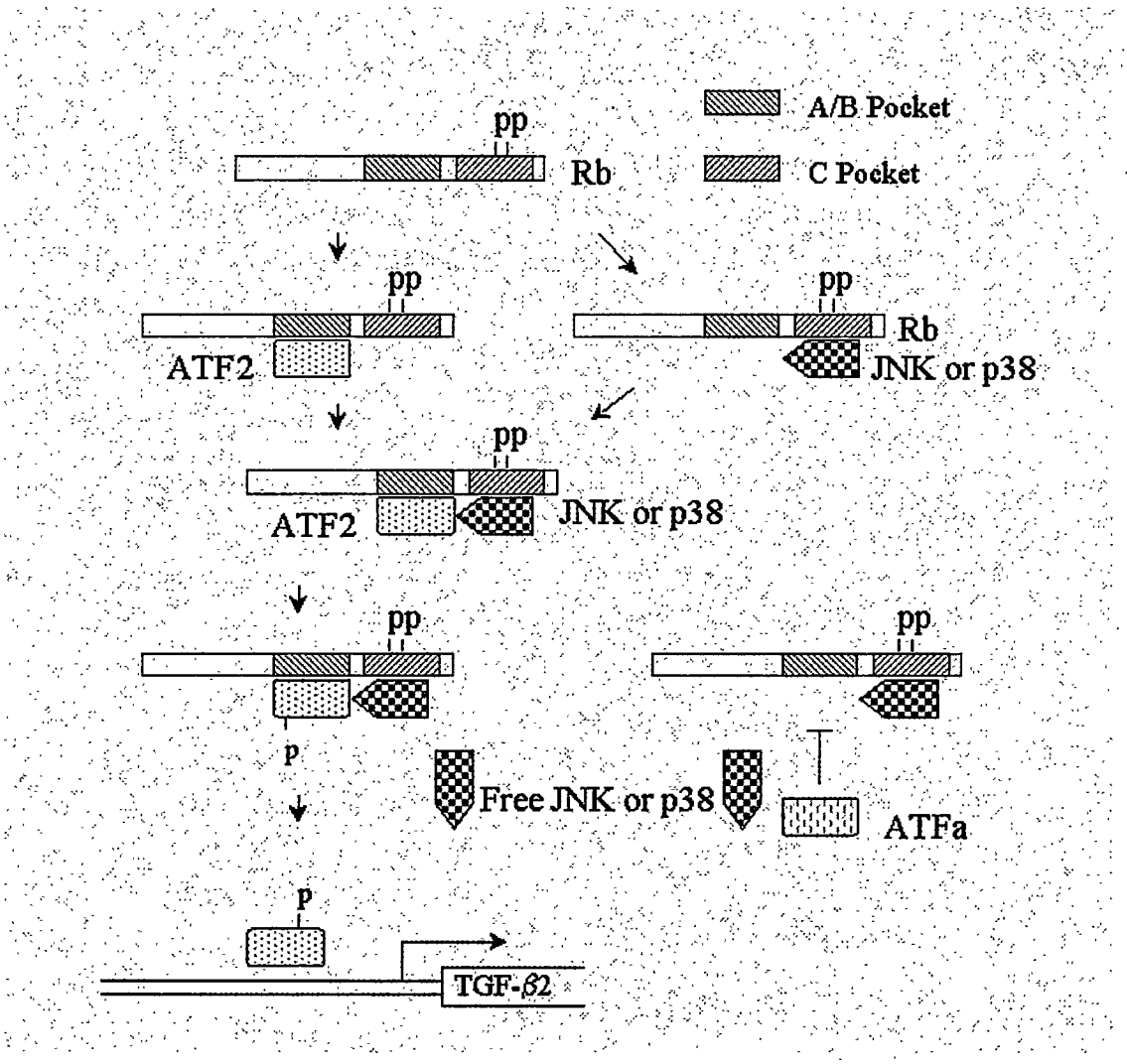

Figure 37. Cartoon diagram of Rb's differential regulation of ATF2 and ATFa. Rb is suggested to bind to JNK or p38 using its C-pocket, and to interact (directly or indirectly) with ATF2 using its A/B pockets in the same complex. These interactions may potentially increase ATF2's phosphorylation, and phosphorylated ATF2 is likely to have higher activity to stimulate TGF-β2 expression (Gupta et al., 1995). On the other hand, ATFa cannot interact with Rb, and hence may only be phosphorylated by free JNK. The presence of a large amount of Rb may sequester JNK away from ATFa, and inhibit its phosphorylation/activation.

VITA

Huo Li was born in Sichuan, People's Republic of China on July 16, 1968. He received his 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.

He entered the Department of Biochemistry at the University of Tennessee, Knoxville in the fall of 1994. He was awarded the Master of Science degree in December, 1996. He was also awarded the Master of Science degree in Statistics in May, 2000 through the Intercollegiate statistics graduate program.

He will be awarded the Ph.D. degree in Biochemistry, Cellular and Molecular Biology in December, 2000. He will work for Biogen, Inc. at Cambridge, MA. as a Bioinformatics Scientist after his graduate study.