To My family
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TABLE OF CONTENTS

DEDICATION .............................................................................. ii
ACKNOWLEDGMENTS ................................................................. iii
VITA ............................................................................................... iv
LIST OF TABLES ........................................................................... xi
LIST OF FIGURES ......................................................................... x

CHAPTER Page

I  INTRODUCTION ................................................................. 1
The ATF/CREB family of transcription factors:
a historical perspective ....................................................... 1
The ATF/CREB family of transcription factors:
definitions and members ................................................... 4
An overview of the rest of the thesis ................................... 12
Perspective ............................................................................ 13

II  The transcriptional activity of ATF3 ...................... 14
Introduction ............................................................................. 14
Material and methods .......................................................... 19
Results ..................................................................................... 26
ATF3 represses transcription in vivo ................................ 26
ATF3 represses transcription in vitro ................................. 30
V  Biochemical approaches to further investigate the
"co-repressor" model .................................................... 102

Introduction ................................................................. 102
Material and methods .................................................... 106
Results ............................................................................ 110
  In vivo assembly assay ................................................ 110
    Stable HeLa cell line expressing ATF3 ....................... 110
    Vaccinia virus expressing ATF3 ............................... 111
    ATF3-affinity column .............................................. 115
Discussion ....................................................................... 119

VI  Future direction ....................................................... 121

LIST OF REFERENCES .................................................... 123
**LIST OF TABLES**

**TABLE**

1. The characteristics of positive cDNA clones encoding ATF3 interacting proteins ........................................ 70
LIST OF FIGURES

FIGURES

2.1 ATF3 represses promoters with the ATF sites .......... 27
2.2 ATF3 represses transcription in dose-dependent manner 28
2.3 ATF3 represses transcription in a DNA-binding dependent manner ...................................... 29
2.4 ATF3 repress the activated E-selectin promoter activity 31
2.5 In vitro transcription assay .................................. 32
2.6 ATF3 specifically represses transcription in vitro .... 34
2.7 ATF3 represses the adenovirus E4 promoter in vitro .. 35
2.8 Vaccinia virus expressed ATF3, but not E. coli expressed ATF3, represses the gadd153/Chop10 promoter in vitro .................................................. 36
2.9 Possible mechanisms to explain the transcriptional repression by ATF3 ...................................... 38
2.10 The "co-repressor" model ...................................... 39
2.11 ATF3(1-100), lacking a functional DNA binding domain, activates reporters with or without ATF sites. 42

2.12 ATF3Δzip, lacking a functional DNA binding domain, activates reporters with or without ATF sites. 43

2.13 The effect of ATF3 deletion mutants on SPI/ATFx4-CAT reporter activity. 44

2.14 The characteristics of positive cDNA clones encoding ATF3 interacting proteins. 45

3.1 λEXlox (+) vector system. 63

3.2 Synthesis of the first and second strands of cDNA. 64

3.3 pET.His and pET.HisK vectors. 67

3.4 An example of library screening. 69

3.5 Keratin7, keratin8, gadd153/Chop10 and small USF2/FIP bind to ATF3 but not to ATF1. 72

3.6 All ATF3 interacting proteins interact with ATF3 through the leucine zipper domain. 73

4.1 The interaction between ATF3 and gadd153/Chop10 was not non-specific and was dependent on the leucine zipper. 89

4.2 gadd153/Chop10 inhibited ATF3 from binding to the ATF consensus sequence and several related sites. 91
4.3 gadd153/Chop10 relieved the repression of E-selectin promoter by ATF3. .............................................. 92

4.4 ATF3 and gadd153/Chop10 were expressed in CCl4-treated liver in an inverse but overlapping manner. . . 95

4.5 Partial hepatectomy but not alcohol treatment decreased the level of gadd153/Chop10 mRNA. ............ 96

4.6 A model for the role of ATF3 in the liver's response to CCI4 ................................................................. 97

4.7 ATF3 interacted with gadd153/Chop10 and c-Jun with similar affinity ................................................. 100

5.1 Fractionation of vaccinia virus expressed his-ATF3 by gel filtration and phosphocellulose columns. . . 113

5.2 Co-immunoprecipitation of vaccinia virus expressed his-ATF3 and viral proteins .............................. 114

5.3 ATF3-GST affinity column functions properly ......................................................................................... 117

5.4 ATF3-GST affinity column bound HeLa cell proteins.. 118
Chapter I

Introduction

The ATF/CREB family of transcription factors: a historical perspective

The regulation of eukaryotic gene expression has been extensively studied since early 1980's. Much progress has been made, particularly, in transcriptional regulation of the protein encoding genes. Some of the initial work was focused on studying the small DNA tumor viruses such as adenovirus, SV40, and herpesvirus. The advantage of studying the small DNA tumor viruses is that viral genomes are much more simple than most of the eukaryotic genomes. In addition, viral gene expression requires the host cellular transcriptional machinery. Therefore, studies of viral gene expression can shed light on the transcriptional machinery of the eukaryotic cells. Several important initial discoveries were made from these studies. For examples, transcriptional enhancers were first identified from SV40 virus (for a review see Serfling et al., 1985), while RNA splicing was first identified from adenovirus (for a review see Sharp, 1985).

Adenovirus is a double-stranded DNA virus containing 36 kb of genomic DNA and encoding 10 transcription regions (Ginsberg, 1984; Doerfler, 1986). Each transcription region is regulated by its own promoter. The expression of adenovirus genes are regulated temporally. Depending on when the genes being expressed, they are referred to as early genes or late genes. One important feature of adenoviral gene expression is that early genes are strongly activated by the early region 1a (E1a) gene product (for a review see Berk, 1986). E1a gene is the first adenoviral gene to be expressed after infection; it encodes two mRNAs, 13S and 12S. The 289-amino-acid E1a protein encoded by the 13S transcript is required to activate the expression of other adenoviral early genes including E1b, E2, E3, and E4 genes. However, the 289-amino-
It is not surprising that additional cellular factors were found to be involved in the regulation of adenoviral early promoters by E1a. A factor referred to as E4F1 was shown to bind to the E4 promoter at a consensus site, ACGT(A/C)AC (Lee and Green, 1987). This consensus sequence was also found in the E1a, E2, and E3 promoters. Similarly, another factor referred to as E2A-EF was also shown to bind to the E1a, E2a, E3, and E4 promoters (Sivaraman et al., 1986). In contrast to E2F, the binding of E4F1 and E2A-EF to DNA is not enhanced by the 289-amino-acid E1a protein. Further analysis indicated that E4F1 and E2A-EF share the same DNA binding specificity and both activate the E2 and E4 promoters in vitro. They were thought to be the same cellular factor and were then referred to as activating transcription factor (ATF) (Lee et al., 1987). The defined binding site for ATF was (A/T)CGTCA. The ATF binding site was not only found in viral promoters but also in many other cellular promoters such as c-Fos, Hsp70, and somatostatin (Lee et al., 1987).

While the ATF site was identified from viral promoters, a DNA binding site that mediates cAMP response was identified from cAMP inducible cellular promoters (Montminy et al., 1986). Significantly, the binding site is similar to the ATF binding sites. It has been known that the 2nd messenger, cAMP, is required to mediate hormones or stimuli induced gene expression. By analyzing cAMP responsive promoters, a palindromic sequence, TGACGTCA, referred to as cAMP responsive element (CRE) was identified (Montminy et al., 1986). Interestingly, the defined CRE site was a subset of the ATF site, (A/T)CGTCA. Therefore, this binding site has been referred to as ATF/CRE site or CRE/ATF site in the literature; for the convenience of discussion, I will refer to this binding site as the ATF site in most cases.
A nuclear protein with a relative molecular mass of 43 kDa was characterized to bind to the CRE site (Montminy and Bilezskijian, 1987). Because the CRE site and the ATF site are identical, it is not surprising that a nuclear protein with the same molecular weight (43 kDa) was identified to bind to the ATF site (Hurst and Jones, 1987). However, in a separate study, two clusters of proteins around 43 kDa and 47 kDa were found to bind to the ATF site (Hai et al., 1988), a first indication that more than one polypeptide can bind to the ATF binding site. Prior to that, it was generally believed that only one transcription factor binds to a given DNA element.

A cDNA clone encoding cAMP responsive element binding protein (CREB) was first isolated from a λgt11 placental library using a radio-labeled ATF binding site as probe (Hoeffler et al., 1988); its rat homolog was isolated based on the amino acid sequence derived from the purified CREB protein (Gonzalez et al., 1989). Using radio-labeled ATF binding site as a probe, a different rat cDNA encoding cAMP responsive element binding protein was isolated; it was referred to as CRE-BP1 (Maekawa et al., 1989). In a separate study, seven human cDNAs encoding seven different ATF proteins were isolated (Hai et al., 1989). This study not only confirmed the previous observation that more than one polypeptide can interact with the ATF site, but also provides the first observation that the number of proteins that can bind to a single DNA site is relatively large. Among the seven proteins, ATF1 is highly homologous to CREB (overall 75% amino acid similarity) and ATF2 is the human homolog of the rat CRE-BP1. At present, more than 10 ATF/CREB proteins have been identified. Identification of the ATF/CREB family of transcription factors, and a number of other transcription factor families, such as nuclear hormone receptors and NF-κB, demonstrated that the phenomenon (multiple transcription factors bind to the similar DNA sequence) is common in higher eukaryotes.

Identification of the family of ATF transcription factors raises questions why the cell produces so many similar transcription factors binding to the same DNA element. Sequence comparison of ATF/CREB proteins indicates that they all contain the similar DNA binding domain, the basic region and the leucine zipper domain (the bZip domain); however, sequences outside the bZip domain are completely different between different ATF/CREB proteins, indicating that they may have different transcriptional properties.
In addition, even within the bZip DNA binding domain, amino acid sequences are quite different between different ATF/CREB proteins indicating that they may have different affinities to the ATF binding sites. Although the ATF binding site has been defined as (A/T)CGTCA or TGACGTCA, the ATF sites identified from different promoters vary from this consensus sequence. Therefore, it is likely that each ATF/CREB family member preferentially binds to a subset of the ATF sites and regulates different promoters. In addition, the ATF binding sites on different promoters have different flanking sequences which are occupied by different transcription factors. Recent evidence suggests that DNA bound transcription factors interact with each other and form some type of protein complex, and the interactions are crucial for the promoter activity (see review by Tjian and Maniatis, 1994). Therefore, subtle differences between different ATF binding sites and adjacent bound transcription factors may determine which ATF/CREB regulates a given promoter. The combination of different DNA elements and different transcription factors within a family greatly increases the possibilities for regulating gene expression.

**The ATF/CREB family of transcription factors: definitions and members**

Members of the ATF/CREB family all share a similar bZip DNA binding domain which is composed of the basic region and the leucine zipper domain (Landschulz et al., 1988). The leucine zipper domain is required to mediate the dimerization between two bZip proteins, while the adjacent basic region is required to recognize the specific DNA sequence. In addition to the ATF/CREB family of transcription factors, the bZip DNA binding domain is also shared by other families of transcription factors including the API (Fos/Jun) family and the C/EBP family. Previously, it was thought that proteins in each family only dimerize with family members and bind to their respective sites. However, this is evidently incorrect. It is clear now that members of each family can dimerize with members from different families, and they can bind to sites other that their own consensus sequence. Even the C/EBP consensus sequence, which is quite different from the ATF and API1 sequence, can be recognized by ATF or API1 proteins (Vinson et al., 1993). Therefore, the distinction between these families of proteins is blurred now; the names reflect more the history of discovery rather than the real difference between them.
Below, I discuss the characteristics and possible functions of genes that belong to the ATF/CREB family. At present, more than 10 ATF/CREB genes have been identified from mammal, aplisia, drosophila, and yeast. Due to the additional similarity outside the bZip domain, some of them can be further organized into a "sub-family" of genes.

CREB subfamily

Thus far, three genes have been identified in the CREB subfamily: CREB (Hoeffler et al., 1988; Gonzalez et al., 1989), ATF1 (Hai et al., 1989), and CREM (Foulkes et al., 1991). Together, they share an overall 75% or greater amino acid similarity. In addition, CREB and CREM genes have similar genomic organization and both produce a variety of alternatively spliced mRNAs. One particularly interesting aspect of these alternatively spliced mRNAs is that they encode not only transcriptional activators but also repressors (for reviews see Foulkes and Sassone-Corsi, 1992; Meyer and Habener, 1993). In addition, they are expressed in a developmental and tissue specific manner. For example, during spermatogenesis, the expression of CREM in testis is switched from antagonists (CREMβ & γ) to an activator (CREMτ) (Foulkes et al., 1992). In comparison to CREB and CREM, the ATF1 gene is not well characterized. Its genomic organization has not been described and it is not clear whether it produces alternatively spliced mRNAs. Due to the high degree of similarity between CREB and CREM, they may be able to complement each other. In a particular case, transgenic mice with a disruption of CREB gene (CREB⁻) appeared healthy and had no obvious defects in growth and development. Interestingly, the expression of CREM was up-regulated in the CREB⁻ mice indicating they may be functionally equivalent and redundant (Hummler et al., 1994).

CREB, ATF1, and CREMτ are all activated by cAMP-dependent protein kinase A (PKA). In the case of CREB, PKA phosphorylation occurs at ser-133 within the kinase-inducible domain (KID) which contains consensus phosphorylation sites for a variety of kinase (for reviews see Brindle and Montminy, 1992; Papavassiliou, 1994). Evidence suggested that phosphorylation at ser-133 induces a conformational change in
the CREB protein and results in the exposure of the glutamine-rich activation domain (Gonzalez et al., 1991). In addition, phosphorylation at ser-133 enables CREB to recruit a transcriptional co-activator, CREB-binding protein (CBP) (Chrivia et al., 1993). The KID and glutamine-rich activation domains are also found in ATF1 and CREM\. Therefore, it is likely that they are also regulated by similar phosphorylation events as found in CREB.

Activation of CREB by cAMP signaling pathway has been implicated in gene regulation of a variety of physiological events. For example, CREB has been shown to be critical for the formation of long-term memory in mammals, aplysia and drosophila (for a review see Frank and Greenberg, 1994). Studies from aplysia indicated that cAMP signaling pathway plays a central role in converting short-term memory to long-term memory (Montarolo et al., 1986); in addition this process requires de novo protein synthesis (Montarolo et al., 1986; Schacher et al., 1988). It was hypothesized that the aplysia CREB-related protein is involved in the cAMP induced gene expression. Subsequently, CREB homologs were identified from aplysia and drosophila (see below). Together with mammalian CREB gene, they have been demonstrated to be directly involved in memory formation (Bourtchuladze et al., 1994; Yin et al., 1994; Bartsch et al., 1995);

**CRE-BP1/ATF2 subfamily**

Thus far, two genes have been identified in this subfamily: CRE-BP1/ATF2 (Maekawa et al., 1989; Hai et al., 1989) and ATFa (Gaire et al., 1990). CRE-BP1/ATF2 and ATFa share greater than 90% homology within the bZip domain and the N\' terminal 100 amino acids. In addition, they both have multiple alternatively spliced isoforms (Gaire et al., 1990; Georgopoulos et al., 1992); similar to that of CREB and CREM, some of the alternatively spliced isoforms act as an antagonist for the activity of the full length form (Pescini et al., 1994). Although they are highly homologous to each other, CRE-BP1/ATF2 and ATFa may not be able to compensate the function of each other: Transgenic mice with a disruption of CRE-BP1/ATF2 showed severe growth
defect and decreased postnatal viability (Reimold et al., 1996), indicating that the function of CRE-BP1/ATF2 is unique and is required for the normal development.

The N terminal 100 amino acids of CRE-BP1/ATF2 has been demonstrated to be the activation domain (Flint and Jones, 1991). In addition, it has been shown to interact with its bZip DNA binding domain and is negatively regulated by this interaction (Li and Green, 1996). One possible mechanism to activate CRE-BP1/ATF2 is to disrupt this intramolecular interaction. Previously, CRE-BP1/ATF2 was reported to be activated by E1a (Liu and Green, 1990; Flint and Jones, 1991), HTLV-1 Tax (Franklin et al., 1993; Wagner and Green, 1993), and HMG I(Y) (Du et al., 1993). Significantly, each of these regulatory proteins interacts with CRE-BP1/ATF2 at the bZip domain. Presumably, when binding to CRE-BP1/ATF2, these proteins prevent the bZip domain of CRE-BP1/ATF2 from interacting with and inhibiting its activation domain. Thereby, these regulatory proteins activate CRE-BP1/ATF2. In addition to being activated by the regulatory proteins, CRE-BP1/ATF2 is activated by phosphorylation. Several studies have shown that the activation domain of CRE-BP1/ATF2 can be phosphorylated by the stress induced c-Jun N terminal kinase/stress-activated protein kinase (JNK/SAPK) (Gupta et al., 1995; Livingstone et al., 1995; van Dam et al., 1995). Mutations at thr69 and thr71 residues, which are phosphorylated by JNK/SAPK, not only abolish the activation of CRE-BP1/ATF2 by JNK/SAPK but also greatly reduce the transcriptional activity of CRE-BP1/ATF2.

ATF3

Unlike CREB and CRE-BP1/ATF2 subfamilies, not much was known about the function and physiological relevance of ATF3 when we initiated this project. Since then, we, as well as several other laboratories, have made some progress in understanding the function of ATF3. Below, I briefly summarize the progress made in the past five years.

ATF3 was originally isolated from a λgt11 cDNA expression library (prepared from serum-induced HeLa cell mRNA) by its ability to bind to the radio-labeled DNA fragment containing the consensus ATF site, TGACGTCA (Hai et al., 1988). The full
length ATF3 cDNA contains a 546 bp open reading frame which encodes a 22-kDa nuclear protein. Thus far, only one alternatively spliced ATF isoform, ATF3Δzip, has been identified (Chen et al., 1994). ATF3Δzip has an extra 145 bp exon replacing the terminal three adenosine residues of the preceding exon, resulting in an in-frame termination codon immediately downstream from the first leucine residue of the leucine zipper domain. Consequently, ATF3Δzip encodes a truncated ATF3 lacking the functional DNA binding domain (Chen et al., 1994). A cDNA encoding the rat homolog of ATF3 was isolated from regenerating liver using a subtraction hybridization approach (Hsu et al., 1991). It was named as liver regeneration factor 1 (LRF-1). The rat LRF-1 and human ATF3 show an overall 95% identity at the amino acid level. At present, it is not clear whether there is an alternatively spliced isoform in rats or not.

The expression of ATF3 is relative low in many cultured cells and most of the tissues examined (skeletal muscle and smooth muscles are exceptions). It is rapidly induced by a variety of stimuli: ATF3 was found to be induced in several cell lines by serum and TPA (Chen et al., 1994), in mouse macrophage cell line by interferon (α, β, γ), PMA, Ca²⁺-influx (Farber, 1992), in neuroblastoma cell by Ca²⁺-influx (Adler and Fink, 1993) and FGF (Tan et al., 1994), and in rat primary glial cells (astrocytes and microglia) by interferon-γ (Vanguri, 1995). In most of these cases, ATF3 mRNA increased to the maximum level within two hours, and then started to decrease. However, when induced by interferon-γ in mouse macrophage cells, ATF3 mRNA level reached the peak within 2 hours and maintained the high level for more than 24 hours. Recently, using in situ hybridization analysis in animals, our laboratory further demonstrated that ATF3 is induced by physiological stresses in several rat tissues: in mechanically injured (partial hepatectomy) or toxin injured (CCl4, alcohol, and acetaminophen) liver, in blood-deprived (ischmic) heart, and in post-seizure brain (Chen et al., 1996; C. Wolfgang and T. Hai unpublished results).

The inducibility of ATF3 by physiological stresses prompted us to postulate that the stress-induced JNK/SAPK signaling pathway may be involved in the activation of the ATF3 promoter (Liang et al., 1996). At present, this notion is supported by three observations: First, the expression of ATF3 is induced in tissue culture cells by anisomycin (Liang et al., 1996), an agent that has been demonstrated to activate the
JNK/SAPK signaling pathways (Mahadevan and Edwards, 1991; Cano et al., 1994). Second, anisomycin treatment activates a CAT reporter gene driven by the ATF3 promoter in both \textit{in vivo} transfection and \textit{in vitro} transcription analyses (Liang et al., 1996). Third, ATF3 promoter was activated by components in the JNK/SAPK pathway. As an example, over-expression of MEKK, a kinase that is thought to be specific to the JNK/SAPK pathway, can activate a CAT reporter driven by the ATF3 promoter (G. Liang and T. Hai unpublished result). However, we do not propose that the JNK/SAPK pathway is the only pathway involved in the activation of the ATF3 promoter. The induction of ATF3 by TPA, Ca\textsuperscript{2+} influx, EGF, and interferon may be mediated by other signaling pathways.

ATF3 is able to form homodimers and bind to DNA. In addition, ATF3 heterodimerizes with a variety of bZip proteins: CRE-BP1/ATF2, c-Jun, JunB, JunD, and gadd153/Chop10 (Hai and Curran, 1991; Hsu et al., 1991; Hsu et al., 1992; Tan et al., 1994; Chen et al., 1996). The ATF3 homodimer, as will be discussed in Chapter II, is a transcriptional repressor. In contrast, ATF3/c-Jun and ATF3/JunB heterodimers have been demonstrated to activate transcription (Hsu et al., 1991; Hsu et al., 1992;). The interaction between ATF3 and gadd153/Chop10 resulted in a heterodimer that does not bind to the ATF site and several ATF-related sites. Consequently, gadd153/Chop10 relieves the transcriptional repression by ATF3 (see Chapter IV for details). However, not much is known about the interaction between CRE-BP1/ATF2 and ATF3.

To understand the physiological relevance of ATF3, it is important to identify the target promoters of ATF3. Thus far, several promoters have been implicated to be regulated by ATF3. As an example, the proenkephalin promoter has been shown to be activated synergistically by fibroblast growth factor (FGF) and cAMP in neuroblastoma (SK-N-MC) cells (Tan et al., 1994). Among many different ATF/CREB and AP1 transcription factors tested, ATF3 and c-Jun stimulated proenkephalin promoter in both Ras- (mediates the EGF effect) and PKA-dependent manner. Evidence from my work indicates that ATF3 represses transcription from the E-selectin, adenoviral E4, and gadd153/Chop10 promoters (see details in Chapter II). However, one intrinsic problem of all these results (obtained from either transfection or \textit{in vitro} transcription assays) is that they are analyzed in an artificial over-expression system, and they may not reflect the real the physiological events occurred in the cell. Therefore, without additional
information from different aspects, it would be difficult to conclude whether a given promoter is the real target promoter for ATF3.

**ATF4**

Not much is known about the function of ATF4. Evidence from our laboratory indicated that ATF4 is a strong transcriptional activator (G. Liang and T. Hai unpublished result). In addition, ATF4 is able to function properly and activate transcription in yeast (G. Liang and T. Hai unpublished result). In contrast to the result from our laboratory, J.M. Leiden and colleagues reported that ATF4 is a transcriptional repressor and negatively regulates transcription from the cAMP responsive element (Karpinski et al., 1992). In that study, the authors showed that ATF4 represses CREB mediated transcriptional activation; however, they did not show that ATF4 by itself can repress transcription. In addition, the authors used excess amounts of an ATF4 expressing plasmid in the transfection experiment. G. Liang, a graduate student in our laboratory, demonstrated that excess amounts of ATF4 results in "squelching" in both transfection and *in vitro* transcription analyses: Instead of activating transcription, excess ATF4 represses transcription by sequestering the limiting amount of general transcription factors or co-factors away from the transcriptional machinery (G. Liang and T. Hai unpublished results). To reconcile the differences, we suggest that J.M. Leiden and colleagues observed the "squelching" effect, instead of "true" transcriptional repression by ATF4 (G. Liang and T. Hai unpublished results).

**Aplysia ATF/CREBs**

Aplysia has been a model system for studying learning and memory. Previously it was known that the cAMP mediated signaling pathway plays a central role in this process (Schacher et al., 1988; Scholz and Byrne, 1988). In addition, to convert short-term to long-term facilitation (memory) requires *de novo* protein synthesis (Schacher et al., 1988). Recent evidence from E.R. Kandel's Laboratory suggests that cAMP induced gene expression in aplysia plays an important role in long-term facilitation. In addition, this process is mediated by a CREB-like transcription factor. Thus far, two
aplysia CREB-like proteins have been isolated; they are referred to as ApCREB1 and ApCREB2 (Bartsch et al., 1995). ApCREB1 is highly homologous to the mammalian CREB in both bZip domain and KID domain (96% and 90% amino acid identity respectively); in addition, it is also phosphorylated and activated by cAMP dependent protein kinase (PKA). ApCREB2 is similar to the mammalian ATF4 in the bZip domain (50% amino acid identity) and it appears to act as an antagonist of ApCREB1: When cotransfected with ApCREB1, ApCREB2 represses ApCREB1 mediated transcriptional activation. In addition, inhibition of ApCREB2 activity by microinjection of ApCREB2 antiserum enhances long-term facilitation in aplysia. Therefore, ApCREB2 is thought to repress long term facilitation (Bartsch et al., 1995).

**Drosophila ATF/CREBs**

Thus far, two drosophila ATF/CREBs have been identified: BBF-2/dCREB-A (Abel et al., 1992; Smolik et al., 1992) and dCREB2 (Yin et al., 1995a). BBF-2/dCREB-A was the first drosophila ATF/CREB identified and was shown to be involved in cell-specific regulation of alcohol dehydrogenase (Abel et al., 1992). dCREB2 is highly homologous to the mammalian CREB and CREM and contains a variety of alternatively spliced isoforms; in addition, it has a region containing the consensus phosphorylation sites for PKA, PKC, casein kinase, and calmodulin-dependent kinases, a domain similar to the KID domain of the mammalian CREB (Yin et al., 1995a). Similar to the mammalian CREB and aplysia CREBs, dCREB2 is involved in learning and memory. T. Tully and colleagues showed that one of the isoform, dCREB2-a, functions as a cAMP-responsive activator and enhances the long-term facilitation (Yin et al., 1995b), while another isoform, dCREB2-b, acts as an antagonist and inhibits the process (Yin et al., 1994).
Thus far, at least four yeast ATF/CREBs have been identified: ACR1/Sko1 (Vincent and Struhl, 1992; Nehlin et al., 1992), HAC1 (Nojima et al., 1994), atfl+ (Takeda et al., 1995), and pcr1+ (Watanabe and Yamamoto, 1996). They are similar to the mammalian ATF/CREB proteins in the bZip domain (especially in the basic region); in addition, they all recognize and preferentially bind to the ATF binding site. ACR1/Sko1 appeared be a transcriptional repressor (Vincent and Struhl, 1992) and is involved in negative regulation of the glucose-repressible SUC2 gene (Nehlin et al., 1992). atfl+ and pcr1+ have been shown to be involved in the regulation of sexual development. Disruption of either gene resulted in inefficient mating and sporulation. This is probably because atfl− or pcr1− cells lost the induction of ste11 which is a key transcription factor for a number of genes required for sexual development (Takeda et al., 1995; Watanabe and Yamamoto, 1996). HAC1 was isolated as a weak suppressor of a S. Pombe cdc10Δ mutant (Nojima et al., 1994). However, its function remains unknown.

An overview of the rest of the thesis

In Chapter II to V, I will describe my findings on ATF3: In Chapter II, I will present evidence that ATF3 is a transcriptional repressor and that it represses transcription presumably by stabilizing the putative co-repressors at the promoter. In Chapter III, I will describe my efforts to investigate the putative co-repressors. I present the results of library screening for the cDNA clones encoding "ATF3-interacting" proteins. Although none of the five clones I isolated appear to encode the putative co-repressors, I further characterize the interaction between ATF3 and one of the "ATF3-interacting" proteins, gadd153/Chop10. In Chapter IV, I describe the interaction between gadd153/Chop10 and ATF3, and their potential roles in the liver during stress responses. Specifically, I will describe their roles in the liver's responses to the xenobiotic agent, carbon tetrachloride (CC14). In Chapter V, I describe two additional approaches to investigate the putative co-repressors: in vivo assembly and ATF3 affinity chromatography. In Chapter VI, I propose some future directions for these projects.
Perspective

My work demonstrated that, contrary to the implication of its name, ATF3 represses transcription. When ATF was named in 1987, it was assumed that there was only one ATF and that it was a transcriptional activator. From the hindsight, the fact that ATF represents a large gene family already indicated the possibility that some of its members may be activators while others may be repressors. It is reasonable that a biological regulatory system has the ability to activate and to repress gene expression. My work also suggested that one possible mechanism by which ATF3 represses transcription is to stabilize the co-repressors on the promoter. This "co-repressor" model is consistent with the emerging observation that certain transcriptional repressors repress transcription through interacting with co-repressors. This observation is reminiscent of the well established co-activator model for transcriptional activation indicating that transcriptional repression and activation could be very similar. Thus far, only a few co-repressors have been identified from yeast and mammalian cells (see Chapter III introduction section). My study on ATF3 provides another example to further investigate the significance of transcriptional co-repressors. Recently, C. Wolfgang in our laboratory demonstrated that ATF3 is induced by a variety of physiological stresses. This has broadened our understanding of stress responses: It induces not only transcriptional activators to turn on certain gene expression but also transcriptional repressor to turn off certain gene expression. Further investigation of the target promoters of ATF3 would give us more insight to the cascade of events occurred in stress responses.

Acknowledgment

I thank G. Liang for sharing his unpublished results.
Chapter II

The transcriptional activity of ATF3

Summary

Activating transcription factor 3 (ATF3) is a member of mammalian ATF/CREB family of transcription factors. In this chapter, I present evidence that, contrary to the implication of its name, ATF3 is not a transcriptional activator; instead, it is a transcriptional repressor. ATF3 represses transcription when it binds to the ATF sites on the promoter. *In vivo* transfection analysis suggests that one possible mechanism by which ATF3 represses transcription is to stabilize the co-repressors at the promoter. This "co-repressor" model further explains that, when not binding to the promoter, ATF3 activates transcription by sequestering the co-repressors away from the promoter.

Introduction

The transcriptional regulatory region of a typical eukaryotic protein-encoding gene contains a TATA box for assembling the basal transcriptional machinery, and a combination of short elements for the binding of upstream transcription factors. The TATA box is recognized by the TATA binding protein (TBP) which recruits other general transcription factors and RNA polymerase II. To date, seven general transcription factors have been identified: IIA, IIB, IID (TBP and TAFs), IIE, IIF, IIH, and IIJ. Together with RNA polymerase II, they assemble into the basal transcriptional machinery around the transcriptional start site. Many lines of evidence indicate that the basal transcriptional machinery produces a low and un-regulated level of transcription.
To precisely regulate transcription requires binding of upstream transcription factors in the promoter.

Most upstream transcription factors contain a DNA binding domain that recognizes specific DNA sequence at the promoter (for a review see Mitchell and Tjian, 1989). Some transcription factors, however, do not bind to DNA directly; instead they are tethered to DNA by interacting with DNA-bound transcription factor. For example, the 289-amino-acid adenovirus E1a protein was shown to bind to ATF2 and activate transcription (Liu and Green, 1990; Flint and Jones, 1991). Once bound to the promoter, the upstream transcription factors may activate or repress transcription through interacting with the basal transcriptional machinery or other transcription factors. Many upstream transcriptional activators contain, in addition to the DNA binding domain, the transcriptional activation domains. Several different classes of transcriptional activation domains have been reported such as the glutamine-rich, proline-rich, and acidic activation domains (Mitchell and Tjian, 1989). These transcriptional activation domains have been implicated to interact directly or indirectly with components of the basal transcriptional machinery. However, the detailed mechanism of transcriptional activation is not fully understood.

In the past decade, much progress has been made in transcriptional activation; only recently did transcriptional repression start to gain more attention. As suggested by Johnson and colleague, the relative unpopularity of transcriptional repression was probably due to two reasons (Herschbach and Johnson, 1993; Johnson, 1995): First, eukaryotic gene expression originally emphasized aspects of transcriptional activation, since in a typical eukaryotic cell only about 7% of the DNA sequences will ever be transcribed. It seems unlikely that the remaining DNA sequences are specifically blocked by transcriptional repressors. Second, most eukaryotic promoters require DNA-bound transcriptional activators to function in vivo.

For a transcriptional repression involves at least three types of strategies: (1) interference with the DNA binding of a transcriptional activator, (2) interference with the activity of a DNA bound transcriptional activator, and (3) interference with the basal transcriptional machinery (Johnson, 1995). First, a transcriptional repressor could inhibit the DNA binding of a transcriptional activator and repress transcription. This can
be achieved by several different mechanisms. A repressor can simply prevent an activator from getting into the nucleus and binding to DNA. One well characterized example is the inhibition of NF-κB nuclear localization by IkB (Nolan et al., 1991; Beg et al., 1992; Inoue et al., 1992). Since a variety of transcription factors have been shown to bind to DNA as a dimer molecule, a repressor can prevent an activator from binding to DNA by forming a non-functional heterodimer with it. For example, drosophila extramacrochaetae (emc) repressor contains the HLH dimerization domain but lacks the basic region for DNA binding. When dimerizing with daughterless (da), which contains the basic region and HLH domain, emc prevents da from dimerizing with other bHLH proteins (encoded by achaete-scute complex) and binding to DNA (Van Doren et al., 1991). In addition, a repressor can directly compete with an activator for the same DNA binding site. For example, ICER, a CREM isoform containing only the minimum bZip domain, can compete for the ATF/CRE binding sites with transcriptional activators such as CREB, CREMt, and ATF1 (Molina et al., 1993). Finally, a repressor can have a binding site adjacent to an activator. When bound to its recognition site, the repressor prevents the activator from binding to the adjacent site. For example, the binding sites of Drosophila krüppel (Kr) repressor have often been found to overlap with that of the activators such as hunchback and bicoid (Small et al., 1991).

Second, a transcriptional repressor can interfere with the activity of a DNA bound transcriptional activator and prevent it from activating transcription. This can be achieved by several ways such as quenching and masking. In the case of quenching, a repressor binds adjacently but not overlappingly with an activator; through direct protein-protein interaction, the repressor inhibits the function of the activator. For example, when bound next to an activator, Drosophila Kr repressor selectively represses transcription mediated by glutamine-rich activation domain presumably through specific protein-protein interaction (Licht et al., 1993). Quenching also happens when two transcriptional activators bind to DNA adjacent to each other. For example, the glucocorticoid receptor and AP-1 (Fos/Jun heterodimer) bind DNA together and prevent mutual transcriptional activation (Diamond et al., 1990). A repressor can also interfere with the activity of a DNA bound activator by masking the activation domain of the activator. One classic example is the masking of Gal 4 C' terminal activation domain by Gal 80 (Lu et al., 1987; Ma and Ptashne, 1987). In addition to quenching and masking.
mechanisms, the activity of a DNA bound transcriptional activator could be inhibited by another transcriptional activator through squelching (or sequestering) mechanism. The "squelching" mechanism originally explained that, when the transcriptional activators are in excess, the excess free activators will compete with DNA-bound activators for the limited transcription factors and result in transcriptional repression (Gill and Ptashne, 1988). Recently, Rosenfeld and colleagues reported that nuclear hormone receptors inhibit AP-1 activity by sequestering the limiting amounts of CBP/P300 away from AP-1 (Kamei et al., 1996).

Third, a transcriptional repressor can interfere with the basal transcriptional machinery and prevent transcription. Some repressors simply bind at or near to the transcription start site and block the access of the general transcription factors or RNA polymerase. This blocking mechanism is common in prokaryotes and is also found in eukaryotes, such as SV40 T antigen inhibiting its own promoter (Hansen and Tenen, 1981). A repressor can also actively repress transcription by interfering with the assembly or the activity of the basal transcription machinery. For example, Dr1/DRAP1 complex binds directly to TBP and prevents TBP from interacting with TFIIB and TFIIB (Inostroza et al., 1992; Mermelstein et al., 1996), while Mot1 prevents TBP from binding to DNA in an-ATP dependent manner (Auble et al., 1994). Consequently, both Dr1/DRAP1 and Mot1 inhibit the assembly of the basal transcriptional machinery and repress transcription. In addition, some repressors have been shown to repress transcription through interacting with a group of co-repressor proteins which will be further discussed in Chapter III.

In this chapter, I will present evidence that, contrary to the implication of its name, ATF3 is not a transcriptional activator; instead, it is a transcriptional repressor. Prior to this work, it was not clear how ATF3 regulated transcription. As a first step to examine the transcriptional activity of ATF3, I carried out co-transfection experiments. I co-transfected ATF3 with the chloramphenicol acetyltransferase (CAT) reporters driven by ATF sites, non-ATF sites, or naturally occurring promoters and examined how ATF3 might regulate these reporters.
Material and methods

Plasmids

pCG-ATF3 was constructed as follows. The ATF3 open reading frame (ORF) was amplified by PCR using *Pfu* polymerase (Stratagene), upstream primer (5'-CGCTCTAGAACCATGATGCTTCAACACCAGGC-3'), and downstream primer (5'-CCGGATCCTTAGCTCTGCAATGTTCCTTC-3'). After PCR amplification, the amplified DNA fragment had an XbaI site introduced at the N' terminus and a BamHI site at the C' terminus of the ATF3 ORF. The XbaI/BamHI DNA fragment was inserted between XbaI and BamHI sites of the pCG expression vector (Tanaka and Herr, 1990) which provided the cytomegalovirus (CMV) promoter, the herpes simplex virus thymidine kinase 5' untranslated region and the ATG initiation codon. pCG-ATF3ΔZip was constructed by the same method using the same previous primers. ATF3 deletion mutants were constructed by inserting the corresponding ATF3 cDNA fragments in frame into the pCG vector: XbaI-PvuII blunt-ended fragments (by AMV reverse transcriptase) for pCG-ATF3(1-100), Aal-I-BamHI blunt-ended fragments (by Mung Bean Nuclease) for pCG-ATF3(72-181), PvuII-BamHI blunt-ended fragments (by AMV reverse transcriptase) for pCG-ATF3(101-181), Pstl-BamHI blunt-ended fragments (by Mung Bean Nuclease) for pCG-ATF3(116-181), and SacI-BamHI blunt-ended fragments (by Mung Bean Nuclease) for pCG-ATF3(136-181). pCGN-ATF2 was constructed as follows. A BamHI blunt-ended fragment (by Mung Bean Nuclease) containing the ATF2 ORF was isolated from pRSET-ATF2 (a gift from Dr. J. Hoeffer) and inserted in frame into the pCGN vector (a modified version of pCG containing the HA tag) between the XbaI and BamHI blunt-ended sites (by AMV reverse transcriptase). pCGN-P50 and pCGN-P65 expressing the NF-κB subunits (p50 and P65) were gifts from Dr. M. Su. pTM1.His-ATF3 and pTM1.His-ATF3Δzip were constructed by cloning the corresponding XbaI/BamHI blunt-ended fragments (by AMV reverse transcriptase) in frame into the pTM1.His vector (modified from pTM1, Moss et al., 1990) into the blunt-ended XhoI site (by Mung Bean Nuclease). The chloramphenicol acetyltransferase (CAT) reporter driven by three tandem ATF sites (ATFx3-CAT) was constructed by inserting a double-stranded oligonucleotide CCCGGGATGACGTCATCCCGGG containing the ATF consensus sequence (underlined) into the Sall blunt-ended site (by AMV reverse transcriptase) of pEC (a gift from Dr.s S. Liang and M. Ptashne) upstream from the E1b TATA and the
CAT gene. SP1/ATFx4-CAT was constructed by inserting a double-stranded oligonucleotide GGTTGCCGCGGGCGGAAGTGACGTTTTCCC containing the SP1 and ATF sites (underlined) into the HindIII blunt-ended site (by AMV reverse transcriptase) of ATFx3-CAT reporter. SP1x3-CAT was constructed by inserting double-stranded oligonucleotide ATTCGATCGGGGCGGGGCGAGC (Promega) containing SP1 consensus sequence (underlined) into the SalI blunt-ended site (by AMV reverse transcriptase) of pEC. ATF/SP1-CAT and mATF/SP1-CAT were constructed as follows. An EcoRI/SphI blunt-ended DNA fragment (by Mung Bean Nuclease) containing six tandem SP1 sites was released from pG6TI-CAT (a gift from Dr.s G. Gill and R. Tjian) and subcloned into the SalI blunt-ended site (by AMV reverse transcriptase) of pEC to create a modified pEC (SP1x6-CAT). Blunt-ended DNA fragments containing either three consensus ATF sites (see above) or four mutant ATF sites ATGAGTTCAT (mutant position underlined) were then inserted into the HindIII blunt-ended site (by AMV reverse transcriptase) of the SP1x6-CAT. pBxSST, a gift received from Dr. O.M. Andrisani, contains the somatostatin promoter (-750 to +50) driven CAT reporter gene (Andrisani et al., 1989). pENKAT-12, a gift received from Dr. M. Comb, contains the human proenkepholin promoter (-193 to +210) driven CAT reporter gene (Comb et al., 1986). pE-selectin-CAT, a gift received from Dr. J. Whelan, contains the human E-selectin promoter (-383 to +80) driven CAT reporter gene (Whelan et al., 1991). E4-CAT was constructed by Guosheng Liang as follows. An EcoRI blunt-ended (by AMV reverse transcriptase)/AluI fragment containing the adenovirus major early 4 (E4) promoter (-330 to +17, with -65 to -138 deleted) was isolated from pE4SM1 and subcloned into pCAT-basic (Promega) at the XbaI blunt-ended site (by AMV reverse transcriptase). gadd153-CAT, a gift received from Dr. N.J. Holbrook, contains the hamster gadd153/Chop10 promoter (-778 to +21) driven CAT reporter gene (Luethy et al. 1990).

Cell culture
COS-1 cells were maintained at 37°C in Dulbecco's modified Eagle media (DMEM) containing Penicillin/Streptomycin (BRL) and 10% fetal bovine serum (FBS) in a CO2 incubator. HeLa cells were maintained at 37°C in DMEM containing Penicillin/Streptomycin and 10% calf serum (CS) in a CO2 incubator. For maintaining the cell lines, the cells were split 1:2 everyday or 1:4 every two days in 100 mm plates. For transfection, the cells were split 1:6 from one 100 mm plate to six 60 mm plates 24
hours prior to the transfection experiment. Every two months, the existing cell line was discarded and replaced with the new cell line revived from a liquid nitrogen-frozen stock to ensure the quality of the cell line.

Transfection
COS-1 cells were transfected by the calcium phosphate method (Graham and van der Eb, 1973). Briefly, a total 7 μg of DNA in 175 μl of 250 mM CaCl2 solution was mixed with an equal volume of 2x HBS buffer (50 mM Hepes, 280 mM NaCl, 10 mM KCl, 1.5 mM Na2HPO4, 12 mM dextrose, pH 7.05). The 2x HBS buffer was added drop by drop to the DNA/CaCl2 solution with gentle vortexing throughout the whole process. After 30 minutes of incubation, the precipitated-DNAs were added to the 60 mm plates of COS-1 cells, at a 60-80% confluency, and incubated for 12 to 16 hours. In the earlier experiments, the transfected COS-1 cells were shocked with 15% glycerol in 1x HBS for 2 minutes, washed twice with phosphate-buffered saline (PBS: 0.8% NaCl, 0.02% KCl, 10 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.4), replenished with fresh media, and incubated for additional 24 hours before harvesting. However, I did not find any obvious increase in the transfection efficiency by the glycerol shock procedure. Therefore, in the later experiments, the transfected COS-1 cells were only washed twice by PBS and replenished with fresh media. Unless otherwise indicated, the amount of each plasmid DNA is: 1.7 μg of reporter, 1.7 μg of each plasmid DNA expressing the appropriate proteins or pCG vector without insert. pGEM3 DNA was added as a carrier to have a total of 7 μg DNA per 60 mm plate.

CAT assay
The transfected cells were washed twice with PBS, harvested using a rubber policeman, transferred to an eppendorf tube, and spun at 4°C for 2 minutes at 14,000 rpm in a microcentrifuge. The cell pellet was resuspended in 200 to 400 μl of Tris buffer (250 mM Tris-HCl [pH 7.8]) and frozen and thawed five times to lyse the cell pellet. After spinning at room temperature for 2 minutes, the supernatant (cell lysate) was transferred to a fresh tube, incubated at 65°C for 10 minutes to inactivate any deacetylases, and spun at room temperature for 2 minutes to remove any precipitation. CAT reactions were carried out in 100 μl of Tris buffer (a final 250 mM Tris-HCl [pH 7.8]) containing 10% to 25% of the cell lysate (depending on the activity of the promoter), 0.2 μCi 14C-chloramphenicol (Amersham or DuPont NEN), and 50 μg butyl coenzyme A (Sigma,
B-1508). The reaction was incubated at 37°C for 30 minutes to 1 hour. The conversion of 14C-chloramphenicol to the butylated form was measured by a phase extraction method (Ausubel et al., 1993). Briefly, the CAT reaction was mixed with 400 μl 2,6,10,14-tetramethylpentadecane (TMPD, Sigma T-7640)/Xylene (1:1) solution, vortexed for 5 minutes to extract the butylated chloramphenicol into the organic TMPD/Xylene phase, and spun at room temperature for 1 minute. 250 μl of the upper TMPD/Xylene phase was transferred to a scintillation vial. The CAT reaction tube was replenished with 250 μl TMPD/Xylene solution and the extraction procedure was repeated for two more times. The extracted butylated 14C-chloramphenicol in the scintillation vial was then mixed with 3 ml ScintiVerse (Fisher) and counted by the scintillation counter.

**Cell labeling and immunoprecipitation**

To examine the expression of ATF3 and ATF3 deletion mutants, 60 mm plates of COS-1 cells were transfected with indicated pCG derivatives by the calcium phosphate method described above. After washing twice with PBS, the transfected cells were replenished with 1 ml of methionine-deficient DMEM containing 10% FBS, and incubated at 37°C in a 5% CO2 incubator for 30 minutes. 100 to 200 μCi of 35S-methionine (Trans35S, >1000 Ci/m mole, ICN) was then added directly to the transfected COS-1 cells. The labeled 60 mm plates were placed inside of a 100 mm plate covered with charcoal paper, and incubated at 37°C in a 5% CO2 incubator for 2 hours. The charcoal paper was used to absorb the 35S compounds released into the air during the cell labeling process. The labeled COS-1 cells were washed twice with ice cold PBS, harvested using a rubber policeman, and spun at 4°C for 2 minutes to collect the cells. The cell pellet was resuspended in 45 μl PBS, mixed with 50 μl of 2x radio-immune precipitation assay (RIPA) buffer (100 mM Tris-HCl [pH 7.5], 300 mM NaCl, 2% NP-40 and 1% sodium deoxycholate) and 5 μl of 20% sodium dodecyl sulfate (SDS) to lyse the cells, boiled for 2 minutes, sonicated briefly to shear the chromosomal DNA, and spun at 4°C for 5 minutes to remove any insoluble material. The supernatant was diluted with 900 μl 1x RIPA buffer to make a final 0.1% SDS solution. Aliquots of the diluted supernatant were incubated with 3 μl of rabbit pre-immune serum or non-immune serum with gentle rocking at 4°C for 2 hours, followed by incubation with 30 μl of protein A agarose (BRL) at 4°C for additional 30 minutes. Material bound to protein A agarose was removed by centrifugation at 4°C for 30 seconds. The
supernatant was incubated with 3 μl of anti-ATF3 antiserum at 4°C overnight, followed by protein A agarose incubation as described above. The protein A bound material was collected by centrifugation at 4°C for 30 seconds. The beads were washed three times with 500 μl of RIPA buffer containing 0.1% SDS at 4°C for 5 minutes, resuspended in 1x SDS gel loading solution (50 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 0.2% bromophenol blue, 0.2% Xylene Cyanole) containing 0.1 M dithiothreitol (DTT), boiled for 3 minutes, and analyzed by electrophoresis on a 10 or 12% SDS-polyacrylamide gel.

Expression and purification of histidine-tagged recombinant proteins from E. coli or vaccinia virus

his-ATF3 and his-ATF1 were produced by expressing pET.His-ATF3 or pET.His-ATF1 in the BL21 (DE3) LysS E. coli strain. Purification under denaturing conditions using a Ni-NTA column and renaturation conditions are described in Chapter III. Expression and purification of his-ATF3 from vaccinia virus is described in Chapter V.

Complete and depleted HeLa nuclear extracts

Suspension HeLa cells (1x10⁶ cells/ml) were collected by centrifugation at 4°C, 2000x g (JS-4.2, 3.5 K RPM) for 15 minutes. The cell pellet was resuspended in a 5x pack cell volume (PCV) of buffer A (10 mM Hepes [pH 7.9], 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.2-0.5 mM PMSF), and spun at 4°C, 2000x g for 10 minutes. The cell pellet was resuspended in 3x PCV of buffer A, incubated on ice for 10 minutes to swell the cells, dounced with a Kontes glass Dounce (pestle B) for 40 strokes to break the cells, and spun at 4°C, 22,000x g (JA-14, 12K RPM) for 15 minutes to pellet the nuclei. The packed nuclei volume (PNV) was roughly estimated. The nuclei were resuspended in 0.5 x PNV of 0.2 M buffer C (20 mM Hepes [pH 7.9], 0.2 M KCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF) and dounced if is necessary. The PNV was re-estimated by measuring the final volume using a glass pipet. According to the re-estimated PNV, the amount of 1.1 M buffer C (similar to 0.2 M buffer C except 1.1 M KCl) required to make the final KCl concentration to 0.325 M was calculated. The nuclei were then transferred to a pre-chilled glass beaker and stirred gently with a magnetic bar at 4°C. While stirring, the nuclear extract was mixed with the calculated amount of 1.1 M buffer C drop by drop, stirred at 4°C for an additional 30 to 60 minutes to extract nuclear proteins, and spun at 4°C, 22,000x g (JA-14, 12K RPM) for 20 minutes. The supernatant (nuclear extract) was transferred to a 12 to 14 kDa cut-off
dialysis tubing and dialyzed against a 200 to 300-volume of 0.1M buffer D (20 mM Hepes [pH 7.9], 0.1 M KCl, 0.2 mM EDTA, 10% glycerol, 0.5 mM DTT, 0.5 mM PMSF) at 4°C for 3 hours. After dialysis, the extract was spun at 4°C, 22,000x g (JA-14, 12K RPM) for 20 minutes to remove any precipitation. The protein concentration was determined (optimal concentration should be around 10 mg/ml), the extract was aliquotted, frozen by liquid nitrogen, and stored at -80°C.

To make deplete endogenous ATFs from the nuclear extract, extracts were passed through an ATF DNA affinity column (see construction below) three times. Briefly, 1 to 2 ml complete HeLa nuclear extract was passed through a 0.2 ml ATF affinity column at the flow rate of 1 to 2 drops (= 30 µl)/minute, collected, and reloaded onto the column two more times. The column was then washed with 0.2 ml of 0.1 M buffer D to elute the remaining nuclear extract in the column. The ATF depleted nuclear extract was aliquotted, frozen by liquid nitrogen, and stored at -80°C.

The ATF DNA affinity column was constructed by coupling a ligated ATF specific oligonucleotide to CNBr-activated Sepharose 4B (Pharmacia) as follows. Three hundred microgram of an ATF oligonucleotide (5'-CCC GGATGACGTCAT-3', the ATF site underlined) in 80 µl H2O was mixed with 10 µl of 10x kinase & ligase buffer (0.5 M Tris-HCl [pH 7.5], 0.5 M KCl, 50 mM MgCl2, 0.1 M DTT) and incubated at 88°C for 2 minutes, at 65°C for 10 minutes, at 37°C for 10 minutes, and at room temperature for 5 minutes to self-anneal the ATF oligonucleotide. The double stranded ATF oligonucleotide was then mixed with 50 units (= 5 µl) of T4 oligonucleotide kinase (BRL) and 5 µl 20 mM ATP (final 1 mM), and incubated at 37°C for 2 hours to phosphorylate the ATF oligonucleotide. The kinase reaction was mixed with 5 µl T4 ligase (BRL, high concentration), 2 µl 20 mM ATP, 2 µl 10x kinase & ligase buffer, 11 µl H2O, and incubated at 14°C overnight to self-ligate the double stranded ATF oligonucleotide. The ligation reaction was continued by adding more ligase, kinase, ATP, and buffer until most of the ligated ATF oligonucleotide are larger than a 10 mer. To couple the ligated double stranded ATF oligonucleotide to CNBr activated Sepharose 4B beads, 0.5 g CNBr activated Sepharose 4B beads (=1.7 ml) was incubated with 200 ml of 1 mM HCl in a sintered glass funnel for 15 minutes and then washed with 40 ml of ice cold 10 mM K2PO4 [pH 8.0]. The beads were then resuspended in 3 ml of 10 mM
K2PO4 [pH 8.0], transferred to a 15 ml screw capped tube containing the ligated double stranded ATF oligonucleotide (in 100 µl K2PO4 [pH 8.0]), and incubated at room temperature with gentle rocking overnight. The beads were then washed with 40 ml of H2O, 20 ml of 1M ethanoamine-HCl [pH 8.0], and incubated with 3 ml of 1M ethanoamine-HCl [pH 8.0] at room temperature for 6 hours to inactivate any un-reacted beads. The beads were washed with 20 ml of 10 mM K2PO4 [pH 8.0], 20 ml of 1 M K2PO4 [pH 8.0], 20 ml of 1 M KCl, 20 ml of H2O, and equilibrated with 20 ml 0.1 M buffer D before use.

**In vitro transcription assay**

HeLa nuclear extract (7.5 or 12.5 µl) was mixed with 0.45 µg template DNA (reporters), the indicated amounts of purified ATF3 or ATF1, and all four ribonucleotide triphosphates (0.5 mM each) in a final 20 µl reaction containing 12.5 mM Hepes-NaOH [pH 7.9], 60 mM KCl, 7.5 mM MgCl2, 0.125 mM EDTA, 0.3 mM DTT, 12.5% glycerol, and 20 units RNasin (Promega). [Note: The amounts of ATF3 or ATF1 were indicated by µl instead of µg in the figure legends because it was not clear that how many percentage of the proteins are transcriptionally active. In addition, using µl to indicate the protein amounts is generally accepted for the *in vitro* transcription assay (for examples, see Abmayr et al., 1988; Shykind et al., 1995).] The transcription reaction was incubated at 30°C for 1 hour. The reaction was then terminated by adding of 10 µg protease K, 5 µg of tRNA, 70 µl of DEP-H2O, 100 µl of PK buffer (200 mM Tris-HCl [pH 7.5], 25 mM EDTA, 300 mM NaCl, 2% SDS), and incubated at 37°C for an additional 30 minutes. After phenol extraction and ethanol precipitation, the in vitro transcribed RNA was subjected to primer extension in order to detect the specific transcript. In some experiments, an end-labeled EcoRI/HindIII polylinker fragment (55 bp) from pGEM3 (Promega) was mixed with the RNA sample after the phenol extraction to serve as an internal control for the ethanol precipitation procedure.

The RNA pellet was resuspended in DEP-H2O, mixed with 0.1 to 0.3 pmole of 32P-labeled primer (>100,000 cpm) in a 10 µl hybridization reaction containing 40 mM Pipes [pH 6.4], 0.4 M NaCl, 1 mM EDTA, and incubated at 37°C overnight. The hybridization mixture was then mixed with 160 µl 2.5 M ammonium acetate and 500 µl 100% ethanol to precipitate the RNA-primer hybrids. The RNA-primer hybrid was
resuspended in a 15 µl reaction containing 50 mM Tris-HCl [pH 8.3], 40 mM KCl, 7 mM MgCl2, 1 mM DTT, 10 units RNasin (Promega), 1 mM each of all four deoxynucleotide triphosphates, 1 µg acetylated BSA, 2.5 units of AMV reverse transcriptase (Promega), and incubated at 42 °C for 2 hours to extend the primer. The primer extension reaction was mixed with 25 µl Maxam-Gilbert dye, boiled for 3 minutes, chilled on ice, and analyzed on a 15% polyacrylamide urea gel.
Results

ATF3 represses transcription in vivo.

To examine the function of ATF3, I carried out a co-transfection experiment as follows. I transfected COS-1 cells with a CAT reporter driven by three tandem ATF sites (ATFx3-CAT) and an ATF3 expressing plasmid (pCG-ATF3) which contains the full length ATF3 open reading frame under the regulation of the CMV promoter. The CMV promoter allows over-expression of ATF3 and examination of its function. In a control without ATF3, pCG vector was added to replace pCG-ATF3. As shown in Figure 2.1, ATF3 repressed transcription. ATF3 consistently repressed the activity of ATFx3-CAT reporter 5 to 10 fold. A similar result was also observed from another reporter construct, SP1/ATFx4-CAT, which was modified from ATFx3-CAT by inserting one additional ATF site and one SP1 site upstream of the three existing ATF sites. SP1/ATFx4-CAT reporter had higher activity than that of ATFx3-CAT. Therefore, the transcriptional repression by ATF3 was more apparent by using SP1/ATFx4-CAT reporter. To further confirm that ATF3 represses transcription, I examined whether increasing concentration of ATF3 could cause a greater repression. I co-transfected COS-1 cells with SP1/ATFx4-CAT reporter and different amount of pCG-ATF3 DNA. As shown in Figure 2.2, transfecting COS-1 cells with increasing amounts of pCG-ATF3 produced more ATF3 protein and resulted in a greater repression.

After showing that ATF3 repressed ATFx3-CAT and SP1/ATFx4-CAT reporters, I then investigated whether DNA-binding was required for repression. I examined the effect of ATF3 on three reporters: ATF/SP1-CAT, SP1x3-CAT, and mATF/SP1-CAT. The ATF/SP1-CAT reporter contains three functional ATF sites; the SP1x3-CAT and mATF/SP1-CAT reporters contain no functional ATF sites. As shown in Figure 2.3, similar to that of ATFx3-CAT and SP1/ATFx4-CAT, ATF3 also repressed the activity of ATF/SP1-CAT reporter. In contrast, ATF3 did not repress the activities of SP1x3-CAT (without ATF sites) or mATF/SP1-CAT (with mutant ATF sites). In fact, ATF3 activated both reporters. Although the level of activation was low, it was reproducible (see interpretation below).
Figure 2.1 ATF3 represses promoters with the ATF sites. COS-1 cells were transfected with a CAT reporter driven by three contiguous ATF sites (ATFx3-CAT) in the presence of either pCG vector(-) or pCG-ATF3(+) producing ATF3. The same experiment was carried using SP1/ATFx4-CAT which contains a CAT reporter driven by one SP1 site upstream of four ATF sites. The result is the average of three experiments.
Figure 2.2 ATF3 represses transcription in a dose-dependent manner. COS-1 cells were transfected with SP1/ATFx4-CAT reporter in the presence of pCG (-) or increasing amounts of pCG-ATF3 as indicated. A parallel experiment was carried out except the proteins were metabolically labeled with $^{[35S]}$methionine. ATF3 was isolated by immunoprecipitation and analyzed on a 15% SDS-polyacrylamide gel. The relative positions of the molecular size markers are indicated on the left and the ATF3 band is indicated by an arrow on the right.
Figure 2.3 ATF3 represses transcription in a DNA-binding dependent manner. ATF3 represses promoter containing ATF sites, but not promoters without functional ATF sites. COS-1 cells were transfected with CAT reporters driven by three ATF sites and six SP1 sites (ATF/SP1-CAT), three contiguous SP1 sites (SP1x3-CAT), or four mutant ATF sites and six SP1 sites (mATF/SP1-CAT) in the presence of either pCG vector(-) or pCG-ATF3 (+) producing ATF3. The result is the average of three experiments.
Thus far, all the reporters tested were driven by the artificial promoters. To determine whether ATF3 also represses transcription from naturally occurring promoters, I examined several promoters which contain ATF or ATF-like sites: proenkephalin (Comb et al., 1986), somatostatin (Andrisani et al., 1989), and E-selectin (Whelan et al., 1991) promoters. Importantly, ATF or ATF-like sites on these promoters have been demonstrated to be important for the function of these promoters. ATF3 neither repressed nor activated these promoters. Because these promoters have low activities prior to induction, it would be difficult to observe any repression. To increase the reporter activity, I induced the E-selectin promoter with NF-κB (p50/p65 heterodimer, Kawakami et al., 1988; Baeuerle and Baltimore, 1989; Nabel and Verma, 1993) and then examined whether ATF3 could repress the activated promoter. As shown in Figure 2.4, ATF3 almost completely abolished the activation of the E-selectin promoter by NF-κB, indicating that ATF3 can repress transcription from a naturally occurring promoter. As a control, I co-transfected ATF2 with NF-κB and found that, in contrast to ATF3, ATF2 further stimulated the E-selectin promoter activity. This result indicated that the repression of the activated E-selectin promoter activity by ATF3 is not a common feature for all the ATF/CREB family of transcription factors.

ATF3 represses transcription in vitro.

In addition to the in vivo transfection analysis, I examined whether ATF3 can repress transcription in vitro in an in vitro transcription assay. The scheme of the in vitro transcription assay is summarized in Figure 2.5. Briefly, to set up the in vitro transcription assay for ATF3, it requires the purified ATF3, DNA templates, and HeLa cell nuclear extract. ATF3, with histidine tag, was expressed and purified from E. coli or vaccinia virus expression systems. DNA templates were those CAT reporters used in the transfection experiments. HeLa cell nuclear extract, providing the transcriptional machinery, allowed the transcription of the DNA templates in vitro in the presence of all four ribonucleotide triphosphates. The in vitro transcribed RNA was then subjected to primer extension to detect the specific transcript from the DNA templates. To examine the transcriptional activity of ATF3, the in vitro transcription reaction was carried out in the absence or presence E. coli expressed ATF3. As shown in Figure 2.6, ATF3
Figure 2.4 ATF3 represses the activated E-selectin promoter activity. E-selectin-CAT reporter was co-transfected into COS-1 cells with the indicated plasmids (pCG (-), pCG-ATF2, or pCG-ATF3), in the absence or presence of plasmids expressing NF-kB (pCGN-P50 and pCGN-P65, 0.5 μg each). The result is the average of three experiments.
**In vitro transcription assay**

1. HeLa cell nuclear extract
2. purified ATF3 protein
3. rNTPs

ATF sites

Hybridize *in vitro* transcribed mRNAs with $^{32}$P labeled primer

Primer extension

---

*Figure 2.5 In vitro transcription assay*

HeLa nuclear extract provides the transcriptional machinery and allows the transcription of the DNA template *in vitro* in the presence of all four ribonucleotide triphosphates. The *in vitro* transcribed RNAs are subjected to primer extension analysis to detect the specific transcript from the DNA template.
repressed transcription from ATFx3-CAT reporter in a dose dependent manner. As a control, ATF3 did not repress transcription from SP1x6-CAT reporter indicating that the \textit{in vitro} transcriptional repression by ATF3 is also dependent on the ATF sites. A similar result was obtained by using vaccinia virus expressed ATF3 (data not shown). As another control, ATF1, expressed and purified from \textit{E. coli} using the same procedure, was tested. In contrast to ATF3, ATF1 did not repress transcription from ATFx3-CAT reporter; instead, ATF1 further activated transcription from ATFx3-CAT reporter indicating that the transcriptional repression by ATF3 is not general to all the ATF family members (Figure 2.6).

The ability of ATF3 to repress transcription \textit{in vitro} was further tested on naturally occurring promoters. Previously, using transfection analysis, I demonstrated that ATF3 repressed the activated E-selectin promoter activity. However, when tested \textit{in vitro}, the E-selectin promoter had relatively low promoter activity, making it difficult to observe any transcriptional repression by ATF3. Therefore, I examined the activity of ATF3 on the adenovirus E4 promoter which has a higher promoter activity \textit{in vitro}. As shown in Figure 2.7, ATF3 repressed E4-CAT reporter, while ATF1 further activated E4-CAT reporter. The ability of ATF3 to repress a naturally occurring promoter was further tested on the gadd153/Chop10 promoter. Previously, Curt Wolfgang, another graduate student in our laboratory, demonstrated that ATF3 repressed gadd153/Chop10-CAT reporter in a co-transfection experiment. When tested in the \textit{in vitro} transcription assay, the activity of gadd153/Chop10-CAT reporter was not repressed by \textit{E. coli} expressed ATF3; however, it was repressed by vaccinia virus expressed ATF3 (Figure 2.8). This result indicated that post-translational modification may regulate the transcriptional activity of ATF3. In addition, it also indicated that the mechanism by which ATF3 represses the gadd153/Chop10 promoter may be different from that by which it represses the adenovirus E4 promoter and the artificial promoter driven by three tandem ATF sites (see discussion for details).

Possible mechanisms of transcriptional repression by ATF3

Having demonstrated that ATF3 is a transcriptional repressor, we then asked what are the mechanisms by which ATF3 represses transcription. We proposed three
Figure 2.6 ATF3 specifically represses transcription *in vitro*.  
(A) ATFx3-CAT and SP1x6-CAT reporters were transcribed by complete HeLa nuclear extract in the absence (−) or presence of increasing amount of *E. coli* expressed ATF3.  
(B) ATFx3-CAT reporter was transcribed by complete HeLa nuclear extract in the absence (−) or presence of increasing amount of *E. coli* expressed ATF1. The arrow (→) indicates the primer extension product. The opened arrow (←) indicates the 55 bp internal control DNA fragment. Note: ATF1 activates the reporter at low dose (0.008 µl and 0.04 µl). However, ATF1 represses the reporter at higher dose (0.2 µl and 1 µl). This repression is probably caused by the squelching effect: excess ATF1 eventually sequesters the limiting transcription factors resulting in transcription repression.
Figure 2.7 ATF3 represses the adenovirus E4 promoter in vitro.
The E4-CAT reporter was transcribed by complete HeLa nuclear extract in the absence (-) or presence of increasing amount of E. coli expressed ATF3 or ATF1 as indicated.
Figure 2.8 Vaccinia virus expressed ATF3, but not *E. coli* expressed ATF3, represses the gadd153/Chop10 promoter *in vitro*. (A). The gadd153/Chop10-CAT reporter was transcribed by complete or ATF depleted HeLa nuclear extract in the absence (-) or presence of ATF3 produced by *E. coli* (E) or vaccinia virus (V) (B). The gadd153/Chop10-CAT reporter was transcribed by complete HeLa nuclear extract in the absence (-) or presence of the indicated amount of vaccinia virus expressed ATF3. The arrow (←) indicates the primer extension product. The opened arrow (↔) indicates the 55 bp internal control DNA fragment.
possible mechanisms to explain the transcriptional repression by ATF3: competition, dominant-negative, and active repression (Figure 2.9). First, ATF3 could repress transcription by competing with endogenous ATF activators for the ATF binding sites. Since there are endogenous ATF activators which bind to the ATF sites and activate transcription, by occupying the ATF sites, ATF3 could prevent endogenous ATF activators from binding to DNA and prevent them from activating transcription. Second, ATF3 could repress transcription by acting as a dominant-negative regulator of the endogenous ATF activators. Since ATF/CREB family of transcription factors bind to DNA as a dimer, through dimerizing with endogenous ATF activators, ATF3 could inhibit their transcriptional activity or change their binding specificity. Third, ATF3 could actively repress transcription. ATF3 not only occupies the ATF sites, but also directly or indirectly interacts with the transcriptional machinery and inhibits transcription.

Although we proposed these three mechanisms to explain the transcriptional repression by ATF3, we did not rule out the possibility that ATF3 may repress transcription by other mechanisms. In addition, these mechanisms are not mutually exclusive. Therefore, it is possible that ATF3 may repress transcription by the combination of several different mechanisms. Alternatively, ATF3 may repress different promoters by different mechanisms. In the following section, I will discuss some evidence to support the notion that ATF3 actively represses transcription by interacting with some "co-repressors".

"Co-repressor" model

As described earlier, using transfection analysis, we observed that ATF3 slightly stimulated reporters lacking a functional ATF binding site (SP1x3-CAT and mATF/SP1-CAT). Although the level of stimulation was low, it was reproducible. To explain these results, we proposed a "co-repressor" model (Figure 2.10) which is a subset of the "active repression" mechanism. The proposed co-repressors loosely interact with the transcriptional machinery and are the "intrinsic" parts of the basal transcriptional machinery. When ATF3 binds to DNA, it stabilizes the co-repressors at the promoter and results in transcriptional repression. When ATF3 does not bind to DNA but still
Figure 2.9 Possible mechanisms to explain the transcriptional repression by ATF3.
(A) ATF3 could repress transcription by competing with the endogenous ATF activators for the ATF binding sites. (B) ATF3 could repress transcription by acting as a dominant-negative inhibitor. Through dimerizing with endogenous ATF activators, ATF3 could inhibit their transcriptional activity or changing their binding specificity. (C) ATF3 could actively repress transcription.
A. 

ATF3 stabilizes co-repressors at promoters with ATF sites and represses transcription.

B. 

ATF3 sequesters co-repressors from promoters without ATF sites and activates transcription.

Figure 2.10 The "co-repressor" model
(A) ATF3 represses transcription from a promoter with ATF sties by stabilizing co-factors at the promoter. (B) ATF3 activates transcription from a promoter without ATF sites by sequestering the co-factors away from the promoter.
interacts with the co-repressors, ATF3 sequesters the co-repressors away from promoter and results in transcriptional activation.

This "co-repressor" model predicts that a mutant ATF3 lacking a functional DNA binding domain should activate transcription from reporters with or without ATF sites. To test this prediction, I constructed an ATF3 deletion mutant, ATF3(1-100), which lacks the leucine zipper domain and half of the basic region. Without the leucine zipper domain, presumably ATF3(1-100) can not dimerize and can not bind to DNA. (Note: ATF3Δzip, which lacks the leucine zipper domain but contains the intact basic region, failed to bind to DNA (Chen et al., 1994). Therefore, it is likely that ATF3(1-100), which is smaller than ATF3Δzip, also can not bind to DNA.) When tested in a co-transfection experiment, ATF3(1-100) activated reporters with ATF sites (SP1/ATFx4-CAT) or without ATF sites (SP1x3-CAT) (Figure 2.11). The "co-repressor" model was further supported by a naturally occurring ATF3 isoform, ATF3Δzip, which encodes a truncated ATF3 lacking the leucine zipper domain (Chen et al., 1994). When tested in the co-transfection experiment, similar to that of ATF3(1-100), ATF3Δzip also activated reporters with or without ATF sites (Figure 2.12).

The fact that ATF3(1-100) and ATF3Δzip activate transcription indicates that both still interact with the putative co-repressors. To further define the domains required for interacting with the co-repressors, I constructed additional ATF3 deletion mutants: ATF3(72-181), ATF3(101-181), ATF3(116-181), and ATF3(136-181). As shown in Figure 2.13; when tested in the co-transfection experiment using reporter driven by ATF sites (SP1/ATFx4-CAT), ATF3(72-181) repressed the reporter. ATF3(101-181), neither repressed nor activated the reporter. Since ATF3(101-181) still contains half of the basic region and the complete leucine zipper domain, it is not clear whether it can bind to the consensus ATF site. ATF3(116-181) and ATF3(136-181), both lack the basic region and presumably should not bind to DNA. When tested in the co-transfection experiment, similar to ATF3Δzip and ATF3(1-100), ATF3(116-181) and ATF3(136-181) both activated the reporter. The protein expression of all ATF3 deletion mutants was examined by cell labeling and immunoprecipitation analyses. As shown in Figure 2.14, except ATF3(136-181), they were expressed in a detectable level. Taken together, these results suggest that ATF3 interacts with the co-repressors through two
distinct domains: one at the N' terminus (1-100) and the other one at the C' terminus (136-181).
Figure 2.11 ATF3(1-100), lacking a functional DNA binding domain, activates reporters with or without ATF sites.

COS-1 cells were transfected with SP1/ATFx4-CAT reporter in the presence of different plasmids: pCG (-), pCG-ATF3 or pCG-ATF3(1-100). The same experiment was carried out using the SP1x3-CAT reporter. The activity of the reporter gene in the absence of ATF3 is arbitrarily defined as 100%. The result is the average of three experiments.
Figure 2.12 ATF3ΔZip, lacking a functional DNA binding domain, activates reporters with or without ATF sites. COS-1 cells were transfected with SP1/ATFx4-CAT reporter in the presence of different plasmids: pCG (-), pCG-ATF3 or pCG-ATF3ΔZip. The same experiment was carried out using the SP1x3-CAT reporter. The activity of the reporter gene in the absence of ATF3 is arbitrarily defined as 100%. The result is the average of three experiments.
Figure 2.13 The effect of ATF3 deletion mutants on SP1/ATFx4-CAT reporter activity. COS-1 cells were transfected with SP1/ATFx4-CAT reporter in the presence of different plasmids: pCG (-), pCG-ATF3, pCG-ATF3 (72-181), pCG-ATF3 (101-181), pCG-ATF3 (116-181), pCG-ATF3 (136-181), pCG-ATF3 (1-100), and pCG-ATF3ΔZip. The activity of the reporter gene in the absence of ATF3 is arbitrarily defined as 100%. The result is the average of two experiments.
Figure 2.14 The expression of ATF3, ATF3Δzip, and ATF3 deletion mutants. COS-1 cells were transfected with the indicated pCG derivatives encoded ATF3, ATF3Δzip, or ATF3 deletion mutants. The transfected COS-1 cells were metabolically labeled with [35S]methionine. All the ATF3 proteins were isolated by immunoprecipitation and analyzed on a 12% SDS-polyacrylamide gel. The relative positions of the molecular size markers are indicated on the left.
Discussion

In this chapter, I present evidence demonstrating that ATF3 is not a transcriptional activator; instead, it is a transcriptional repressor: ATF3 represses transcription in a DNA-binding dependent manner both in vivo and in vitro. The in vivo transfection analysis suggests that one possible mechanism by which ATF3 represses transcription is to stabilize co-repressors at the promoter. This "co-repressor" model was supported by three observations: First, ATF3 activated transcription from a reporter without ATF sites. Second, ATF3 deletion mutants lacking a functional DNA binding domain activated transcription from reporters with or without ATF sites. Third, ATF3Δzip, a naturally occurring ATF3 isoform without a functional DNA binding domain, also activated transcription from reporters with or without ATF sites.

Co-repressor model

This "co-repressor" model is similar to the "squelching" model but with an opposite effect. The "squelching" model explains that, when the transcriptional activators are in excess, the excess free activators will compete with DNA-bound activators for the limited transcription factors (Gill and Ptashne, 1988). The result is that the activator, instead of activating transcription, inhibits transcription by sequestering the limited transcription factors away from the promoters. By the same logic, the "co-repressor" model explains, when not binding to DNA, ATF3 activates transcription presumably by sequestering the co-repressors away from the promoters. Taking the parallel of these two models, one would expect when ATF3 is in large excess, it will sequester the co-repressors away and activate transcription from not only the promoters without ATF sites but also the promoters with ATF sites. When co-transfecting SPI/ATFx4-CAT reporter with increasing amount of pCG-ATF3, I did observe occasionally that the reporter activity decreased to a certain point and then started to increase (data not shown). However, it was not consistent. One explanation is that, once bound to DNA, ATF3 interacts with co-repressors and DNA at the same time and forms a tightly associated complex. Consequently, the co-repressors are committed to the promoter and can not be sequestered by the excess free ATF3. ATF3(1-100) and ATF3Δzip, however, can activate transcription from both promoters.
with ATF sites and promoters without ATF sites, because they differ from the full length ATF3 in a significant manner: They do not bind to DNA and will not be committed to the promoter. Consequently, they can sequester the co-repressors away from even the promoters with ATF sites. This hypothesis is supported by the observation that, when co-transfected with ATF3 (1 μg) and different amounts of ATF3Δzip (0.15 μg, 0.5μg, 1.5 μg and 5 μg), the SP1/ATFx4-CAT reporter was always repressed by ATF3 (data not shown). Presumably, ATF3 helps the co-repressors to commit to the promoter. Therefore, even in excess amounts, ATF3Δzip can never sequester the co-repressors away from the promoter in the presence of ATF3.

The proposed co-repressors are presumably components of the basal transcriptional machinery but loosely interact with the transcriptional machinery. Recently, new evidence suggest that RNA polymerase II and some of the general transcription factors form a so-called "Pol II holoenzyme" before being recruited to the promoter (Koleske and Young, 1994). The purified "Pol II holoenzyme" from HeLa cells has a relative molecular mass of ~2,000 kDa and contains RNA polymerase II, TFIIE, THIIF, mammalian SRBs (adaptor proteins), and others (Chao et al., 1996; Maldonado et al., 1996). Although most of the components of the "Pol II holoenzyme" described are involved in transcriptional activation, it is possible that some unidentified components are involved in transcriptional repression, such as the putative co-repressors. When the co-repressors are removed from the holoenzyme, the transcriptional activity of the "Pol II holoenzyme" may be enhanced. This may explain how ATF3 activates transcription from promoters without ATF sites.

Deletion studies of ATF3 indicated that ATF3 interacts with the co-repressors through two distinct domains: one at the N' terminus (1-100) and the other one at the C' terminus (136-181). This notion is based on indirect evidence that ATF3 mutants, containing either of these two domains but lacking the functional DNA domain, activated SP1/ATFx4-CAT reporter. One way to further substantiate this notion is to demonstrate that these two domains can mediate the transcriptional repression when fused to a heterologous DNA binding domain. However, when fused with the Gal 4 DNA binding domain, ATF3 did not transfer its inhibitory activity to the Gal 4 DNA binding domain (data not shown). It appears that the inhibitory activity of ATF3 is not transferable. ATF3 may need to bind to DNA directly to be able to repress transcription.
It is possible that, when bound to DNA, ATF3 may change conformation to help stabilize the co-repressors at the promoter. An ATF3 fusion protein with a heterologous DNA binding domain may not have the proper conformation and therefore can not repress transcription. If this is the case, point mutation studies of these two domains may give more insights to the interaction between ATF3 and the co-repressors.

An interesting observation of these two domains is that they are also required for ATF3 to interact with Tax. Tax is a viral transcription factor encoded by human T-cell leukemia virus 1 (HTLV-1) and is required to transactivate HTLV-1 LTR. Similar to adenovirus E1a, Tax is unable to bind to DNA directly (Giam and Xu, 1989; Marriott et al., 1989). Therefore, Tax was thought to be tethered to DNA through interacting with a DNA-binding transcription factor. Several cellular transcription factors, including ATF3, have been shown to interact with Tax (Low et al., 1994). Evidence indicated that ATF3 interacts with Tax through two distinct domains: ATF3 (1-66) and ATF3 (139-181). These two Tax-interacting domains overlap with the "co-repressor interacting" domains defined in this work. However, contrary to the interaction between ATF3 and co-repressors, the interaction between ATF3 and Tax does not result in repression of transcription; instead, it activates transcription. It is possible that, upon HTLV-1 infection, Tax competes with the co-repressors for interacting with ATF3. Consequently, Tax may alter the function of ATF3 from repressing transcription to activating transcription.

The evidence that I have described thus far for the "co-repressor" model is only indirect. To prove the model, it is necessary to isolate the co-repressors and demonstrate their ability to support the transcriptional repression by ATF3. According to the model, one would expect that the co-repressors are among those proteins that interact with ATF3. Screening cDNA expression library or detecting by biochemical approaches for the ATF3-interacting proteins will help to identify the co-repressors. Alternatively, we could examine whether ATF3 interacts with any of the known general transcription factors or co-factors, and test whether they can support ATF3 to repress transcription.
In vitro transcription assay

Although my results from the in vivo transfection analysis are consistent with the "co-repressor" model, we can not rule out the possibility that ATF3 may repress transcription by other mechanisms. In fact, evidence from in vitro transcription analysis indicates that ATF3 could repress transcription by the "competition" mechanism. Previously, I demonstrated that ATF3 represses the ATFx3-CAT and the E4-CAT reporters in vitro. Further analysis indicates that the activities of these two reporters are dependent on endogenous ATF proteins in the HeLa nuclear extract; depletion of endogenous ATF proteins from HeLa nuclear extract greatly reduces the activities of ATFx3-CAT and the E4-CAT reporters (data not shown). This result suggests that, simply by occupying the ATF sites, ATF3 can prevent endogenous ATF proteins from binding and from activating these two reporters. In addition to the "competition" mechanism, ATF3 may also repress transcription by other mechanisms. One indication came from the study of the gadd153/Chop10 promoter. The activity of gadd153/Chop10 promoter was not affected by the depletion of endogenous ATF proteins from HeLa nuclear extract (data not shown). Therefore, the repression of this promoter by ATF3 can not be due to the simple replacement of the endogenous ATF proteins by ATF3. In addition, even in the ATF-depleted HeLa nuclear extract, vaccinia virus expressed ATF3 still repressed the gadd153/Chop10 promoter. The result suggested that ATF3 probably represses gadd153/Chop10 promoter through mechanism other than competing with the endogenous ATF proteins for binding to the DNA. Therefore, gadd153/Chop10 promoter may be a good candidate to test whether ATF3 can actively repress transcription in vitro.

The observation that gadd153/Chop10 promoter was repressed by vaccinia virus expressed ATF3 but not E. coli expressed ATF3 suggests that post-translational modification may be important for the activity of ATF3. Apparently, post-translational modification is not required for ATF3 to repress all the promoters because E. coli expressed ATF3 can repress ATFx3-CAT and E4-CAT reporters well. As discussed earlier, ATF3 represses both reporters by preventing the endogenous ATF proteins from binding to the promoter. Therefore, it is not surprising that E. coli expressed ATF3 can repress these promoters, because E. coli expressed ATF3 can bind to DNA. It is possible that post-translational modification affects the ability of ATF3 to interact with
other proteins such as co-repressors. This hypothesis explains why gadd153/Chop10 was only repressed by vaccinia virus expressed ATF3 but not E. coli expressed ATF3. In addition, it is also consistent with the notion that ATF3 represses the gadd153/Chop10 promoter through different mechanisms. However, this hypothesis is only based on few observations. These observation need to be confirmed and the hypothesis needs to be tested by further experiments.
Chapter III

Isolation and identification of cDNA clones encoding ATF3 interacting proteins

Summary

One method to investigate the "co-repressor" model is to identify ATF3-interacting proteins. Using protein-protein blot screening approach, I screened a COS-1 cDNA expression library and identified five previously known proteins that can interact with radiolabeled ATF3: keratin 7, keratin 8, USF2/FIP, C/EBPγ, and gadd153/Chop10. All five proteins contain leucine zipper or leucine zipper like domain. In addition, protein-protein blot analysis further demonstrated that they all interact with ATF3 through the leucine zipper domain. Although none of them appears to be the putative co-repressor, the potential consequences of their interaction with ATF3 are discussed.

Introduction

As described in Chapter II, transcriptional repressors can function by actively interfering with the basal transcription machinery: The transcriptional repressor may interact directly with the basal transcriptional machinery or indirectly through adapter proteins (co-repressors), resulting in transcriptional repression. In Chapter II, I presented evidence suggesting that ATF3 may repress transcription by stabilizing co-repressors at the promoter. However, the evidence is only indirect. To test this hypothesis, it is necessary to identify the co-repressors that can support the transcriptional repression by ATF3.
Thus far, several different co-repressors have been identified. Among them, yeast Ssn6 (or Cyc8)/Tup1 complex is the best characterized. The Ssn6/Tup1 complex is required to repress at least four unrelated classes of yeast genes which are regulated by glucose, oxygen, cell type, and DNA damage (Keleher et al., 1992; Tzamarias and Struhl, 1995 and references therein). Because Ssn6/Tup1 complex does not bind to DNA directly, it was thought to be recruited to the promoters by different pathway-specific transcription factors (Keleher et al., 1992). Further evidence suggested that Tup1 is required to mediate the general transcriptional repression while Ssn6 (occasionally Tup1) is required to interact with different pathway-specific transcription factors (Tzamarias and Struhl, 1995). The mechanism by which Ssn6/Tup1 complex is thought to involve positioning of a nucleosome around the transcription start site. The nucleosome then blocks the access of the basal transcriptional machinery to the promoter and consequently inhibits transcription (Cooper et al., 1994).

Sin3 (also referred to as UME4, SDI1, and RPD1) is another co-repressor type of transcriptional regulatory protein identified from yeast. Similar to Ssn6/Tup1 complex, Sin3 does not bind to DNA (Sternberg et al., 1987; Nasmyth et al., 1987) and is required to repress genes regulated by stimuli such as external signals, cell type, and cell differentiation process (Vidal et al., 1991). Recently, a mammalian Sin3 (mSin3) was identified by its ability to interact with the basic-helix-loop-helix (bHLH) family of transcription factors, Mad and Mxi1 (Ayer et al., 1995; Schreiber-Agus et al., 1995). Mad and Mxi1 are similar to the c-myc proto-oncogene for heterodimerizing with Max (another bHLH protein) and binding to the same E-box sequence, CACGTG. However, c-Myc/Max heterodimer activates transcription, whereas Mad/Max and Mxi1/Max heterodimers repress transcription. Using yeast two-hybrid screening approach, two independent laboratories reported that Mad (Ayer et al., 1995) and Mxi1 (Schreiber-Agus et al., 1995) interact with mSin3. Mutating the "mSin3-interacting" domain abolished the inhibitory activities of Mad and Mxi1 indicating that mSin3 is required to mediate the transcriptional repression by Mad and Mxi1.

Adenovirus E1b oncoprotein has also been shown to function as a transcriptional co-repressor. One important activity of E1b is to inactivate the tumor suppresser gene, p53. p53 is a sequence-specific DNA-binding transcriptional activator and is the most
commonly mutated gene in human tumors (for a review see Vogelstein and Kinzler, 1992). p53 has also been shown to be the target for cellular and viral oncoproteins such as MDM-2 (Momand et al., 1992; Oliner et al., 1993), SV40 large T antigen (Bargonetti et al., 1992; Farmer et al., 1992), and adenovirus E1b (Sarnow et al., 1982). Some of the oncoproteins inactivate p53 by inhibiting its DNA binding activity or by masking its transcriptional activation domain. However, E1b inactivates p53 by neither mechanism. Instead, E1b functions as a co-repressor to repress transcription from p53 target promoters (Yew and Berk, 1992; Yew et al., 1993). This conclusion is based on two observations: First, some E1b mutants that fail to repress transcription can still interact with p53 indicating that E1b does not mask the transactivation domain of p53 (Yew and Berk, 1992). Second, when fused to Gal 4 DNA binding domain, E1b represses transcription in a Gal 4 binding site dependent but p53 independent manner (Yew et al., 1993), indicating that E1b can actively repress transcription when bound to DNA. Taken together, these results suggest that E1b functions as a transcriptional co-repressor.

Another example of co-repressor proteins is N-CoR (Hörlein et al., 1995) and SMRT (Chen and Evans, 1995) in mediating the transcriptional repression by several steroid hormone receptors. In the case of thyroid hormone receptor (TR), prior to hormone binding, TR/RXR heterodimer binds to DNA and represses transcription from the target promoter. This transcriptional repression is dependent on the interaction between TR and either N-CoR or SMRT. Upon ligand binding, TR changes the conformation and no longer interacts with N-CoR or SMRT. Instead, the ligand bound TR interacts with co-activator proteins, such as p160 or CBP, and activates transcription from the target promoters (Kamei et al., 1996).

One common feature for these transcriptional co-repressors is that they lack the DNA binding ability. Therefore, to repress the target promoter, they tether to DNA through interacting with another DNA-bound transcription factor. One advantage is that, through interacting with different transcription factors, each co-repressor may regulate a variety of genes involved in different aspects. However, the mechanisms by which co-repressors inhibit transcription are largely unknown. As proposed in Chapter II, ATF3 represses transcription by interacting and stabilizing the putative co-repressors at the
promoter. It is possible that ATF3 may interact with some of the known co-repressors. At present, we have not tested this hypothesis. Instead, to further investigate ATF3 interacting co-repressors, our approach was to identify ATF3-interacting proteins. In this chapter, I present the results from screening a COS-1 cDNA expression library for the cDNA clones encoding ATF3-interacting proteins. I took a protein-protein blot screening approach by using radiolabeled ATF3 as a probe to screen the library. Using this approach, I identified five previously known proteins that can interact with ATF3. Although none of them appears to be the putative co-repressors, I discuss the specificity of the interaction and the potential consequence of their interaction with ATF3.
Material and methods

Plasmids

pET.His and pET.Hisk (Chen and Hai, 1994) were modified from the pET3 vector developed by Studier et al. (Studier et al., 1990). The details of the construction are described in the results section. pET.Hisk-ATF3 was constructed by cloning the full-length ATF3 XbaI/BamHI blunt-ended fragment (see Chapter II, material and methods section) in-frame into the pET.HisK vector at the BamHI blunt-ended site (by AMV reverse transcriptase). pET.HisK-ATF3 N' was constructed by cloning the ATF3 XbaI/PvuII blunt-ended fragment (by AMV reverse transcriptase) in-frame into the pET.HisK vector at the BamHI blunt-ended site (by AMV reverse transcriptase). pET.HisK-ATF3 C' was constructed by cloning the ATF3 AvaI/BamHI blunt-ended fragment (by Mung Bean nuclease) in-frame into the pET.HisK vector at the XhoI blunt-ended site (by AMV reverse transcriptase). pET.His-ATF1 was constructed as follows. The ATF1 ORF was amplified by PCR using Taq polymerase (Promega), upstream primer (5'-GGCATATGCATCACCATCACCATCACCTGCCACAAACTGTGGTGATG-3'), and downstream primer (5'-GGGAGATCTTCAAACACTTATTGGAATA -3'). After PCR amplification, the amplified DNA fragment contained an NdeI site and six histidine codons at the N' terminus and a BglII site at the C' terminus. The NdeI/BglII DNA fragment was inserted into the pET3 vector between the NdeI and BamHI sites.

Cell culture

The maintenance of the COS-1 cells was described in the material and methods section of Chapter II.

Total RNA and poly(A+)RNA purification

Total RNA was isolated by the guanidine isothiocyanate method (Strohman et al., 1977). Briefly, ten 150 mm plates of COS-1 cells were collected using a rubber policeman and homogenized with a Tissumizer (Tekmar) in 6 ml of guanidine isothiocyanate homogenization buffer (4 M guanidine isothiocyanate, 0.1 M Tris-HCl [pH 7.5], 1% β-mercaptoethanol). After homogenization, sodium lauryl sarcosinate (sarkosyl) was added to the lysate at a final concentration of 0.5% The homogenized lysate was spun at room temperature, 15,000x g for 10 minutes to remove any insoluble debris. The
supernatant was transferred to a fresh tube, mixed with solid CsCl (0.15 g per ml of the lysate), gently overlaid on top of 3 ml CsCl/EDTA solution (5.7 M CsCl, and 10 mM EDTA) in a 10 ml UltraTube (Nalgene 3410-1489), and spun in an ultracentrifuge using a SW41 swing-bucket rotor at 20°C, 30,000 rpm for 23 hours. The RNA pellet was collected, transferred to a clean eppendorf tube, washed once by 70% ethanol, dissolved in 400μl diethyl pyrocarbonate (DEP)-treated H2O, followed by phenol extraction and ethanol precipitation.

Poly(A+) RNA was purified from total RNA using a poly(dT) cellulose column (Pharmacia). Total RNA was adjusted to a concentration of 0.5 mg/ml in 1x poly(dT) cellulose column loading buffer (0.5 M NaCl, 20 mM Tris-HCl [pH 7.6], 1 mM EDTA, 1% sarkosyl) and loaded onto a 1 ml poly(dT) cellulose column at the flow rate of 1 drop (= 30 μl)/ 40 seconds. The column was then washed with 10 ml of 1x loading buffer at the flow rate of 1 drop/ 5 seconds. The poly(A+) RNA was eluted with 1x TE buffer (10 mM Tris-HCl [pH 7.6], 1 mM EDTA) at the flow rate of 1 drop/ 25 seconds. The eluted poly(A+) RNA was collected and re-purified by the poly(dT) cellulose column a second time using the same procedure.

cDNA synthesis

cDNA was synthesized by the Riboclone cDNA synthesis kit (Promega) according to the manufacturer's instructions. Briefly, 4 μg poly(A+) RNA was mixed with 2 μg oligo-dT primer and DEP-treated H2O in a final volume of 20 μl, heated at 70°C for 10 minutes, and chilled quickly on ice to anneal the oligo-dT primer to the poly-A tail of the poly(A+) RNA. First strand cDNA was synthesized by adding 10 μl of 5x first strand buffer (250 mM Tris-HCl [pH 8.3], 375 mM KCl, 15 mM MgCl_{2}, 50 mM DDT), 5 μl methylation dNTPs mix (10 mM each of dATP, dGTP, dTTP, and 5-methyl-dCTP), 2 μl RNasin, 200 units of MMLV reverse transcriptase, and DEP-treated H2O to a final volume of 50 μl. The reaction was incubated at 42°C for 2 hours. The second strand cDNA was synthesized by mixing 50 μl of the first strand reaction with 50 μl of 5x second strand buffer (200 mM Tris-HCl [pH 7.5], 425 mM KCl, 22 mM MgCl_{2}, 12 mM DDT), 2 μl methylation dNTPs mix, 50 units of DNA polymerase I, 1.6 units of RNase H, and DEP-treated H2O to a final volume of 250 μl. The mixture was incubated at 15°C for 90 minutes, and stopped by heating at 70°C for 10 minutes. The cDNA ends were flushed
by adding 8 units of T4 DNA polymerase to the reaction and incubated at 37°C for 10 minutes followed by phenol extraction and ethanol precipitation.

**Ligation of synthesized cDNA with the λEXlox(+) arms**

100 pmol of HindIII/EcoRI linkers (Novagen, 5'-GCTTGAATTCAAGC-3') were kinased using 5 units of T4 polynucleotide kinase (BRL) in 20 μl of ligation buffer (50 mM Tris-HCl [pH 8.0], 5 mM MgCl2, 10 mM DTT, 0.1 mM ATP, 50 μg/ml acetylated BSA) at 37°C for 1 hour. The kinased HindIII/EcoRI linkers were ligated to the cDNA by T4 DNA kinase at 16°C for 16 hours using the same buffer. After phenol extraction and ethanol precipitation, the cDNA was digested with HindIII for 4 hours and then by EcoRI overnight.

To remove any excess HindIII/EcoRI linkers and short cDNA fragments (<300 bp), the digested cDNA was passed through a Sepharose CL-4B spin column (Pharmacia) as follows. Sepharose CL-4B column was equilibrated with STE buffer (10 mM Tris-HCl [pH 7.6], 1 mM EDTA, 150 mM NaCl), and spun in clinical centrifuge at setting 2 (=320 x g) for 2 minutes. The cDNA was carefully applied to the center of the bed surface, and spun for 2 minutes to elute the cDNA. The eluted cDNA was then ligated with the λEXlox (+) arms at different insert:vector ratios (see details in the results section) using 0.5 to 1 units of T4 DNA ligase in 5 μl of ligation buffer (50 mM Tris-HCl [pH 8.0], 5 mM MgCl2, 10 mM DTT, 100 μM ATP, 50 μg/ml acetylated BSA). The ligation mixture was incubated at 16°C overnight.

**Packaging the λEXlox (+) DNA to make the primary library**

The ligation mix was packaged into the λ phage particle using Gigapack II packaging extract (Stratagene) according to the manufacturer's instructions.

**Titration of the primary λEXlox (+) library**

Small aliquots of the in vitro packaged λEXlox (+) library was diluted in a 10-fold series dilution scheme using SM buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 8 mM MgSO4, 0.01% gelatin). The serial dilutions of the λEXlox (+) library were introduced into the ER1647 E. coli strain as follows. Aliquots of ER1647 frozen stock were streaked onto a LB plate. This plate was then wrapped in aluminum foil and incubated at 37°C overnight (ER1647 has a high mutation rate caused by light. Wrapping the plate
with aluminum foil is to reduce the light-induced mutagenesis). The ER1647 plate was then stored at 4°C and used within two weeks. A single colony of ER1647 was picked from the plate, inoculated into 5 ml of LB media (1% NaCl, 1% tryptone, 0.5% yeast extract) containing 0.2% maltose and 10 mM MgSO4, and incubated at 37°C on a rotary drum for 6 hours. The cells were collected by centrifugation at 4°C, 2000x g for 10 minutes and resuspended in 10 ml of 10 mM MgSO4 solution (the OD600 reading should be around 0.5). Aliquots of the cells (200 to 400 µl) were mixed with the λEXlox (+) phage in a 15 ml centrifuge tube and incubated at 37°C for 20 minutes to allow the λ phage to infect the cells. The infected cells were then mixed with 3 ml of melted and cooled (at 48°C) top agarose (0.5% NaCl, 1% bacto-tryptone, 0.6% agarose), quickly poured on top of a pre-warmed 100 mm LB plate, and incubated at 37°C for 8 hours to overnight. The titer of the λEXlox (+) library was calculated by multiplying the number of plaques on the plate by the dilution factor.

**Amplification of the λEXlox (+) library**

Since the primary library was methylated, it needed to be amplified in the ER1647 E. coli strain (which has a deletion of six genes involved in methylation restriction) before it can be introduced into the BL21 (DE3) LysS E. coli strain used to express the T7 gene 10 fusion proteins. The primary library (1×10^6 pfu) was introduced into the ER1647 E. coli strain at the titer of 5 x 10^4 plaques per 150-mm plate (total 20 plates), and incubated at 37°C for 6 to 8 hours, before the plaques started to overlap. Each plate was overlaid with 10 ml SM buffer, and placed at 4°C with gentle rocking overnight. The phage suspension was collected, mixed, and spun at 4°C, 2000x g for 5 minutes to remove any insoluble cell debris. The supernatant (phage suspension) was transferred to a fresh tube. For short term storage, chloroform was added to the supernatant at a final concentration of 0.3%. For long term storage, DMSO was added to the supernatant at a final concentration of 7%. Small aliquots of the supernatant were frozen on dry ice and stored at -80°C.

**Purification, renaturation, and labeling of hisk-ATF3**

To make the hisk-ATF3 fusion protein, BL21 (DE3) LysS competent cells were freshly transformed with the pET.HisK-ATF3 construct (freshly transformed cells give better protein production), inoculated in 5 to 25 ml M9ZB media (0.5% NaCl, 1% tryptone, 1
mM MgSO4, 0.4% glucose, 0.6% Na2HPO4, 0.3% KH2PO4, 0.1% NH4Cl) containing 50 μg/ml of ampicillin (Amp) and 50 μg/ml of chloramphenicol (Chl), and incubated at 37°C on a rotary drum or shaker incubator overnight. The overnight culture was diluted 1:20 with M9ZB (with Amp and Chl) in 1.8 liter flasks (no more than 250 ml in each flask, for better aeration and better protein production), incubated at 37°C on a shaker incubator until the OD600 reading reached 0.6 to 0.7 (takes 1.5 to 2 hours), and induced with 0.1 to 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 hours. The cell pellet was collected by centrifugation at 4°C, 2000x g (JS-4.2, 3.5 K RPM) for 15 minutes. The cell pellet obtained from a 500 ml culture was resuspended in 15 to 20 ml Ni-NTA buffer A (6 M guanidine-HCl, 10 mM Tris-HCl, 100 mM NaH2PO4, pH 8.0), stirred at 4°C for 30 minutes to denature the total proteins, sonicated to shear the chromosomal DNA, and spun at 4°C, 18,000x g (JA-14, 11K RPM) for 20 minutes to remove the insoluble debris. The supernatant was passed through 5 ml Ni-NTA agarose beads (Qiagen), washed with 5 to 10 column volumes of Ni-NTA buffer B (8 M urea, 10 mM Tris-HCl, 100 mM NaH2PO4, pH 8.0) and 5 to 10 column volumes of Ni-NTA buffer C (same as buffer B, but the pH is adjusted to 6.3 by HCl). hisk-ATF3 was eluted by Ni-NTA buffer D (same as buffer B, but the pH is 5.0). Denatured-purified hisk-ATF3 was augmented with BSA to a final concentration of 1 mg/ml, transferred into a 12 to 14 kDa cut-off dialysis tubing, and dialyzed against a series of 200- to 300-volume of 0.1 M buffer D containing 1 M urea for 2 to 3 hours, 0.1 M urea for 2 to 3 hours, and 0 M urea for another 2 to 3 hours. The dialysis procedure was performed at 4°C with gentle stirring of the dialysis buffer with a magnetic bar (the dialysis tubing should just be floating and whirling on the surface of the buffer). After dialysis, the renatured hisk-ATF3 was transferred to an eppendorf tube, spun in the eppendorf centrifuge at 4°C, 14,000 rpm for 5 minutes to remove any precipitated proteins. The soluble protein was aliquotted, frozen using liquid nitrogen, and stored at -80°C. his-ATF1, hisk-ATF3 N', and hisk-ATF3 C' fusion proteins were produced and purified from E. coli by the same procedure using pET.His-ATF1, pET.HisK-ATF3 N', and pET.HisK-ATF3 C' constructs.

The renatured hisk fusion proteins were labeled in vitro by Heart Muscle Kinase (HMK, Sigma P-2645) with [γ-32P]ATP. To reconstitute the HMK, one vial (250 units) of HMK was resuspended in 50 μl of 40 mM DTT and incubated at room temperature for 10 minutes. The reconstituted HMK can only be stored on ice and used within 2 to 3
days. To save the material, aliquots (= 20 to 30 %) of the HMK from the vial can be reconstituted in 10 to 20 μl 40 mM DTT. The phosphorylation reaction was carried out in 50 μl of kinase buffer (20 mM Tris-HCl [pH 7.5], 100 mM NaCl, 12 mM MgCl2) containing 0.5 to 5 μg of renatured hisk fusion proteins, 3 μl [γ-32P]ATP (3000 Ci/mmol, 10 μCi/μl), 25 to 50 units of reconstituted HMK, and incubated at room temperature for 30 minutes. The labeled proteins were separated from un-incorporated [γ-32P]ATP by G-50 exclusion chromatography (see below).

**G-50 exclusion chromatography**

10 g of Sephadex G-50 superfine (Pharmacia) was swelled in 100 ml of 0.1 M buffer D (for protein work) in an autoclaved bottle. To set up a 1 ml column, a 1 ml syringe was plugged with siliconized glass wool and filled with the G-50 slurry to the top of the syringe without creating air bubbles. The syringe was placed in a 12 ml conical tube, spun in the clinical centrifuge at setting #4 (~ 800 g) for 2 minutes. The eluant was discarded. An eppendorf tube without a cap was then placed into the conical tube underneath the syringe. The kinase reaction was mixed with 50 μg acetylated BSA and 0.1 M buffer D to a final volume of 100 μl, loaded onto the column, and spun for 2 minutes. Another 100 μl 0.1 M buffer D was loaded onto the column and spun for 2 minutes to elute the remaining labeled protein from the column.

**Protein-protein blot screening**

The amplified λEXlox(+) library was introduced into the BL21 (DE3) LysS E. coli strain at the titer of 2.5 x 10^4 plaques per 150-mm plate and incubated at 37 °C for 6 to 8 hours. A piece of nitrocellulose membrane impregnated with 10 mM IPTG was overlaid on the plate and incubated for another 4 to 6 hours to induce the expression of the T7 gene 10 fusion proteins. The plates were then chilled at 4°C for 30 minutes to help pealing off the nitrocellulose membranes. The following treatments for the nitrocellulose membranes were all performed at 4°C. The nitrocellulose membranes were incubated twice with 6 M guanidine-HCl in renaturation buffer (25 mM HEPES [pH 7.5], 25 mM NaCl, 2.5 mM MgCl2, and 1 mM DTT) for 10 minutes to denature the proteins, followed by incubation in a series of renaturation buffers with decreasing concentrations of guanidine-HCl (3 M, 1.5 M, 0.75 M, 0.375 M, 0.19 M, 0.1 M and 0 M) for 10 minutes intervals to renature the proteins. The membranes were then incubated with pre-binding buffer (renaturation buffer with 5% non-fat milk, 0.1 % NP-40, 1 mM EDTA, and protease inhibitors) for
one hour, then with the binding buffer (same as pre-binding buffer except 75 mM NaCl and 1% non-fat milk) for 30 minutes to block the membranes. The membranes were then incubated with the binding buffer containing $^{32}$P-labeled hisk-ATF3 ($>2.5 \times 10^5$ cpm/ml) at 4°C for 4 hours followed by washing with PBS containing 0.1% NP-40 and 0.1% TX-100 at room temperature for 10 to 20 minutes with three changes of the solution. The washing steps should not extend for too long to avoid losing the signal. The membranes were then air dried and exposed to X-ray film for 8 to 48 hours without the intensive screen to identify the potential positive plaques. After aligning the plate with X-ray film, the area containing the positive plaque was transferred to an eppendorf tube by a pasteur pipet, incubated with 0.5 ml SM buffer at 4°C overnight to elute the λ phage particles from the agar. The eluted λ phages were re-introduced into the BL21 (DE3) LysS E. coli strain for the next round of screening until the pure plaque was obtained. The confirmed positive λ phages were introduced into the BM 25.8 E. coli strain to excise the pEXlox(+ ) plasmids. The pEXlox(+) plasmids were purified and re-transformed into the DH5α E. coli strain to amplify the plasmids for sequencing the inserted cDNA or re-transformed into the BL21 (DE3) LysS E. coli strain to express the T7 gene 10 fusion proteins.

**Sequencing**

Regular dideoxy sequencing was carried out by *Taq* DNA polymerase (Promega) and deaza-GTP according to the manufacturer's instructions.
Results

To construct a cDNA expression library, we chose the \( \lambda\text{EX} \text{lox} (+) \) vector (Novagen) three reasons. First, the synthesized cDNA is directionally inserted into \( \lambda\text{EX} \text{lox} (+) \) vector. This can eliminate cloning of the cDNA in the wrong orientation and double the chance of the cDNA to be expressed. Second, the inserted cDNA can be automatically excised from the \( \lambda\text{EX} \text{lox} (+) \) vector by the P1 \text{cre} recombinase (Figure 3.1). The excised DNA can re-circularize as pEX\text{lox} (+) plasmid. Third, \( \lambda\text{EX} \text{lox} (+) \) vector contains the T7 promoter and the full length T7 gene 10 coding sequence. When inserted into the \( \lambda\text{EX} \text{lox} (+) \) vector, the cDNA is fused downstream from the T7 gene 10. This T7 expression system allows high levels of protein production to facilitate the library screening.

Construction of the COS-1 cDNA expression library

Because all the previous transfection experiments were performed in COS-1 cells, COS-1 cell poly(A+)RNA was used to synthesize the cDNA for constructing the cDNA expression library. Approximately, 2.3 mg of total RNA was isolated from 2.2 x 10^8 COS-1 cells (ten 150-mm plates) by the guanidine-thiocyanate method. Poly(A+)RNA was purified by oligo(dT) cellulose column (Pharmacia) twice. Four \( \mu \)g of poly(A+)RNA was used to synthesized the cDNA by Riboclone cDNA synthesis kit (Promega). In both first and second strand cDNA synthesis, 5-methyl-dCTP together with regular dATP, dGTP, and dTTP was used to synthesize the methylated cDNA. The methylation of the cDNA protects any internal HindIII and EcoRI sites from digestion by restriction enzymes in the reaction. In addition, trace amount of [\( \alpha^{32} \text{P}- \)]dCTP was added in the cDNA synthesis reaction to estimate the yields and sizes of the cDNA. Approximately, 2.2 \( \mu \)g double stranded cDNA was synthesized from 4 \( \mu \)g poly(A+) RNA. The synthesized cDNA was examined on an alkaline agarose gel. The sizes of the cDNA ranged from 500 bp to 5 kb (Figure 3.2).

The synthesized cDNA was blunt-ended by T4 DNA polymerase in the presence of deoxynucleotides. The blunt-ended cDNA was then ligated with HindIII/EcoRI linkers (Novagen) by T4 DNA ligase, and digested by HindIII and EcoRI restriction enzymes.
Figure 3.1 IEXlox (+) vector system.
The cDNA is inserted directionally (EcoRI and HindIII) into IEXlox(+) vector downstream from the full length T7 gene 10 coding sequence which is regulated by T7 promoter. loxP is a 34 bp recognition sequence for P1 cre recombinase which allows the excision of pEXlox(+) plasmid from the IEXlox(+) vector. This figure is modified from the Novagen 1993 catalog.
Figure 3.2 Synthesis of the first and second strands of cDNA.
In separate experiments, trace amount of [α-32P]dCTP was added in either the first or the second strand cDNA synthesis reaction. An aliquot of each reactions was analyzed on a 1% alkaline agarose gel. Molecular size markers (SM) in kilo base-pair or base-pair are indicated on the left.
enzymes. The excess HindIII/EcoRI linkers and small cDNA fragments (< 300 bp) were separated from the larger cDNA by CL-4B spun column (Pharmacia, ~60% recovery). The vector-ready cDNA was then ligated with λEXlox (+) arms. To optimize the ligation efficiency, I tested different insert:vector ratios in the ligation reaction. In two separate experiments, 0.5 μg (0.017 pmole) of λEXlox (+) arms were ligated with 0.05 μg or 0.15 μg vector-ready cDNA. Presumably, the average size of cDNA is 1.5 kb. Therefore, 0.05 μg and 0.15 μg cDNA corresponds to 0.05 pmole and 0.15 pmole of double stranded cDNA molecules. Ligation of 0.05 μg cDNA with 0.5 μg vector corresponds to an insert:vector ratio of 3, and ligation of 0.15 μg cDNA with 0.5 μg vector corresponds to an insert:vector ratio of 9. The ligated cDNA and λEXlox (+) arms were packaged into λ phage particle using Gigapack II packaging extract (Stratagene) to generate the primary libraries. The primary libraries were then introduced into the ER1647 E. coli strain to examine their titers. Although the libraries were constructed with different insert to vector ratios, they had a similar titer (= 0.5 x 10^6 pfu). To enhance the titer of the library, I did the following treatments: The remaining cDNA was re-digested by EcoRI and HindIII, passed through CL-4B spun column, ligated with λEXlox (+) arms, and packaged into λ phage particle. The estimated insert to vector ratio was greater than 10. The approximate titer for the third library was about 1x10^6 pfu. Since the primary libraries contained the methylated cDNA, they could not be introduced directly into the BL21 (DE3) LysS E. coli strain to express the cDNA. Therefore, the primary libraries were introduced and amplified in the ER1647 E. coli strain (with deletion of six genes involved in methylation restriction). The amplified libraries were then introduced into the BL21 (DE3) LysS E. coli strain to express the T7 gene 10 fusion proteins.

**Purification and radiolabeling of HisK-ATF3**

To radiolabel ATF3 for the library screening, I expressed and purified, from E. coli, an ATF3 fusion protein (hisk-ATF3) containing histidine tag and two Heart Muscle Kinase (HMK) phosphorylation sites. Both the histidine tag and the HMK phosphorylation sites were provided by the pET.HisK expression vector (see below). The histidine tag makes it possible to affinity purify hisk-ATF3 in one step by the Ni-
NTA agarose beads (Qiagen), and the HMK phosphorylation sites makes it possible to radiolabel hisk-ATF3 \textit{in vitro} by HMK in the presence of \([\gamma-^{32}\text{P}]\text{ATP.}\)

pET.HisK vector was modified from pET3 \textit{E. coli} expression vector (Studier et al., 1990) through two steps. A double stranded oligonucleotide containing Ndel/BamHI sticky ends, six histidine codons, and two additional cloning sites (XhoI, and NcoI) was used to replace the Ndel/BamHI fragment of pET3 vector (Figure 3.3.A). The modified vector, pET.His, had the initial methionine codon (within the Ndel site) followed by six histidine codons and three cloning sites (XhoI, NcoI, and BamHI). These three cloning sites allow cloning of the blunt-ended DNA fragment in three different reading frames. For an example, if the pET.His vector is linearized with BamHI and blunt-ended by AMV reverse transcriptase, the last nucleotide (C residue) of the resulting DNA can be fused with the first two nucleotides of the insert DNA to form an in-frame codon (CNN). I refer to this vector as a +1 vector. Depending on the sequence of the insert DNA, the fusion amino acid can be leucine, proline, histidine, arginine, or asparagine. If linearized with XhoI and blunt-ended by AMV reverse transcriptase, it becomes a +2 vector. The fusion amino acid (GAN) can be aspartic acid, or glutamic acid. If linearized with NcoI and blunt-ended by AMV reverse transcriptase, it becomes a +0 vector. The pET.His vector was further modified into pET.HisK vector by inserting at the XhoI site with another double stranded oligonucleotide containing XhoI sticky ends and two HMK phosphorylation sites (Figure 3.3.B). After modification, the downstream XhoI site was intact but the upstream XhoI site was destroyed. Therefore, pET.HisK vector maintains the same multiple cloning sites as that of pET.His vector and allows the cloning of blunt-ended DNA fragment in three different reading frame.

To express hisk-ATF3 fusion protein, full length ATF3 open reading frame was subcloned into pET.HisK vector. The resulting construct, pET.HisK-ATF3, was then transformed into the BL21 (DE3) LysS \textit{E. coli} strain to express the hisk-ATF3 fusion protein. hisk-ATF3 fusion protein was purified by the Ni-NTA agarose beads under the denaturing condition. After renaturation, hisk-ATF3 was radiolabeled \textit{in vitro} by HMK and used as a probe to screen the COS-1 cDNA expression library for cDNA encoding ATF3-interacting proteins.
Figure 3.3 pET.His and pET.HisK vector.

(A) Double stranded oligonucleotides for converting pET3 vector to pET.His vector. Amino acid sequence between NdeI and BamHI are shown. The nucleotide sequences before the cloning junction in +0, +1, and +2 vectors are specified. (B) Double stranded oligonucleotides for converting pET.His vector to pET.HisK vector. The amino acid sequences for HMK phosphorylation site are indicated. (C) The map of pET.HisK (vector: only some of the unique sites are indicated).
Isolation of ATF3-interacting proteins

In four screening experiments, ten positive clones were isolated from a total 1.5 x 10^6 plaques. As an example, Figure 3.4 showed one typical positive clone and one negative clone in the second round of screening. The positive clones were then introduced into the BM25.8 E. coli strain, which expresses P1 cre recombinase, to excise the pEXloxo (+) plasmids. The excised pEXloxo (+) plasmids were then transformed into the DH5α E. coli strain to amplify the plasmids for sequencing or transformation into the BL21(DE3) LysS E. coli strain to express the T7 gene 10 fusion proteins.

The sequencing result showed that four positive clones (317-5, 317-6, 319, and 324-3) encode an identical cDNA derived from a basic-helix-loop-helix (bHLH) family of transcription factors: USF2/FIP. Because these four clones have exactly the same sequence at both N' terminal and C' terminal cloning junctions, they are most likely to be derived from the same original primary plaque. It is possible to screen out the same clone more than once from the amplified library. However, by statistics, the chance to screen out the same clone for four times is low. I speculated that they resulted from cross contamination during the 2nd or 3rd round of screenings through pipetman. To avoid missing any potential positive clones, all the original λ phages (obtained from the first round of screening) of these identical clones were re-examined. However, no additional positive clone was found in this attempt. In addition to these four clones, another two positive clones (317-8 and 317-18) also contain identical inserted cDNA. By the same logic, it is likely that they are also resulted from cross contamination. These two clones also encode USF2/FIP, but are shorter than that of the above clones. The remaining four positive clones encode cDNA derived from different genes. Two clones (324-4 and 314-2) contain cDNA derived from the intermediate filament genes (keratin 7 and keratin 8), and another two clones (324-1 and 324-2) contain cDNA derived from the C/EBP family of genes (C/EBPγ and gadd153/Chop10). The characteristics of these clones are summarized in Table 1.

All positive clones were transformed into the BL21 (DE3) LysS E. coli strain to express the T7 gene 10 fusion proteins. Their abilities to interact with ATF3 were confirmed by protein-protein blot experiment. As shown in Figure 3.5A, T7 gene 10
Figure 3.4 An example of library screening
The figure shows a typical result from a second round screening. The positive clone indicated is keratin 8.
<table>
<thead>
<tr>
<th>Name</th>
<th>cDNA I.D. #</th>
<th>cDNA insert size</th>
<th>size of T7 gene 10 fusion protein</th>
<th>Cloning junction &amp; Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>C/EBPγ</td>
<td>#548</td>
<td>≈ 2kb</td>
<td>≈ 43 kDa</td>
<td>5′-G AAT TCA AGC AAG GAA TTA AGT GTT ATC-3′. This clone encodes a truncated C/EBP γ, missing 13 a.a. from the N′ terminal. It may also contain more than one cDNA insert.</td>
</tr>
<tr>
<td>gadd153 Chop10</td>
<td>#553</td>
<td>≈ 500 bp</td>
<td>≈ 39 kDa</td>
<td>5′-G AAT TCA AGC CAG AGC CCT CAC-3′, This clone encodes a truncated gadd153/Chop10, missing 76 a.a. from the N′ terminal.</td>
</tr>
<tr>
<td>keratin 7</td>
<td>#555</td>
<td>≈ 300 bp</td>
<td>≈ 43 kDa</td>
<td>5′-G AAT TCA AGC AAG TTG GAG GCC-3′, This clone encodes a truncated keratin7, missing 330 a.a. from the N′ terminal.</td>
</tr>
<tr>
<td>keratin 8</td>
<td>#544</td>
<td>≈ 650 bp</td>
<td>≈ 42 kDa</td>
<td>5′-G AAT TCA AGC CTG CAG CGG GCC-3′, This clone encodes a truncated keratin 8, missing 155 a.a. from the N′ terminal.</td>
</tr>
<tr>
<td>USF2/FIP (large)</td>
<td>#542</td>
<td>≈ 1.5 kb</td>
<td>≈ 65 kDa</td>
<td>5′-G AAT TCA AGC CCG GGT CTG CTG GAT-3′, This clone encodes a truncated USF2/FIP, missing 5 a.a. from the N′ terminal.</td>
</tr>
<tr>
<td>USF2/FIP (small)</td>
<td>#543</td>
<td>≈ 700 bp</td>
<td>≈ 33 kDa</td>
<td>5′-G AAT TCA AGC CTG CAG ATG GAC-3′, This clone encodes a truncated USF2/FIP, containing only the C′ terminal 40 a.a. which starts from the first leucine of the leucine zipper.</td>
</tr>
</tbody>
</table>

Table 1. The characteristics of positive cDNA clones encoding ATF3 interacting proteins. The underline indicates the EcoRI cloning junction. The arrow (↑) indicated the start of the cDNA insert.
fusion proteins encoded by these positive clones were able to interact with $^{32}$P-labeled hisk-ATF3. The specificity of the interactions were examined by a similar protein-protein blot analysis using $^{32}$P-labeled his-ATFl as a probe. As shown in Figure 3.5B, Keratin 7, Keratin 8, small USF2/FIP, and gadd153/Chop10 did not interact with ATF1. Only C/EBPγ and large USF2/FIP were able to interact with ATF1. The binding of C/EBPγ to ATF1 was stronger than that to ATF3, whereas, the binding of large USF2/FIP to ATF1 was weaker than that to ATF3. Because all positive clones contain the leucine zipper or leucine zipper like domains, it is likely that they interact with ATF3 through the leucine zipper dimerization domain. To test this hypothesis, I repeated the protein-protein blot analysis using the bZip domain (ATF3 72-181, with the leucine zipper domain) or the N' terminal domain (ATF3 1-100, without the leucine zipper domain) of ATF3 as probes. As shown in Figure 3.6, all positive clones interacted with ATF3 bZip domain but not with N' terminal domain-indicating that they interact with ATF3 through the leucine zipper domain.
Figure 3.5 Keratin 7, Keratin 8, small USF2/FIP, and gadd153/Chop10 bind to ATF3 but not to ATF1.
keratin 7, keratin 8, USF2/FIP large (USF2-L), USF2/FIP small (USF2-S), C/EBPγ, gadd153/Chop10 (gadd153) were expressed in E. coli and analyzed by protein-protein blot using radiolabeled His-ATF3 (A) or His-ATF1 (B) as probes. All the clones produce the similar amount of T7 gene 10 fusion proteins except USF2-L (data not shown). Size markers in kilodaltons are indicated on the left.
Figure 3.6 All ATF3-interacting proteins interact ATF3 through the leucine zipper domain.

keratin 7, keratin 8, USF2/FIP large (USF2-L), USF2/FIP small (USF2-S), C/EBPγ, gadd153/Chop10 (gadd153) were expressed in E. coli and analyzed by protein-protein blot using radiolabeled Hisk-ATF3 C' (A) or Hisk-ATF3 N' (B) as probes. Size markers in kilodaltons are indicated on the left.
Discussion

To further investigate the "co-repressor" model, I screened the cDNA expression library for clones encoding ATF3-interacting proteins. Using protein-protein blot approach, I screened 1.5 x 10^6 plaques from an amplified COS-1 cDNA expression library and identified five previously known proteins that can interact with ATF3: USF2/FIP (two clones encoding different length of cDNA), Keratin 7, Keratin 8, C/EBP γ, and gadd153/Chop10. As demonstrated by protein-protein blot analysis, they all interacted with ATF3 through the leucine zipper domain. In addition, Keratin 7, Keratin 8, gadd153/Chop10, and the small USF2/FIP clone interacted with ATF3 but not with ATF1. However, the large USF2/FIP clone interacted with ATF1 with a low affinity. It is not clear whether there is additional domain in the large USF2/FIP to assist its interaction with ATF1.

As discussed in Chapter II, ATF3 interacts with the putative co-repressors through two distinct domains, ATF3 (1-100) and ATF3 (136-181). However, all five proteins described here interacted with ATF3 through the leucine zipper domain. Therefore, it is likely that they are not the putative co-repressors. Below, I offer several explanations for the library screening result: First, I might not have screened enough cDNA clones. One indication is that I did not isolated any previously known ATF3 dimerizing partners such as ATF2 (Hai and Curran, 1991). Although I screened total 1.5 x 10^6 plaques, theoretically, only one third of them have cDNA inserts being fused in the correct reading frame. In addition, the λEXlox (+) library was amplified once in the ER1647 E. coli. It is not clear whether each of the original plaques has similar representative number in the amplified library. Therefore, further screening of the COS-1 library or other libraries may yield additional ATF3-interacting proteins.

Second, I used ATF3 that is expressed and purified from E. coli as a probe to screen the library. E. coli expressed ATF3 lacks proper post-translational modification that may be necessary for the interaction between ATF3 and the co-repressors. One such indication came from the study of in vitro transcription assay. When tested in an in vitro transcription assay, ATF3 expressed and purified from vaccinia virus expression system was able to repress the transcription from ATFx3-CAT and gadd153/Chop10-CAT reporters, whereas ATF3 expressed and purified from E. coli could only repress ATFx3-
CAT reporter but not gadd153/Chop10-CAT reporter (see Chapter II for details), indicating that there are some differences between the proteins isolated from *E. coli* and vaccinia virus expression systems. The most likely reason for the different activity is the post-translational modification. However, it is not clear how post-translational modification may affect the activity of ATF3.

Third, it is possible that the putative co-repressors are composed of multiple subunits. Without proper assembling, each individual subunit may interact with ATF3 only weakly. However, despite this concern, cDNA clones expressing weak interacting proteins have been isolated. As an example, although E2F-1 needs to dimerize with DP-1 in order to interact with retinoblastoma gene product (Rb), E2F-1 cDNA was isolated by the protein-protein screening approach using radio-labeled Rb as a probe (La Thangue, 1994). In addition, we expect that the high levels of protein production by the *λEXIlox(+) system* should be able to drive the reaction.

Fourth, it is possible that the interaction between ATF3 and the co-repressors is not strong enough to be detected by this protein-protein screening approach. In comparison with Southern or northern blots, the background of the protein-protein blot analysis is relatively high. If the interaction between ATF3 and the co-repressors is not strong enough, it may not stand out from the background and can not be detected. If this is the case, yeast two hybrid screening approach may help to identify the ATF3 interacting co-repressors. Using yeast two hybrid approach, several co-repressors have been identified, such as N-CoR (Hörlein et al., 1995), SMRT (Chen and Evans, 1995), and mSin3 (Ayer et al., 1995; Schreiber-Agus et al., 1995).

Possible significance of the interactions between ATF3 and ATF3-interacting proteins

Although none of the five proteins appears to be the putative co-repressor, it is possible that their interactions with ATF3 are physiologically relevant. Below, I discuss the potential significance of their interactions with ATF3.
USF2/FIP

USF2/FIP is a member of bHLH family of transcription factors. In addition to the HLH dimerization domain, USF2/FIP contains the leucine zipper domain for stabilizing the dimer interaction. It was named for its similarity to another family member, USF1, and for its ability to interact with c-Fos (FIP stand for c-Fos interacting protein) (Sirito et al., 1992; Blanar and Rutter, 1992). The interaction between USF2/FIP and c-Fos requires the dimerization through the leucine zipper domains. Similarly, USF2/FIP also interacts with ATF3 through the leucine zipper domain. This is further supported by the fact that the small USF2/FIP clone encodes only the leucine zipper domain.

When co-transfected with c-Fos, USF2/FIP was reported to activate an AP1 site driven CAT reporter in a c-Fos and AP-1 site dependent manner (Blanar and Rutter, 1992). However, no enhancement of c-Fos binding nor the c-Fos-USF2/FIP complex were detected in a band shift analysis using $^{32}$P-labeled AP-1 binding site as a probe. Only one follow-up report was found in the MedLine database indicating that c-Fos is present in a complex bound to the USF binding site (Lewin et al., 1993). In light of the similarity between ATF3 and c-Fos (see review by Meyer and Habener, 1993), it was not too surprising that ATF3 interacted with USF2/FIP. At present, it is not clear whether the interaction between ATF3 and USF2/FIP is physiologically relevant.

Keratin 7 & Keratin 8

Keratin 7 (Glass et al., 1985) and keratin 8 (Leube et al., 1986) belong to the family of intermediate filament proteins which are required for the construction of the cytoskeleton. The intermediate filament proteins have a large central $\alpha$-helical "rod" domain (>300 amino acids.) which contains heptad repeats and apolar residues at the "a" and "d" positions (see review by Steinert and Roop, 1988) for forming two-chain coiled-coil dimer molecule. The coiled-coil dimer molecule is further assembled into the higher ordered intermediate filament structure. The intermediate filament then forms the complex interconnecting networks (cytoskeleton) from nuclear surface to the cytoplasmic membrane.
At present, it is not clear whether the interaction between ATF3 and keratin 7 or keratin 8 has any physiological relevance. Although it has never been reported that keratins interact with any transcription factors thus far, it remains possible that the interaction between ATF3 and keratins is physiologically relevant. For an example, the localization of ATF3 may be regulated by keratins. Since keratins are abundant cytoplasmic proteins, they could serve as a sink and retain ATF3 in the cytoplasm. For ATF3 to translocate into the nucleus and execute its transcriptional function, it may require specific post-translational modifications to disrupt the interaction between ATF3 and keratins. To test this hypothesis, one can over-express ATF3 alone or together with either of the keratins in mammalian cultured cells and examine whether the nuclear localization of ATF3 affects by the presence of keratins using immunofluorescence analysis.

C/EBPγ

C/EBPγ is a member of the C/EBP family of transcription factors and is also referred to as Ig/EBP because of its ability to bind to immunoglobulin enhancer region (Roman et al., 1990; Thomassin et al., 1992). Similar to ATF/CREB proteins, members of the C/EBP family of transcription factors contain the bZip DNA binding domain. They also form selective heterodimers with family members and other bZip proteins. However, the interaction between ATF3 and C/EBPγ was not further pursued for two reasons. First, C/EBPγ not only interacted with ATF3, but also interacted with ATF1 (see Figure 3.5B) and ATF4 (Vinson et al., 1993). Second, the affinity of the interaction between C/EBPγ and ATF3 was weaker than that between C/EBPγ and ATF1 (Figure 3.5) or that between C/EBPγ and ATF4 (Vinson et al., 1993).

gadd153/Chop10

gadd153/Chop10 is also a member of the C/EBP family of transcription factors. It was first identified by the fact that its mRNA level is induced by growth arrest and DNA damaging (gadd) agents (Fornace et al., 1989). It was also refereed to as C/EBP homolog protein (Chop) because it is homologous to other C/EBP family proteins in the
bZip domain (Ron and Habener, 1992). Although gadd153/Chop10 is probably not the putative co-repressor, the interaction between ATF3 and gadd153/Chop10 appeared to be interesting for the following reasons. First, the interaction between gadd153/Chop10 and ATF3 is not non-specific because both gadd153/Chop10 and ATF3 do not interact with ATF1 (see Figure 4.1). Second, both gadd153/Chop10 and ATF3 are inducible genes. The expression of gadd153/Chop10 was reported to be induced by a variety of growth arrest and DNA damaging conditions. Curt Wolfgang in our laboratory demonstrated that the expression of ATF3 can be induced by a variety of stress conditions (Chen et al., 1996). It is possible that, under certain conditions, ATF3 and gadd153/Chop10 may be co-induced. They may then dimerize and regulate the expression of certain target promoters. Third, the C/EBP family of transcription factors has been implicated to play important roles in regulating the expression of liver specific genes (for reviews see Lai and Darnell, 1991; McKnight, 1992). Results from our laboratory (Chen et al., 1996) and R. Taub's laboratory (Hsu et al., 1991) also suggested that ATF3 may play a role in regulating the expression of liver specific genes. Since the interaction between gadd153/Chop10 and ATF3 may be meaningful, I further investigated this interaction. The results of this line of research will be described in Chapter IV.
Chapter IV

gadd153/Chop10, an ATF3 dimerizing partner, negatively modulates the function of ATF3

Summary

In this chapter, I present evidence that gadd153/Chop10, a member of C/EBP family of transcription factors, dimerizes with ATF3 through the leucine zipper dimerization domain. The consequence of this interaction is that gadd153/Chop10 negatively modulates the function of ATF3: gadd153/Chop10 inhibits ATF3 from binding to the ATF or ATF-related sites, and thereby inhibits ATF3 from repressing transcription. Using northern blot and RT-PCR analyses, I demonstrated that the expression of ATF3 and the expression of gadd153/Chop10 are inverse but overlapping in the CC14 treated rat liver: The level of gadd153/Chop10 mRNA is high in the normal liver and decreases upon CC14 treatment, while the level of ATF3 mRNA is low in the normal liver and increases upon CC14 treatment. We hypothesize that, in normal liver, the high level of gadd153/Chop10 is able to functionally inhibit the trace amount of ATF3. In response to CC14 treatment, the level of gadd153/Chop10 decreases to allow the increased level of ATF3 to carry out its function.

Introduction

gadd153/Chop10, a growth arrest and DNA damage-inducible gene, was originally isolated from a hamster cDNA library by subtraction hybridization based on its rapid induction by UV irradiation (Fornace et al., 1989). Subsequently, gadd153/Chop10 was shown to be induced by a variety of cellular stresses, such as
hypoxia (Price and Calderwood, 1992), calcium influx (Bartlett et al., 1992), nutrient
deprivation (Carlson et al., 1993; Marten et al., 1994), inflammation response (Sylvester
et al., 1994). etc. Thus far, several signaling pathways have been implicated in the
induction of gadd153/Chop10. Ca\(^{2+}\) may play a role in regulating the expression of
gadd153/Chop10 because buffering intracellular and extracellular Ca\(^{2+}\) by combined
treatment with BAPTA-AM and EDTA prevented the induction of gadd153/Chop10 by
calcium ionophore (A23187) and DNA damaging agent (methyl methanesulfonate)
(Bartlett et al., 1992). In addition, studies from kinase and phosphatase inhibitors
indicated that the induction of gadd153/Chop10 is dependent on an un-identified
serine/threonine kinase but independent on protein kinase C and tyrosine kinases (Bartlett
et al., 1992, Leuthy and Holbrook, 1994).

gadd153/Chop10 encodes a C/EBP-homologous protein and was thought to
predominantly heterodimerize with members of the C/EBP family (Ron and Habener,
1992). However, in comparison with other C/EBP proteins, gadd153/Chop10 has a
unusual basic region: Several conserved amino acid residues in this region are
substituted by helix-breaking glycine and proline residues. Therefore, gadd153/Chop10
was predicted to have a non-functional DNA binding domain. When dimerizing with
other C/EBP proteins, such as C/EBP\(\alpha\) and C/EBP\(\beta\), gadd153/Chop10 was shown to
act as a dominant negative inhibitor and prevent them from binding to the C/EBP sites
(Ron and Habener, 1992). However, three lines of evidence described below suggest
that gadd153/Chop10 does not merely function as an inhibitor of the C/EBP proteins;
instead, it may have certain functions.

The first evidence came from the study of gadd153/Chop10 in the regulation of
cell cycle progression. Previously, C/EBP\(\alpha\) and C/EBP\(\beta\) have been shown to inhibit cell
proliferation and initiate differentiation in adipocytes and hepatoma cells (Umek et al.,
1991, Buck et al., 1994). Microinjection of C/EBP\(\alpha\) or C/EBP\(\beta\) into NIH 3T3-L1
adipoblast blocked the cell cycle progression and caused growth arrest (Barone et al.,
1994). However, co-injection of gadd153/Chop10 did not affect C/EBP\(\alpha\) or C/EBP\(\beta\)
induced growth arrest in NIH 3T3-L1 cells. In fact, microinjection of gadd153/Chop10
alone also blocked the cell cycle progression at the G1/S checkpoint, resulting in growth
arrest. This ability of gadd153/Chop10 to block cell cycle progression requires an intact
bZip domain (Barone et al., 1994). When injected into NIH 3T3 L1 cells, the
gadd153/Chop10 mutants lacking the leucine zipper dimerization domain or the basic region failed to induce growth arrest. These results argue against the notion that gadd153/Chop10 has a non-functional DNA binding domain. If gadd153/Chop10 only functions as an inhibitor to regulate the DNA binding ability of other C/EBP proteins, deletion of the basic region should not affect the activity of gadd153/Chop10.

The second evidence indicating that gadd153/Chop10 has a functional DNA binding domain came from the identification of the tumor-specific form of gadd153/Chop10 (Crozat et al., 1993, Barone et al., 1994). gadd153/Chop10 was shown to be disrupted in human myxoid sarcomas by a particular chromosomal translocation. Interestingly, the chromosomal rearrangement created a novel breakpoint fusion protein, TLS-Chop. This fusion protein consists of the N terminus of TLS, which has a strong transcriptional activation domain, and the full length coding region of gadd153/Chop10. In contrast to the wild type gadd153/Chop10, microinjection of TLS-Chop failed to cause growth arrest. In fact, TLS-Chop stably introduced into NIH 3T3 cells transformed the cells. Similar to gadd153/Chop10 induced growth arrest, TLS-Chop induced transformation also required the intact bZip domain. Microinjection of TLS-Chop mutants lacking the leucine zipper dimerization domain or the basic region failed to transform the NIH 3T3 cells (Zinszner et al., 1994). This observation is consistent with the notion that basic region is not dispensable for the function of gadd153/Chop10. It is more likely that the basic region contributes to the DNA binding ability of gadd153/Chop10.

Third, using random site selection analysis, D. Ron and colleagues demonstrated that gadd153/Chop10 is able to bind to DNA (Ubeda et al., 1996). The selected binding sites for C/EBPα-gadd153/Chop10 heterodimer contain a core sequence of PuPuPuTGCAAT(A/C)CCC. In addition, by fusing gadd153/Chop10 with Gal 4 DNA binding domain, the authors demonstrated that gadd153/Chop10 contains a stress-inducible transcriptional activation domain. However, it is not clear whether C/EBPα-gadd153/Chop10 heterodimer can bind to the selected sites and mediate stress-induced transactivation in vivo. Furthermore, it remains to be determined whether the selected sites can be found on any naturally occurring promoters and whether the sites can be regulated in a stress-dependent manner. Taken together, these observations suggest that
gadd153/Chop10 does not merely function as an inhibitor of the C/EBP proteins; it may also function as a direct regulator of transcription.

In addition to dimerizing with the C/EBP family proteins, gadd153/Chop10 also dimerizes with the ATF/CREB family members. As described in Chapter III, in the process of screening the COS-1 cell cDNA expression library for the cDNA clones encoding the putative co-repressors, I identified the interaction between ATF3 and gadd153/Chop10. Although gadd153/Chop10 is probably not the putative co-repressor, its interaction with ATF3 was further investigated for the following reasons. First, the interaction between gadd153/Chop10 and ATF3 is not non-specific: gadd153/Chop10 interacted with ATF3 but not with ATF1. Second, both gadd153/Chop10 and ATF3 are stress-inducible genes. As described earlier, gadd153/Chop10 is induced by a variety of cellular stresses. Using animal experiments and in situ hybridization, C. Wolfgang in our laboratory demonstrated that ATF3 is induced in a variety of stressed-tissues: mechanically injured liver, toxin-injured liver, blood-deprived heart, and post-seizure brain (Chen et al., 1996). It is possible that, under certain stress conditions, ATF3 and gadd153/Chop10 may be co-induced and heterodimerize to regulate specific target promoters. Third, the C/EBP family of transcription factors have been implicated to play important roles in the regulation of liver specific gene expression (for review see Lai and Darnell, 1991; McKnight, 1992). It is possible that gadd153/Chop10 is also involved in regulating gene expression in the liver. In addition, gadd153/Chop10 is induced in liver by amino acid starvation (Marten et al., 1994) and inflammation (Sylvester et al., 1994). Evidence from ours laboratory and Dr. R. Taub's laboratory also indicated that ATF3 may play a role in regulating gene expression in the liver (Hsu et al., 1991; Chen et al., 1996). Therefore, the interaction between ATF3 and gadd153/Chop10 may be physiologically relevant.

In this chapter, I present evidence that gadd153/Chop10 negatively regulates the function of ATF3. By dimerizing with ATF3, gadd153/Chop10 inhibits ATF3 from binding to the ATF or ATF related sites and from repressing transcription. Using CCl4 treated liver as a model, I demonstrated that the expression of ATF3 and gadd153/Chop10 are inverse but overlapping with each other. We propose a model to explain the role of ATF3 and gadd153/Chop10 in the liver's response to stress.
Material and methods

Plasmids

pCG-gadd153/Chop10 was constructed as follows. The gadd153/Chop10 ORF was amplified by a combination of reverse transcription and PCR (RT-PCR, see below) from HeLa cell total RNA using AMV reverse transcriptase (Promega), pfu polymerase (Stratagene), upstream primer 5'-GCTCTAGAGCCATGGCAGCTGACTC-3', and downstream primer 5'-GCGGATCCTGTTCATGCTTGGTGCAG-3'. The amplified fragment has the introduction of an XbaI site at the N terminus and a BamHI site at the C terminus. The XbaI/BamHI DNA fragment was inserted in frame into the pCG vector (Tanaka and Herr, 1990) between the XbaI and BamHI sites for expressing gadd153/Chop10 in mammalian culture cells. pET.His-gadd153/Chop10 was constructed by cloning the above XbaI/BamHI blunt-ended fragment (by AMV reverse transcriptase) in frame into the pET.His vector at the BamHI blunt-ended site (by AMV reverse transcriptase). pTM1-gadd153/Chop10 was constructed by cloning the above XbaI/BamHI blunt-ended fragments (by AMV reverse transcriptase) in frame into the pTM1 vector (Moss et al., 1990) at the SmaI site. pTM1.GST was constructed as follows. The GST ORF was amplified by PCR using Pfu polymerase (Stratagene), upstream primer (5'-GCTCTAGAACATCCC CTATACTAGGTTA-3'), and downstream primer (5'-TGACCCGGGTTTTGGAGGA TGGTCGCCAC-3'). After PCR amplification, the amplified DNA fragment has the introduction of an XbaI site at the N terminus and a SmaI site at the C terminus. The XbaI/SmaI DNA fragment was inserted between XbaI and SmaI sites of the pGEM3 vector (Promega). A HincII/SmaI fragment containing the GST open reading frame was then isolated from pGEM3-GST and inserted in frame into the pTM1 vector between the NcoI blunt-ended (by AMV reverse transcriptase) and SmaI sites. pTM1-gadd153/Chop10 was constructed by cloning gadd153/Chop10 XbaI blunt-ended (by AMV reverse transcriptase)/BamHI fragment in frame into the pTM1.GST vector between the Sall blunt-ended (by Mung Bean Nuclease) and BamHI sites. pTM1.His-ATF3, pCG-ATF3, pCGN-P50, pCGN-P65, and pElam-CAT were described in Chapter II. pET.His-ATF1, pET.HisK-ATF3 C, and pET.HisK-ATF3 N were described in Chapter III.

Cell culture

The maintenance of COS-1 and HeLa cells was described in Chapter II.
Expression and purification of histidine-tagged recombinant proteins from *E. coli*

The expression and purification of his-ATF1, hisk-ATF3 N, and hisk-ATF3 C fusion proteins were described in Chapter III. his-gadd153/Chop10 fusion protein was also expressed and purified from the BL21 (DE3) LysS *E. coli* strain using the pET.His-gadd153/Chop10 construct and the same procedure for other histidine fusion proteins.

**Protein-protein blot**

Protein-protein blot analysis was performed using the same procedure for the COS-1 cDNA expression library screening described in Chapter III. Briefly, *E. coli* expressed recombinant proteins were analyzed by electrophoresis on a 10 or 12 % of SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, denatured, renatured, and incubated with the indicated $^{32}$P-labeled proteins.

**In vitro protein synthesis and band shift analysis**

ATF3 and gadd153/Chop10 proteins were synthesized *in vitro* by the TNT coupled reticulocyte lysate system (Promega) using pTM1 derivatives containing the corresponding cDNA as follows. Briefly, a 10 µl reaction containing 0.2 µg of a pTM1 derivative was mixed with 0.4 µl TNT buffer (500 mM Tris-HCl [pH 8.0], 15 mM MgCl2, and 1 mM each of ribonucleotide), 0.2 µl T7 RNA polymerase (Promega), 0.2 µl 1 mM amino acid mix (minus methionine), 0.2 µl 1 mM methionine, 4 units RNasin (Promega), 5 µl reticulocyte lysate, and DEP-treated H2O. The reaction was incubated at 30°C for 90 minutes.

Band shift analysis was carried out using *in vitro* synthesized proteins as follows. DNA fragments containing different binding sites and flanking sequences were isolated from pGEM3 or pBluescript derivatives (see below), and end-labeled as probes for the band shift analysis. A EcoRI/HindIII fragment containing the ATF consensus sequence (ggAGACGTCAAtt) was isolated from pATF(cat)x1. An EcoRI/HindIII fragment containing the AP1 consensus sequence (gggTGACTCAccc) was isolated from pAPx1. An EcoRI/HindIII fragment containing the Elam-1 site (ttcTGACATCAttg) was isolated from pELAM(24A+B)x1. An EcoRI/HindIII fragment containing the E4F site (gggTGACGTAACgt) was isolated from pE4F2core x1. An EcoRI/HindIII fragment
containing the Enk-2 site (ttcTGCCTGCAgcgc) was isolated from pGEM3-Enk-2. An Xbal/HindIII fragment containing the C/EBP consensus sequence (tgaTTGCGCAATac) was isolated from pBSKS-CEBP. An Xbal/HindIII fragment containing the ATF and C/EBP chimeric site (tgcTGACGCAATac) was isolated from pBSKS-CEBP/CRE.

The end-labeling reaction was performed using 0.1 µg (= 2 pmoles) of an isolated DNA fragment, 3 µl [α-32P]dCTP (3000 Ci/ mmole, 10 µCi/µl), 2.5 units of AMV reverse transcriptase (Promega) in a 20 µl reaction containing 50 mM Tris-HCl [pH 8.3], 40 mM KCl, 7 mM MgCl2, 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dTTP, 2 µg acetylated BSA, and incubated at 42°C for 30 minutes. The labeled DNA fragment was separated from un-incorporated [α-32P]dCTP by G-50 exclusion chromatography (see below).

The binding reaction was performed using 2 µl of the in vitro translation reaction mixture, 0.5 µg of poly[d(I-C)], and 0.02 pmole probe in 20 µl of 0.5 x buffer D (10 mM Hepes [pH 8.0], 10 % glycerol, 50 mM KCl, 0.1 mM EDTA, and 0.25 mM DTT). All components except the labeled DNA fragment (probe) were mixed together and pre-incubated at room temperature for 5 minutes. After adding the probe, the binding reaction was incubated at room temperature for an additional 20 minutes and subjected to electrophoresis on a 5% (1:40 cross linking) native polyacrylamide gel containing 0.5 x TBE (45 mM Tris-borate, 45 mM boric acid, and 1 mM EDTA pH 8.3) at 4°C for 4 hours (run at 200V) or 8 hours (run at 100V). After electrophoresis, the gel was transferred to Whatman 3 mm paper, dried on a gel dryer for 1 hour, and exposed to X-ray film for 2 hours to overnight depending on the experiment.

G-50 exclusion chromatography
G-50 exclusion chromatography was carried using the same the procedure described in Chapter III except replacing 50 µg acetylated BSA with 5 µg tRNA and using D.D. water (instead 0.1 M buffer D) to reconstitute the Sephadex G-50 beads.

Transfection and CAT assay
Transfection and CAT assay were carried out using the same procedure described in Chapter II.
**Northern blotting**

Total RNA was isolated from rat liver using guanidine isothiocyanate method described in Chapter III. Poly(A+) RNA was further purified by poly(dT) cellulose column (Pharmacia) using a batchwise procedure as follows. Five hundred to eight hundred microgram of total RNA in poly(dT) column loading buffer (0.5 M NaCl, 20 mM Tris-HCl [pH 7.6], 1 mM EDTA, 1% sarkosyl) was incubated with 100 µl poly(dT) cellulose beads at 4°C with gentle rocking for 2 hours. The beads were washed thrice with 1 ml loading buffer at 4°C for 10 minutes. The beads were then incubated with 50 µl TE buffer at 4°C with gentle rocking for 10 minutes to elute the poly(A+) RNA. The elution procedure was repeated one more time. After phenol extraction and ethanol precipitation, 3 µg of batchwise-purified poly(A+) RNA was resuspended in 4.5 µl DEP-H2O and mixed with 2 µl 5x formaldehyde gel running buffer (0.1 M MOPS [pH 7.0], 40 mM sodium acetate, and 5 mM EDTA), 3.5 µl formaldehyde (Baker Analyzed, 37% solution), and 10 µl deionized formamide (100%). The RNA mixture was incubated at 65°C for 15 minutes, chilled on ice, mixed with 2µl formaldehyde gel loading buffer (50% glycerol, 1 mM EDTA, 0.25% bromophenol blue, and 0.25% xylene cyanol), and subjected to electrophoresis on a 1% formaldehyde agarose gel. The RNA was then transferred to a Duralon membrane (Stratagene) and cross-linked to the membrane by the UV cross-linker (Stratagene) for 30 seconds or baked in a vacuum oven at 80°C for 2 hours. The membrane was then pre-hybridized with hybridization buffer (50% formamide, 5 x SSC, 2 x Denhardt's reagent, 1% SDS, 100 µg/ml salmon sperm DNA) at 42°C for one hour to block the membrane, and hybridized with 32P-labeled DNA probe in hybridization buffer at 42°C for 16 to 24 hours, washed thrice with 0.2x SSC containing 0.1% SDS at 68°C for 20 minutes, and exposed to the phosphor (Molecular Dynamics) overnight or X-ray film with an intensifying screen for 3 to 7 days.

The 32P-labeled DNA probe was synthesized by random primer labeling reaction using the DNA templates containing rat ATF3, rat gadd153/Chop10, or cyclophilin cDNA (Danielson et al., 1988). Briefly, 25 ng DNA template in 15 µl H2O was heated to 100°C for 3 minutes, and chilled on ice to denature the DNA template. The denatured DNA template was mixed with 300 pmole (=1 µg) random 9 mer oligonucleotide (Stratagene), 3 to 5 µl [α-32P]dCTP (3000 Ci/m mole, 10 µCi/µl), and 1 unit klenow fragment (BRL) in a 50 µl reaction containing 50 mM Tris-HCl [pH 7.8], 200 mM HEPES-NaOH [pH 6.6], 5 mM MgCl₂, 10 mM β-mercaptoethanol, 30 µM dATP, 30
μM dGTP, 30 μM dTTP, and 0.4 μg/μl BSA (nuclease free). The reaction was performed at 37°C for 30 minutes. The un-incorporated [α-32P]dCTP was removed by Sephadex G-50 spun column (Pharmacia) as described in Chapter II.

**RT-PCR**

Reverse transcription reaction was carried out as follows. 1 μg poly(A+) RNA or 5 to 10 μg total RNA in 5 μl DEP-H2O was heated at 90°C for 5 minutes, and chilled on ice. The RNA was then mixed with 100 pmole random hexamer, 20 units Rnasin (Promega), and 2.5 units AMV reverse transcriptase (Promega) in a 20 μl reaction containing 10 mM Tris-HCl [pH. 9.0], 50 mM KCl, 1.5 mM MgCl2, 1 mM DTT, 0.1% TX-100, and 0.2 mM of all four deoxynucleotide triphosphates. The reaction was incubated at 23°C for 10 minutes and then incubated at 42°C for one hour.

Five microliter of the reverse transcription reaction was mixed with 4.5 μl 10 x Taq buffer (100 mM Tris-HCl [pH. 9.0], 500 mM KCl, 25 mM MgCl2, 1% TX-100), 4.5 μl 2 mM deoxynucleotides mix, 20 pmole of upstream and downstream primers, and 2.5 units Taq DNA polymerase (Promega) in a final volume of 50 μl. The PCR reaction was carried out for 30 cycles at 95°C for 1 minute, at 55°C for 1 minute, and at 72°C for 1 minute. If the PCR reaction was carried by Pfu DNA polymerase (Stratagene), 10 x Pfu buffer (200 mM Tris-HCl [pH. 8.2], 100 mM KCl, 60 mM (NH4)2SO4, 20 mM MgCl2, 1% TX-100, and 100 μg/ml BSA) was replaced with the 10 x Taq buffer in the PCR reaction.
Results

gadd153/Chop10 dimerizes with ATF3.

As described in Chapter III, a T7 gene 10-gadd153/Chop10 fusion protein isolated from the COS-1 cell cDNA expression library interacted with $^{32}$P-labeled ATF3 but not $^{32}$P-labeled ATF1 in the protein-protein blot analysis. To confirm this result, I expressed and purified the full length gadd153/Chop10 from E. coli, and examined its ability to interact with ATF3 by the protein-protein blot analysis. As shown in Figure 4.1B, $^{32}$P-labeled ATF3 C terminal domain, containing only the bZip domain, bound to gadd153/Chop10 and to itself but not to ATF1. The binding of ATF3 C terminal domain to gadd153/Chop10 was consistently greater than that to itself. The specificity of the interaction was further demonstrated by the fact that $^{32}$P-labeled ATF1 did not bind to gadd153/Chop10. These results indicated that the interaction between ATF3 and gadd153/Chop10 was not non-specific. The leucine zipper domain, as expected, was required for this interaction: As shown in Figure 4.1C, $^{32}$P-labeled ATF3 N terminal domain, lacking the leucine zipper domain, failed to interact with gadd153/Chop10.

In a collaborative effort, C. Wolfgang further demonstrated that ATF3 and gadd153/Chop10 interacted with each other in vivo: When co-expressed with GST-gadd153/Chop10 in HeLa cells, ATF3 was co-purified with GST-gadd153/Chop10 by the glutathione conjugated agarose beads. In addition, when co-expressed with gadd153/Chop10 in HeLa cells, ATF3 was co-immunoprecipitated with gadd153/Chop10 by the anti-gadd153/Chop10 antibodies but not by a non-immune serum. Previously, gadd153/Chop10 has been demonstrated to be a nuclear protein (Ron and Habener, 1992). Using immunofluorescence analysis, C. Wolfgang also demonstrated that ATF3 is a nuclear protein. These results suggested that, when present in the same cell, gadd153/Chop10 and ATF3 can heterodimerize with each other.
Figure 4.1 The interaction between ATF3 and gadd153/Chop10 was not non-specific and was dependent on the leucine zipper.

A) Coomassie blue stain to ensure that similar amounts of protein were used. his-ATF1 (lane 2), hisK-ATF3 C' (lane 3, ATF3 C' terminal domain), and his-gadd153/Chop10 (lane 4) expressed from *E. coli* were analyzed by electrophoresis on a 12% SDS-PAGE and stained. Lane 1 shows rainbow size marker (Amersham). (B) Interaction between ATF3 and gadd153/Chop10 was not non-specific. his-ATF1, hisK-ATF3 C' or his-gadd153/Chop10 were analyzed by protein-protein blot using radio-labeled hisK-ATF3 C' (lanes 1-3) or his-ATF1 (lanes 4 and 5) as a probe. (C) Leucine zipper was necessary for the interaction between ATF3 and gadd153/Chop10. Increasing amounts of gadd153/Chop10 was analysis by protein-protein blot using radio-labeled hisK-ATF3 N' (ATF3 N' terminal domain), which lacks the leucine zipper, as a probe.
gadd153/Chop10 inhibits ATF3 from binding to DNA.

Because the interaction between gadd153/Chop10 and ATF3 is relatively stable (can be detected both in vitro and in vivo), and also because the leucine zipper domain is required for ATF3 to interact with gadd153/Chop10, it is likely that, upon interaction, gadd153/Chop10 and ATF3 form a stable heterodimer. We then asked how this interaction affects ATF3 from binding to DNA. As indicated by Ron and Habener, gadd153/Chop10 has an amino acid substitution of three conserved residues in the basic region (Ron and Habener, 1992). These amino acid substitutions render gadd153/Chop10 unable to bind to the known C/EBP sites. When dimerizing with other C/EBP proteins, such as C/EBPα and C/EBPβ, gadd153/Chop10 acts as a dominant negative inhibitor and blocks binding to the C/EBP sites. In light of these observations, I examined whether gadd153/Chop10 could also inhibit ATF3 from binding to the ATF site by band shift analysis. As shown in Figure 4.2A, gadd153/Chop10 inhibited the binding of ATF3 to the consensus ATF site. Presumably, gadd153/Chop10 formed a non-functional heterodimer with ATF3 and prevented ATF3 from binding to the ATF site. However, gadd153/Chop10 has never been proven to be unable to bind to DNA. It is possible that, when dimerizing with ATF3, gadd153/Chop10 may alter the binding specificity of ATF3. To test this hypothesis, I examined the binding of ATF3-gadd153/Chop10 heterodimer on several ATF-related sites: AP1 site, Elam-1 site, E4F site, and Enk-2 site. In addition, I also examined the C/EBP site and a chimeric site composed of an ATF half site and a C/EBP half site (Figure 4.2B). As shown in Figure 4.2C, ATF3 homodimer bound to the AP1-, Elam-1, E4F, and chimeric sites specifically. The binding of ATF3 to these sites was inhibited by gadd153/Chop10. ATF3 homodimer did not bind to the Enk-2 and C/EBP sites. In the presence of gadd153/Chop10, gadd153/Chop10 apparently did not assist ATF3 to bind to either of the sites. Taken together, these results indicated that, when dimerizing with ATF3, gadd153/Chop10 inhibits ATF3 from binding to ATF and several ATF-related sites.

gadd153/Chop10 relieves the transcriptional repression by ATF3.

The band shift result predicts that dimerization between gadd153/Chop10 and ATF3 should also inhibit the transcriptional function of ATF3. Since ATF3 homodimer
Figure 4.2  gadd153/Chop10 inhibited ATF3 from binding to the ATF/CRE consensus sequence and several related sites.

(A) gadd153/Chop10 inhibited ATF3 from binding to the ATF consensus site. 1 μl of ATF3 generated by reticulocyte lysate was mixed with radio-labeled DNA fragment containing the consensus ATF/CRE site in the absence (lane 2) or presence of gadd153/Chop10 generated by reticulocyte lysate (lanes 3-5; 1, 3, and 9 μl respectively). Lane 6 shows the result of mixing 9 μl gadd153/Chop10 and DNA together, indicating that gadd153/Chop10 by itself did not bind to the ATF site. Reticulocyte lysate not programmed to generate any specific protein was included in the reactions to make sure that each reaction contained the same amount of reticulocyte lysate. (B) The sequences of the ATF consensus and related sites. (C) gadd153/Chop10 inhibited ATF3 from binding to several ATF related sites. Radio-labeled DNA fragments containing the indicated sites were incubated with 1 μl of ATF3, 9 μl of gadd153/Chop10 or both. Reticulocyte lysate not programmed to generate any specific protein was included so that each reaction contained the same amount of reticulocyte lysate. 200 fold of specific (sp) or non-specific (ns) competitor DNA was included as indicated to demonstrate the specificity of binding.

91
Figure 4.3  gadd153/Chop10 relieved the repression of E-selectin promoter by ATF3.

(A) E-selectin-CAT reporter was transfected into COS-1 cells with different plasmids: pCGN-p50 and pCGN-p65 (NF-κB), pCG-ATF3, and pCG-gadd153. pCG vector containing only the CMV promoter was included in some calcium phosphate/DNA mixes to make sure that each transfection mix contained the same amount of promoter. (B) Mutant E-selectin-CAT reporter containing mutated ELAM-1 site (TCACGACGGT) was co-transfected with DNA expressing ATF3 or NF-κB as indicated. The results are the average of three experiments.
represses transcription when bound to DNA, I examined whether co-expression of gadd153/Chop10 could relieve the transcriptional repression by ATF3. As shown in Chapter II, ATF3 was able to repress the activated E-selectin promoter activity. When co-transfected with ATF3, gadd153/Chop10 relieved the repression of the E-selectin promoter by ATF3 (Figure 4.3). Importantly, gadd153/Chop10 alone did not further stimulate the activated E-selectin promoter. This result suggested that the apparent relief of repression was not due to the masking of repression by transcriptional activation.

The expression of ATF3 and gadd153/Chop10 in CCl4 treated liver is inverse but overlapping.

Having demonstrated that gadd153/Chop10 negatively regulates the function of ATF3, we then asked whether this regulation is physiologically relevant. As described earlier in the introduction section, ATF3 and gadd153/Chop10 may play roles in the liver. Therefore, we examined the expression kinetics of ATF3 and gadd153/Chop10 in CCl4 treated rat liver using northern blot analysis. As shown in Figure 4.4A, ATF3 mRNA was not detectable in the saline-treated liver. Upon CCl4 treatment, ATF3 mRNA increased to a high level at 1.5 hours, decreased at 3.5 and 7.5 hours, and returned to the non-detectable level at 17.5 hours. On the other hand, gadd153/Chop10 mRNA level was relative high in the saline-treated liver. Upon CCl4 treatment, gadd153/Chop10 mRNA decreased to a low level at 1.5 and 3.5 hours, and returned to the high level again at 7.5 hours and 17.5 hours. This result indicated that, in the CCl4-treated rat liver, the expression of ATF3 is inversely correlated to that of gadd153/Chop10: The expression of ATF3 was induced by CCl4, whereas the expression of gadd153/Chop10 was repressed by CCl4. Since gadd153/Chop10 negatively regulates the function of ATF3, we proposed that the decrease of gadd153/Chop10 would allow the increased ATF3 to function (see details in discussion).

The expression of gadd153/Chop10 mRNA in livers collected at 1.5 hours and 3.5 hours post CCl4 treatment decreased to a low level but was detectable by northern blot analysis. However, the expression of ATF3 mRNA in saline-treated liver and liver collected at 17.5 hours post CCl4 treatment was not detectable by northern blot analysis. Because northern blot is not very sensitive, to better characterize the expression of ATF3,
I re-examined the level of ATF3 mRNA by RT-PCR analysis. As shown in Figure 4.4C, ATF3 mRNA was clearly detectable in saline-treated liver and liver collected at 17.5 hours post CCl4 treatment. Therefore, we concluded that the expression of ATF3 and gadd153/Chop10 are inverse but overlapping in the CCl4 treated liver. In addition to my analysis of the mRNA levels, C. Wolfgang examined ATF3 and gadd153/Chop10 proteins by immunohistochemistry and immunoprecipitation analyses. Using liver collected at 17.5 hours post CCl4 treatment as an example, he demonstrated that ATF3 and gadd153/Chop10 proteins do exist in an overlapping manner.

The inverse expression of ATF3 and gadd153/Chop10 is not a general phenomenon.

After showing that the expression of ATF3 and gadd153/Chop10 is inversely correlated in the CCl4 treated liver, we then asked whether this is a general phenomenon in the liver's response to stresses. To this end, I used RT-PCR analysis to examine the mRNA level of ATF3 and gadd153/Chop10 in livers treated with alcohol or partial hepatectomy. As shown in Figure 4.5A, consistent with C. Wolfgang’s in situ hybridization results, the mRNA level of ATF3 increased in the rat liver after alcohol or partial hepatectomy treatments. However, gadd153/Chop10 behaved differently in the alcohol and partial hepatectomy treated livers: Similar to that of the CCl4 treated liver, the mRNA level of gadd153/Chop10 also decreased after partial hepatectomy treatment; in contrast, its mRNA level did not change after alcohol treatment (Figure 4.5B). These results indicate that the inverse expression pattern of ATF3 and gadd153/Chop10 is not a general phenomenon of the liver's response to stresses.
Figure 4.4 ATF3 and gadd153/Chop10 were expressed in CCl4-treated liver in an inverse but overlapping manner.

(A) Northern blot analysis indicated an inverse expression of ATF3 and gadd153/Chop10. Rats intragastrically injected with CCl4 were sacrificed at the indicated times; poly(A) RNA was prepared from liver and analyzed (3 μg/lane) by northern blot using radio-labeled ATF3, gadd153/Chop10 or cyclophilin cDNA as probe. Cyclophilin is abundant and ubiquitous in tissue and phylogenetic distribution (Danielson et al., 1988). (B) Quantitation of northern blot. The signals in (A) were quantitated by PhosphorImager (Molecular Dynamics). ATF3 and gadd153/Chop10 signals were normalized against cyclophilin, and the highest expression point was arbitrarily defined as 100%. (C) RT-PCR analysis indicated an overlapping expression of ATF3 and gadd153/Chop10: ATF3 mRNA was present in the normal liver and in the livers collected at 7.5 and 17.5 hours after CCl4 treatment. Total RNA (10 μg) or poly(A) RNA (1 μg) from saline-treated liver (-) or CCl4-treated livers collected at indicated times was analyzed by RT-PCR using primer set to amplify ATF3. An aliquot of the product was loaded on the gel. As a control, water was used to replace RNA (H2O). Size markers in base pair are indicated on the left.
Figure 4.5. Partial hepatectomy but not alcohol treatment decreased the level of gadd153/Chop10 mRNA.
Total RNA (10 μg) from the control livers, from the livers collected at 3.5 hours after alcohol treatment or from the livers collected at 2 hours after partial hepatectomy was analyzed by RT-PCR using primer sets to amplify ATF3 (A), gadd153/Chop10 (B), or GAPDH (C) cDNAs as indicated. Water control for each primer set is indicated (H2O). Size markers in base pair are indicated on the left.
Figure 4.6. A model for the role of ATF3 in the liver's response to CCl4.
The active promoter is indicated by a thick arrow; the inactive promoter is indicated by a thin arrow and an "X". For simplicity, other upstream transcription factors and the basic transcription machinery are not shown.
Discussion

In this chapter, I present evidence that gadd153/Chop10 dimerizes with ATF3 and negatively modulates the function of ATF3: gadd153/Chop10 inhibits ATF3 from binding to the ATF and several ATF-related sites. Consequently, gadd153/Chop10 relieves the transcriptional repression by ATF3. Using northern blot and RT-PCR analyses, I demonstrate that the expression of ATF3 and gadd153/Chop10 in the liver respond to the CCl4 treatment in an opposite way: The expression of ATF3 is induced by CCl4 treatment, whereas the expression of gadd153/Chop10 is repressed by CCl4 treatment. Based on these observations, we proposed a model to explain the roles of ATF3 and gadd153/Chop10 in the liver's response to CCl4 stimulation (Figure 4.6). In the normal liver, the level of ATF3 is low. However, the level of its inhibitor, gadd153/Chop10, is high to functionally inhibit the trace amounts of ATF3. Upon CCl4 treatment, ATF3 is induced while gadd153/Chop10 is repressed. ATF3 forms a homodimer and represses transcription from certain target promoters. One potential target promoter for the ATF3 homodimer is the gadd153/Chop10 promoter (see explanation below). Also, we can not rule out the possibility that ATF3 may form heterodimers with other bZip proteins ("protein X"). The ATF3/protein X heterodimers may act as transcriptional activator of a different set of target promoters. One possible candidate for protein X is c-Jun (see explanation below). In the post-stress liver, the level of ATF3 decreases while the level of gadd153/Chop10 increases. The increased gadd153/Chop10 protein is able to inhibit the activity of ATF3 by forming a non-functional heterodimer with it. This process sequesters ATF3 away from the functional pool and provides another mechanism, in addition to decreasing the level of ATF3, to down regulate the activity of ATF3.

The relatively high level of gadd153/Chop10 in normal liver contradicts a previous study. N.J. Holbrook and colleagues reported that the expression of gadd153/Chop10 was not detectable in the normal rat liver and was induced by lipopolysaccharide (LPS) which caused an acute phase response (Sylvester et al., 1994). In my northern blot analysis, the level of gadd153/Chop10 was clearly detectable in the normal rat liver. This discrepancy may be explained by the fact that the human gadd153/Chop10 cDNA was used as a probe to detect the rat gadd153/Chop10 mRNA in the study by Sylvester et al. In fact, in a separate study, J. Habener and colleague also
demonstrated that gadd153/Chop10 expresses in a relative high level in normal rat liver (Ron and Habener, 1992)

**The potential target promoter for ATF3 homodimer**

According to the model (Figure 4.6), upon CCl4 treatment, the level of gadd153/Chop10 decreases to allow the increased ATF3 to function. ATF3 may form a homodimer and repress transcription from the target promoters. One of the potential target promoters for ATF3 homodimer appear to be the gadd153/Chop10 promoter which contains ATF3 binding sites. First, ATF3 is able to repress the gadd153/Chop10 promoter in an *in vitro* transcription assay (see Chapter II). Second, C. Wolfgang demonstrated that ATF3 is able to bind to gadd153/Chop10 promoter by band shift and footprinting analyses, and ATF3 represses gadd153/Chop10 promoter in a co-transfection experiment (C. Wolfgang and T.Hai unpublished results). Third, using *in situ* hybridization analysis, C. Wolfgang demonstrated that the sub-tissue expression pattern (location) of ATF3 and gadd153/Chop10 were complementary to that of each other: Upon CCl4 treatment, ATF3 mRNA was induced around the central veins, while gadd153/Chop10 mRNA disappeared around the central veins (C. Wolfgang and T.Hai unpublished results). These results suggest that ATF3 may negatively regulate the expression of its inhibitor, gadd153/Chop10.

**The possible candidate for protein X**

In the proposed model (Figure 4.6), we hypothesize that ATF3, in addition to dimerizing with itself, dimerizes with other bZip proteins, such as protein X. The resulting ATF3/protein X heterodimer may activate instead repress transcription. At present, one possible candidate for protein X is c-Jun for the following reasons. First, c-Jun has been demonstrated to dimerize with ATF3 previously (Hai and Curran, 1991); the affinity of the interaction between ATF3 and c-Jun is comparable to that between ATF3 and gadd153/Chop10 (Figure 4.7). Second, in contrast to ATF3 homodimer, ATF3/c-Jun heterodimer was shown to activate transcription (Hsu et al., 1991; Hsu et al., 1992). Third, using *in situ* hybridization analysis, C. Wolfgang demonstrated that c-
Figure 4.7. ATF3 interacted with gadd153/Chop10 and c-Jun with similar affinity.
(A) Coomassie blue stain to ensure that similar amounts of protein were used.
(B) ATF3 showed similar affinity toward gadd153/Chop10 and c-Jun. Increasing amounts of gadd153/Chop10 or c-Jun were analyzed by protein-protein blot analysis using radio-labeled hisK-ATF3 C' as a probe.
Jun is also induced in the CCl4 treated liver and has the similar expression kinetics and pattern to that of ATF3: The mRNA of c-Jun not only increases to the high level at the same time (1.5 hours) with ATF3 but also localizes around the central veins where ATF3 is expressed (C. Wolfgang and T.Hai unpublished results). Taken together, these results suggest that upon CCl4 stimulation, ATF3 and c-Jun may heterodimerize with each other and activate transcription from certain target promoters.

Additional significance of the interaction between ATF3 and gadd153/Chop10

The band shift analysis indicated that gadd153/Chop10 inhibits ATF3 from binding to the ATF and several ATF-related sites. However, this experiment did not rule out the possibility that ATF3 and gadd153/Chop10 heterodimer may bind to sequences unrelated to ATF or C/EBP sites. In fact, using random site selection analysis, D. Ron and colleagues recently demonstrated that C/EBPα-gadd153/Chop10 heterodimer is able to bind to DNA indicating that gadd153/Chop10 is not merely an inhibitor of the C/EBP proteins; it may also function as a direct regulator of transcription (Ubeda et al., 1996). By the same logic, it is possible that ATF3-gadd153/Chop10 heterodimer may bind to DNA and regulate specific target promoters. As indicated by the RT-PCR analysis, the inverse expression of ATF3 and gadd153/Chop10 is not a general phenomenon. Therefore, under certain conditions, ATF3 and gadd153/Chop10 may co-exist. However, it remains to be determined whether ATF3-gadd153/Chop10 heterodimer binds to DNA. It is possible that we can identify the binding sites for ATF3-gadd153/Chop10 heterodimer by the same method. With the binding sites, we may then be able to identify potential target promoters and further investigate the additional physiological relevance of this interaction.

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Chapter V

Biochemical approaches to further investigate the "co-repressor" model

Summary

One first step to investigate the "co-repressor" model is to study the ATF3-interacting proteins. In addition to the protein-protein blot screening approach described in Chapter III, I carried out two biochemical approaches to further investigate the ATF3-interacting proteins: \textit{in vivo} complex assembly assay and ATF3 affinity column assay. Although I did not obtain any success using the \textit{in vivo} complex assembly assay, using ATF3 affinity column, I repeatedly observed two polypeptides, 80 kDa and 90 kDa, and occasionally a third polypeptide, 50 kDa, that interacted with ATF3. However, the specificity of their binding to ATF3-GST affinity column remains to be determined.

Introduction

Transcription factors usually do not function by themselves; to perform their activities, they need to interact with other proteins. Therefore, identification of the interacting proteins will give us the clues to the function of a known transcription factor. One commonly used method to identify the interacting proteins is the protein-protein blot (or far western blot) analysis. In this method, a radiolabeled protein is used as a probe to identify its interacting proteins. For example, CBP was identified from a cDNA expression library by its ability to interact with radiolabeled CREB (Chrivia et al., 1993). As described in Chapter III, using this method, I identified several ATF3 interacting proteins; however, they do not appear to be the desired co-repressor (see discussion in Chapter III). In addition to the protein-protein blot analysis, there are several biochemical
and genetic approaches to study the interacting proteins. Below, I discuss some of the commonly used methods.

**Protein affinity column:** This method is to immobilize the protein of interest to some type of matrix to generate a protein affinity column. This column can then be used to "capture" the interacting proteins. The conventional approach is to covalently cross-link the protein of interest to the matrix. A more recent method is to fuse the protein of interest with glutathione S-transferase (GST) (Smith and Johnson, 1988). Because GST binds to glutathione with extremely high affinity, the fusion protein will bind to the agarose beads conjugated with glutathione avidly. The GST fusion approach is fast and convenient, and has been widely used to study protein-protein interaction. For example, using GST-RB affinity column, D.M. Livingston and colleagues reported that multiple cellular proteins also bind to RB at the "E1a-binding region" (Kaelin et al., 1991). One potential problem of this approach, however, is that, when fused with the GST domain, the protein of interest may change the conformation. This may affect its ability to interact with the interacting proteins.

**Co-immunoprecipitation:** This method is to immunoprecipitate the protein of interest under conditions allowing protein-protein interaction. Under such conditions, proteins which interact and associate with the protein of interest may be co-immunoprecipitated and detected. One classic example is the study of adenovirus E1a interacting proteins (Harlow et al., 1986). Using co-immunoprecipitation analysis, E1a was found to interact with multiple cellular factors including E2F, cyclin A, RB, p107 (RB related), p130 (RB related), and p300 (see review by Zantema and van der Eb, 1995). One advantage of the co-immunoprecipitation approach is that it may detect proteins both directly or indirectly associated with the protein of interest. By investigating the whole complex of associated proteins, one can have a more complete picture for the function of the protein of interest. However, this approach largely relies on the quality of the antibodies. If the epitope recognized by the antibodies is blocked by protein-protein interaction, the antibodies may not be able to precipitate the complex.

**In vivo assembly:** This method requires over-expression of the epitope tagged protein of interest in the cells. The epitope usually contains only a few amino acids and should not affect the function of the protein of interest; therefore, the epitope tagged
protein of interest is expected to associate normally with its interacting proteins and assemble into complex with them. The epitope then allows the isolation of the assembled protein complex by specific antibody against the epitope. The advantage of this approach over co-immunoprecipitation is that there is no need to develop the antibodies. In addition, the epitope tag can be fused at either terminus of the protein of interest or different components of the protein complex to prevent being blocked by the protein-protein interaction. Using this approach, A.J. Berk and colleagues successfully isolated several TBP-associated factors (TAFs): TAF 250, TAF 125, TAF 95, TAF 78, and TAF 50 (Zhou et al., 1992).

Cross-linking: This method is to covalently cross-link two interacting proteins by cross-linking reagent which contains two reactive moieties to react with both proteins. The cross-linked complex can then be isolated by co-immunoprecipitation or other methods to identify the interacting proteins. One recent example is the identification of the interaction between RNA polymerase II carboxyl terminal domain (CTD) and two general transcription factors, TFIIE-β (TFIIE small subunit) and RAP74 (TFIIF large subunit) (Kang and Dahmus, 1995). The advantage of the cross-linking method is that it may be able to detect the weak or transient protein-protein interaction. Weak interactions sometimes could be physiologically relevant; however, they may not be detected by co-immunoprecipitation or protein affinity column approaches. The disadvantage is that proteins indirectly associated with the protein of interest may not be detected by this method. In addition, because it is highly sensitive, the cross-linking method often detects non-specific protein-protein interactions.

Yeast two-hybrid: This method is a genetic approach to detect the interaction between two protein (Fields and Song, 1989): One protein (X) is fused with a given DNA binding domain and another protein (Y) is fused with a transcriptional activation domain; they are then introduced into a yeast strain that contains a reporter gene driven by the binding sites of the given DNA binding domain. The interaction between X and Y brings the transcriptional activation domain to the DNA binding domain and results in activation of the reporter gene. This method is also widely used as a library screening approach to identify the interacting proteins. For example, using yeast two-hybrid screening method, Rosenfeld and colleagues identified N-CoR, a co-repressor protein, which binds to nuclear hormone receptors and mediates the transcriptional repression by
nuclear hormone receptors (Hörlein et al., 1995). In comparison to the protein-protein blot screening method, one advantage of the yeast two-hybrid screening method is that the host cells provide some type of post-translational modification which may assist protein-protein interactions. Another advantage is that it is probably more sensitive than the protein-protein blot screening method (Guarente, 1993). Therefore, yeast two-hybrid screening method may detect weak protein-protein interaction. However, it is more time consuming than the protein-protein blot screening method. In addition, due to its sensitivity, yeast two-hybrid screening method may produce more false positive clones.

Selection of suppressor mutants: A suppressor mutant is a mutated gene which can suppress the mutation of another gene. Selection of suppressor mutants is another genetic approach to identify the interacting proteins. For example, VP16, one of the strongest eukaryotic transcriptional activators, can activate transcription not only in a mammalian system but also in a yeast system. Over-expression of VP16 in yeast is toxic to the cells. Presumably, VP16 sequesters the limiting transcription factors or co-factors away from the transcriptional machinery, and results in the inhibition of the expression of the host genes. By selecting the suppressor mutants, L. Guarente and colleagues identified several adapter proteins (ADA1, ADA2, and ADA3) that mediate the transcriptional activation by VP16 (Bergere et al., 1992). However, this method has several limitations. For example, some mammalian transcription factors can not function properly in the yeast. Therefore, it is not possible to study them by this approach.

According to the "co-repressor" model, ATF3 interacts with co-repressors. To investigate the "co-repressor" model, one first step is to study the ATF3-interacting proteins. As described in Chapter III, using protein-protein blot approach, I screened a COS-1 cDNA expression library for clones encoding ATF3-interacting proteins. In this Chapter, I present two biochemical approaches to investigate the ATF3-interacting proteins from the HeLa cells: in vivo complex assembly assay and ATF3 affinity column assay. Although I did not obtain any successful result from the in vivo complex assembly assay, I identified at least three HeLa cellular proteins that interact with ATF3 by the affinity column approach.
Material and methods

Plasmids

pRc/CMV-HA-ATF3 was constructed as follows. The HA-ATF3 DNA fragment was amplified from pCGN-ATF3 (for expressing HA tagged ATF3 in mammalian culture cells) by PCR using \textit{Pfu} polymerase (Stratagene), the HA primer (5'-ATGGCTTCTAGCTATCC-3'), and an ATF3 downstream primer (5'-CCGGATCCTTAGCTCTGCAATGTTCCTTC-3'). The blunt-ended/BamHI DNA fragment was cloned into the pGEM3 vector between the Smal and BamHI sites to generate pHA-ATF3. A Kpnl blunt-ended/BamHI fragment (by Mung Bean Nuclease) isolated from pHA-ATF3 was inserted in frame into pRc/CMV (Invitrogen) at the HindIII blunt-ended site (By AMV reverse transcriptase) to create pRc/CMV-HA-ATF3. pGEX.2T-ATF3 was constructed by cloning the full-length ATF3 Xbal/BamHI blunt-ended fragment (see Chapter II, material and methods section) in-frame into the pGEX-2T vector (Pharmacia) at the Aval blunt-ended site (by AMV reverse transcriptase). pET3-ATF3-GST was constructed as follows. To express ATF3-GST fusion protein, the GST ORF was amplified by PCR using \textit{Pfu} polymerase and cloned into the pGEM3 vector (Promega). The ATF3 C' terminus deletion mutant, ATF3(1-171) which lacks the termination codon, was fused in frame at the N' terminus of GST open reading frame. The ATF3-GST fragment was excised from the pGEM3 vector and cloned into the pET3 vector. pTM1-ATF1 was constructed by Curt Wolfgang as follows. A blunt-ended Ndel/HindIII fragment (by AMV reverse transcriptase) was isolated from pET.His-ATF1 and subcloned into pTM1 (Moss et al., 1990) at the EcoRI blunt-ended site (by Mung Bean nuclease). The construction of pTM1.His-ATF3 was described in Chapter II. The construction of pET.His-ATF1, and pET.His-ATF3 were described in Chapter III. The construction of pTM1-gadd153/Chop10 was described in Chapter IV.

Cell culture

The maintenance of COS-1 cells and monolayer HeLa cells was described in the Chapter II. Suspension HeLa cells were maintained in Minimum Essential Media modified for suspension cultures (S-MEM) with Penicillin/Streptomycin (BRL), non-essential amino acids (BRL), ferric nitrate (0.1 μg/ml), and 10% CS at 37°C in a spindle flask (no requirement for CO2). 143B human osteosarcoma cells, without thymidine kinase (TK-), were maintained in Minimum Essential Media (MEM) with Earl's salt, Penicillin/
Streptomycin, non-essential amino acids, 50 μg/ml bromodeoxyuridine (BUdr or BrdU, Sigma B9285), and 10% FBS at 37°C in a 5% CO2 incubator.

To generate the stable HeLa cell line expressing HA-tagged ATF3, pRc/CMV-HA-ATF3 was linearized by ScaI and transfected into monolayer HeLa cells by the calcium phosphate method described in Chapter II. Sixteen hours after transfection, the HeLa cells were split into three different ratios: 1:10, 1:30, and 1:90. Immediately after splitting, G418 (BRL) was added directly to the media at a final concentration of 400 ng/ml for selecting HeLa cells with the stable integration of pRc/CMV-HA-ATF3 and expression of neo resistant gene. The selective media was changed every three days. Two weeks after the transfection, the individual G418-resistant colonies were subcloned and expanded.

Expression of histidine-tagged ATF3 by vaccinia virus
Recombinant vaccinia virus encoding histidine-tagged ATF3 was generated as follows. COS-1 cells were infected with wild type vaccinia virus at a titer of 0.05 pfu/cell for 2 hours, and then transfected with calcium phosphate-precipitated pTM1.His-ATF3 plasmid for 4 hours at 37°C in a CO2 incubator. The infected/transfected cells were washed twice with PBS, replenished with fresh media, and incubated at 37°C in a CO2 incubator for an additional 48 hours to allow recombination between vaccinia virus genome and pTM1.His-ATF3. The infected/transfected COS-1 cells were harvested using a sterile rubber policeman inside the tissue culture hood, transferred to a sterile eppendorf tube, spun at 4°C for 2 minutes to collect the cells. The cell pellet was resuspended in 0.5 ml media (also operated in the tissue culture hood), frozen and thawed five times in order to lyse the cell pellet and release the vaccinia virus.

Osteosarcoma 143B (TK-) cells were used to select the thymidine kinase minus recombinant vaccinia virus as follows. The vaccinia virus obtained from the infected/transfected COS-1 cells was diluted in a 10-fold series using MEM containing 2.5% FBS (MEM-2.5). The virus dilutions were then used to infest 60 mm plates of 143B cells, and incubated at 37°C in a CO2 incubator for 2 hours. After removing the media, each of the 60 mm plates was overlaid with 3 ml selective agarose (MEM-2.5 media, 1% low melting agarose, 50 μg/ml BrdU, melted and cooled at 42°C), placed at room temperature for 5 minutes, and incubated at 37°C in a CO2 incubator for 48 hours.
Each of the plates was then overlaid with 2 ml second agarose (EMEM-2.5 media, 1% low melting agarose, 100 μg/ml neutral red, melted and cooled at 42°C), and incubated at 37°C in a CO2 incubator for an additional 24 hours (neutral red will help to visualize the plaques). The well separated plaques were picked using a sterile pasteur pipet and transferred to an eppendorf tube (operated in the tissue culture hood) containing 400 μl MEM-2.5 media, and frozen and thawed five times to release the recombinant virus. Aliquots of the recombinant virus were analyzed by PCR using T7 and ATF3 primers. Recombinant virus (vA3) with the correct size of inserted his-ATF3 was amplified and expanded.

To express his-ATF3, confluent suspension HeLa cells (~ 1x10^6 cells/ml) were collected by centrifugation at room temperature, 2000x g (JS-4.2, 3.5K RPM) for 15 minutes. The cell pellet was resuspended in S-MEM media (= 30 ml per liter of cells), infected with vTF 7-3 and vA3 (< 1 pfu/cell of each virus), and incubated at 37°C for 2 hours with gentle stirring. The infected HeLa cells were diluted with S-MEM media to a concentration of 5x10^5 cells/ml, and incubated at 37°C for 48 hours before harvesting. The infected cells were collected by centrifugation at 4°C, 2000x g (JS-4.2, 3.5K RPM) for 15 minutes and subjected to nuclear extract preparation (described in Chapter II) or his-ATF3 purification (using the same procedure for purifying histidine-tagged recombinant proteins from E. coli as described in Chapter III).

Expression and purification of GST fusion proteins from E. coli
To express GST and GST-ATF3 proteins, pGEX.2T or pGEX.2T-ATF3 was freshly transformed into the DH5α E. coli strain followed by IPTG induction as described for histidine-tagged recombinant proteins expression in Chapter III. To express the ATF3-GST fusion protein, pET3-ATF3-GST was freshly transformed into the BL21 (DE3) LysS E. coli strain and induced with IPTG as described in Chapter III. After IPTG induction, cells expressing GST or GST fusion proteins were collected by centrifugation at 4°C, 2000x g (JS-4.2, 3.5 K RPM) for 15 minutes. The cell pellet obtained from one liter of cell culture was resuspended in 30 ml STE buffer (25 mM Tris-HCl [pH 8.0], 150 mM NaCl, and 1 mM EDTA) containing 100 μg/ml lysozyme, and incubated on ice for 15 minutes. The cell suspension was then mixed with sarkosyl to a final concentration of 1.5% (to lyse the cells and to help solubilize the recombinant proteins), sonicated briefly to shear the chromosome DNA, and spun at 4°C, 18,000x g (JA-14,
IK RPM) for 20 minutes to remove the insoluble debris. The supernatant was mixed with TX-100 to a final concentration of 2% (TX-100 will form mixed micelles with sarkosyl), aliquotted, frozen on the dry ice, and stored at -80°C. To generate the GST or ATF3-GST affinity column, supernatants containing GST or ATF3-GST proteins were incubated with glutathione agarose beads at room temperature for 15 minutes with gentle rocking, and washed briefly with PBS 3 to 5 times to remove all the detergents.

**Labeling of suspension HeLa cells and ATF3-GST affinity column purification**

To label the suspension HeLa cells, 40 to 50 ml confluent cells (=1x10^6 cells/ml) was collected by centrifugation in the clinical centrifuge at setting #4 (= 800 g) for 5 minutes, washed twice with 20 ml PBS, and resuspended in 1 ml methionine deficient DMEM containing 10% CS. The cells were then transferred to a 35 mm plate, and incubated at 37°C in a CO2 incubator for 30 minutes. Five hundred μCi of 35S-methionine (Tran^35S, >1000Ci/mmole, ICN) was then added directly to the methionine deficient DMEM in the 35 mm plate. The labeled 35 mm plate was placed inside of a 100 mm plate covered with charcoal paper, and incubated at 37°C in a CO2 incubator for 2 hours. The labeled suspension HeLa cells were transferred to an eppendorf tube, spun at 4°C for 2 minutes to collect the cells, washed twice with PBS, and resuspended in 0.5 ml GST beads buffer (20 mM Hepes [pH 7.5], 75 mM KCl, 2.5 mM MgCl2, 0.1 mM EDTA, 0.05% NP-40, 1% non-fat milk). The cells were then sonicated briefly (setting #2, 40% duty cycle, no more than 10 pulses) to lyse the cells, and spun at 4°C for 5 minutes to remove any insoluble debris. The whole cell extract was pre-incubated with GST beads at 4°C with gentle rocking for one hour and spun for 30 seconds to pellet the beads. The pre-cleaned whole cell extract was split and incubated with GST or ATF3-GST columns at 4°C for 6 hours. The GST or ATF3-GST column bound proteins were washed thrice with 1 ml GST beads buffer at 4°C for 5 minutes, resuspended in SDS gel loading solution containing 0.1 M DTT, boiled for 3 minutes, and analyzed by electrophoresis on a 10% SDS-polyacrylamide gel.

**Expression and purification of histidine-tagged recombinant proteins from E. coli**

Expression and purification of his-ATF3 and his-ATF1 from *E. coli* is described in Chapter II.
Results

I. In vivo assembly assay

According to the "co-repressor" model described in Chapter II, ATF3 interacts with the co-repressors: When ATF3 binds to DNA, it stabilizes the co-repressors at the promoter and results in transcriptional repression; when ATF3 does not bind to DNA, it still interacts with the co-repressors and sequesters the co-repressors away from the promoter, resulting in transcriptional activation. This model predicts that ATF3 and co-repressors form a complex in the presence or absence of DNA. Therefore, by isolating the ATF3-containing complex, we may be able to identify the putative co-repressors. Since the endogenous level of ATF3 is low, it would be difficult to isolate endogenous ATF3-containing complexes. To facilitate the purification, I took two approaches to over-express ectopic ATF3 in the HeLa cells: stable expression and transient expression. Presumably, the over-expressed ATF3, together with the co-repressors, may assemble into some type of protein complex. As describe in the introduction, this approach has been used successfully to study the TBP associated proteins, TAFs (Zhou et al., 1992).

Stable HeLa cell line expressing ATF3

To express ectopic ATF3, my first approach was to generate a stable HeLa cell line expressing HA-tagged ATF3. I transfected HeLa cells with pRc/CMV-HA-ATF3 plasmid which contains neo resistant gene driven by the RSV promoter and HA-tagged ATF3 driven by the CMV promoter. The HeLa cell with stable integration of pRc/CMV-HA-ATF3 expresses neo resistant gene and can survive in the G418 selective media. After two weeks of G418 selection, individual G418-resistant colonies were subcloned and expanded. Thirty-five G418-resistant colonies were subcloned but only 14 colonies were able to be expanded and stabilized.

Although these HeLa cell lines were resistant to G-418, when examined by northern blot analysis, none of them expressed detectable HA-ATF3. One explanation is that these cell lines have pRc/CMV-HA-ATF3 integrated at the loci with low
transcriptional activity. Therefore, more colonies must be screened to identify lines expressing high level HA-ATF3. Another explanation is that over-expression of ATF3 may be toxic to the cells. As discussed in Chapter IV, the level of ATF3 is low in the normal cells and is rapidly induced by physiological stresses in a variety of tissues. This observation indicated that, normally, cells do not require the function of ATF3; only under certain stressful conditions, cells express ATF3 transiently to regulate certain genes in response to the stresses. Therefore, it is possible that continuously over-expressing ATF3 may disturb the normal transcriptional activity in cells and may be detrimental. At present, it is not clear why we failed to generate a stable over-expressing line. To avoid the potential detrimental effect of continuously over-expressing ATF3, I used the vaccinia expression method to transiently over-express ATF3 in HeLa cells.

**Vaccinia virus expressing ATF3**

One advantage of the vaccinia virus expression system is that it gives high levels of protein production. This allows us to follow and purify the recombinant protein easily. The vaccinia virus expression system we chose requires co-infecting the HeLa cells with two recombinant vaccinia viruses (Moss et al.). One recombinant virus, vTF 7-3, expresses T7 RNA polymerase, and the other recombinant virus encodes the gene of interest under the control of T7 promoter and encephalomyocarditis (EMC) virus 5'-end untranslated region. T7 RNA polymerase can recognize the T7 promoter and transcribe the target gene with high efficiency, while EMC 5'-end untranslated region enables the mRNA to be translated efficiently.

To generate the recombinant virus encoding ATF3, I infected HeLa cells with wild type vaccinia virus and then transfected the same HeLa cells with pTM1.His-ATF3 plasmid. pTM1.His-ATF3 contains histidine-tagged ATF3 (his-ATF3) under the control of T7 promoter and EMC 5'-end untranslated region. The T7-EMC-his-ATF3 expression cassette is flanked by segments of the vaccinia virus TK gene which allows homologous recombination between the expression cassette and the TK gene of the wild type vaccinia virus. After homologous recombination, the recombinant virus loses the TK gene and can be selected in 143B cells (also TK minus) by TK minus selective media. Using this procedure, I constructed the recombinant virus, vA3. To express his-
ATF3, I infected suspension HeLa cells with vTF 7-3 and vA3 recombinant viruses for 24 hours, and then purified the nuclear extract from the infected HeLa cells. The nuclear extract contained high levels of his-ATF3 which can be detected easily by immuno-blot analysis.

To examine whether his-ATF3 interacts with any endogenous HeLa cell proteins, my first approach was to purify his-ATF3 by the Ni-NTA column under native conditions. If there were endogenous HeLa cell proteins associated with his-ATF3, they should be co-purified with his-ATF3 by the Ni-NTA column. However, under native conditions, his-ATF3 could not be purified by the Ni-NTA column. The unsuccessful purification of his-ATF3 by the Ni-NTA column was probably because the histidine tag is masked. This notion was supported by the fact that, under denaturing conditions, the same his-ATF3 is able to be purified by the same Ni-NTA column. The masking of the histidine tag could be caused by protein-protein interaction or folding of his-ATF3 polypeptides.

Since native his-ATF3 could not be purified by Ni-NTA column, I then examined his-ATF3 by gel filtration column. If his-ATF3 did not interact with endogenous HeLa cell proteins, it should elute from the gel filtration column in fractions corresponding to its molecular weight (44 kDa for ATF3 homodimer). If his-ATF3 associated with endogenous HeLa cell proteins, it should elute from the column in fractions corresponding to larger molecular weight. When examined by Sephacryl S-200 gel filtration column (Pharmacia, with separation range from 5 to 250 kDa), his-ATF3 eluted from the column in the exclusion fraction indicating that his-ATF3 was present in some type of complex with molecular weight larger than 250 kDa. To improve resolution, I re-examined his-ATF3 containing complex by Sephacryl S-300 gel filtration column (separation range from 10 to 1,500 kDa). As shown in Figure 5.1A, his-ATF3 eluted from the Sephacryl S-300 column in several fractions indicating that his-ATF3 was present in a heterogeneous population of complexes. Based on comparison to molecular weight standards, they ranged from 150 kDa to 500 kDa. In addition, I also analyzed his-ATF3 containing complexes by P11 phosphocellulose column (Whatman). As shown in Figure 5.1B, his-ATF3 eluted from P11 column in different salt concentrations (from 100 mM to 500 mM). This observation further strengthened the notion that ATF3
Figure 5.1 Fractionation of vaccinia virus expressed his-ATF3 by gel filtration and phosphocellulose columns.

(A) Immuno-blot analysis of vaccinia virus expressed his-ATF3 in fractions from a Sephacryl S300 (Pharmacia) gel filtration column. The column was calibrated with molecular weight markers as indicated on the bottom. Size marker in kilodaltons are indicated on the right.

(B) Immuno-blot analysis of vaccinia virus expressed his-ATF3 in fractions from a P11 (Whatman) phosphocellulose column. Size markers in kilodaltons are indicated on the right.
Figure 5.2 Co-immunoprecipitation of vaccinia virus expressed his-ATF3 and viral proteins.

(A) Multiple proteins were co-immunoprecipitated with his-ATF3. HeLa cells were co-infected with vTF7-3 and vA3 to express ATF3. Twenty-four hours after infection, the cells were metabolically labeled with 35S-methionine. ATF3 and associated proteins were immunoprecipitated by anti-ATF3 antibodies and analyzed on a 12% SDS-polyacrylamide gel. The arrow (←) indicates the position of ATF3. Size markers in kilodaltons are indicated on the left. (B) Viral proteins were immunoprecipitated in the presence or absence of his-ATF3. HeLa cells were treated with anisomycin at sub-inhibitory concentration (50 ng/ml) for 2 hour, or infected with vTF7-3 alone or together with vA3 for 24 hours. HeLa cells with different treatments were metabolically labeled with 35S-methionine. Whole cell extracts were subjected to immunoprecipitation by anti-ATF3 (α-ATF3) or anti-gadd153/Chop10 (α-gadd) antibodies as indicated. The immunoprecipitated proteins were analyzed on a 12% SDS-polyacrylamide gel. The arrow (←) indicates the position of ATF3. Size markers in kilodaltons are indicated on the left.
was present in several different multi-protein complexes.

Because the his-ATF3 containing complex eluted from Sephacyr S-300 or p11 columns is in a crude fraction, it is not possible to determine the specific polypeptides that interact with ATF3 by gel analysis. Therefore, I took the cell labeling and co-immunoprecipitation approach to detect the components of his-ATF3 containing complex: I infected HeLa cells with vTF7-3 and vA3 viruses for 24 hours, metabolically labeled the infected cells with $^{35}$S-methionine for two hours, isolated whole cell extract, and immunoprecipitated ATF3 containing complex by anti-ATF3 antibodies under the native condition. As shown in Figure 5.2A, ATF3 interacts with multiple polypeptides. However, further analysis indicated that these polypeptides are not specific. First, as shown in Figure 5.2B, even when the cells were infected with vTF7-3 virus only, these polypeptides were immunoprecipitated by anti-ATF3 antibodies and non-specific antibodies. Second, when the cells were infected with vTF7-3 and vA4 viruses (for the expression of ATF4), these polypeptides were immunoprecipitated by anti-ATF3 and anti-ATF4 antibodies (data not shown). Third, these polypeptides were not found in cells treated with anisomycin, which induces the expression of endogenous ATF3 (Figure 5.2B). Taken together these results indicate that the polypeptides observed in the co-immunoprecipitation experiments are probably viral proteins. The co-immunoprecipitation of viral proteins and his-ATF3 could be resulted from non-specific interaction between viral proteins and his-ATF3 or between viral proteins and anti-ATF3 antibodies. Because of the abundance of the viral proteins, even if some endogenous co-repressors do interact with his-ATF3, it would be difficult to distinguish them from the viral proteins. In addition, because viral proteins are actively translated, majority of the $^{35}$S-methionine would be incorporated into viral proteins instead of endogenous cellular proteins. Therefore, the in vivo complex assembly assay does not appear to be promising. Consequently, I took another biochemical approach, ATF3 affinity column, to further investigate the ATF3 interacting proteins.

II. ATF3-affinity column assay

I constructed an ATF3 affinity column and examined whether endogenous HeLa cell proteins interact with ATF3 and bind the column. To generate the ATF3 affinity
column, I used the GST fusion approach. The GST domain was fused to the N' terminal of the full length ATF3. The resulting construct, GST-ATF3, was expressed at relatively high levels in *E. coli*; however, it was not stable and degraded easily. When purified by glutathione conjugated agarose beads, greater than 50% of the fusion proteins contained only the GST domain; only less than 10% of purified fusion proteins had the expected molecular weight of the full length GST-ATF3 (data not shown). To solve this degradation problem, I constructed another fusion protein, ATF3-GST, by fusing the GST domain to the C' terminal of ATF3. Although the expression level of ATF3-GST was much lower than that of GST-ATF3, it was more stable. When purified by glutathione conjugated agarose beads, the major purified polypeptides had the expected molecular weight of ATF3-GST and was recognized by the anti-ATF3 antibodies in immuno-blot analysis (data not shown). ATF3-GST was then incubated with glutathione conjugated agarose beads to generate ATF3 affinity column. To evaluate the affinity column, I examined binding of gadd153/Chop10 (an ATF3 interacting protein) to the column. As shown in Figure 5.3, 20% of the loaded gadd153/Chop10 was retained by the ATF3-GST column; in contrast, less than 2% of the loaded ATF1 was retained by the ATF3-GST column.

After demonstrating that ATF3-GST column works properly, I examined whether any endogenous HeLa cell proteins can bind to the column as follows: I metabolically labeled HeLa cells with $^{35}$S-methionine, prepared a whole cell extract, passed the $^{35}$S-labeled HeLa cell extract through the ATF3-GST column, and analyzed bound proteins by an SDS-PAGE. As shown in Figure 5.4A, I repeatedly observed two polypeptides, around 80 kDa and 90 kDa, bound specifically to the ATF3-GST column but not to the GST column. I also observed another polypeptide, around 50 kDa, bound to the ATF3-GST column occasionally. However, in a competition experiment, the binding of these polypeptides to the ATF3-GST column can not be competed by excess free ATF3 (without GST domain). In fact, the binding was enhanced in the presence of ATF3 (Figure 5.4B). One possible explanation is that ATF3 dimerizes with ATF3-GST and enhances the binding of these polypeptides to the ATF3-GST column. Because of the unsuccessful attempt of the competition experiment, further analysis is necessary to determine the specificity of the interaction. In addition, even if the interaction with ATF3 is specific, it remains to be determined whether these polypeptides are the putative corepressors.
Figure 5.3 ATF3-GST affinity column functions properly.
(A) ATF3-GST affinity column preferentially interacted with gadd153/Chop10 over ATF1. ATF1 and gadd153/Chop10 (gadd) were produced by reticulocyte lysate in the presence of $^{35}$S-methionine. Twenty μl of $^{35}$S-labeled ATF1 or gadd153/Chop10 was incubated with ATF3-GST column. Purified proteins (Lane 3 and 4) were analyzed on a 12% SDS-polyacrylamide gel. Lane 1 and 2 show 1 μl of unpurified ATF1 or gadd153/Chop10. Size markers in kilodaltons are indicated on the left. (B) Percentage of the ATF3-GST column bound ATF1 and gadd153/Chop10 (gadd).
Figure 5.4 ATF3-GST affinity column bound HeLa cell proteins
(A) Three HeLa cell proteins (50 kDa, 80 kDa, and 90 kDa) interacted specifically to ATF3-GST column but not GST column. HeLa cells were metabolically labeled with 
$^{35}$S-methionine for 2 hours. Whole cell extract was precleaned with GST column, and then incubated with either GST or ATF3-GST columns. Column bound proteins were analyzed on a 10% SDS-polyacrylamide gel. Size markers in kilodaltons are indicated on the left. (B) Excess of free ATF3 but not ATF1 enhanced the binding of "ATF3 interacting proteins" to ATF3-GST column. $^{35}$S-methionine labeled whole cell extract was incubated with GST column or ATF3-GST column in the absence or presence of proteins isolated from E. coli as indicated. Column bound proteins were analyzed on a 10% SDS-polyacrylamide gel. Size markers in kilodaltons are indicated on the left.
Discussion

In vivo assembly assay

Because ATF3 probably associates with the putative co-repressors in the presence or absence of DNA, by isolating the ATF3 containing complex, we may be able to identify the co-repressors. To facilitate the purification of ATF3 containing complex, I took two approaches (stable expression, transient expression) to over-express ATF3 in HeLa cells. Presumably, over-expressed ATF3, together with the co-repressors, may assemble into some type of protein complex. However, neither approaches gave successful results. As described earlier, vaccinia virus expression system not only produced high levels of ATF3, but also a variety of viral proteins. Due to the complication of the non-specific protein-protein interaction, even if some endogenous HeLa cell proteins interact with ATF3, it would be difficult to study them using this expression system. Therefore, using a stable cell line to over-express ATF3 may be a better approach. However, the attempt to make the ATF3 expressing cell line was not successful. I speculate that this is because continuous over-expression of ATF3 is detrimental to the cells. If that is the case, one possible solution is to generate a stable cell line containing ectopic ATF3 under the control of an inducible promoter. The expression of ATF3 can then be regulated to circumvent detrimental effects. Several mammalian expression systems have been development for this purpose. For example, H. Bujard and colleagues developed a "reverse Tet system" which allows the expression of an ectopic gene to be tightly regulated (Gossen et al., 1995): The ectopic gene is driven by the E. coli tetracyclin (tet) operator and an eukaryotic TATA box. A transcriptional activator containing the VP16 activation domain and a mutant tet repressor will bind to the tet operator in the presence of tetracyclin and induce the expression of the ectopic gene. Using this "reverse Tet system", the authors observed greater than 10^3-fold induction of the ectopic gene in HeLa cells. Alternatively, we can investigate whether endogenous ATF3 interacts with other proteins. As described in Chapter I, the expression of ATF3 is induced by a variety of stimuli. It is possible that, upon induction, ATF3 may form a complex with its interacting proteins. However, the induction is transient (only lasts for one or two hours), and there may not be much ATF3 accumulated during this short
period of time. Therefore, to study endogenous ATF3, it is necessary to enlarge the operation scale and identify the most efficient method to induce the expression of ATF3.

**ATF3 affinity column**

Using ATF3 affinity column approach, I repeatedly observed two polypeptides, 80 kDa and 90 kDa, that can interact with ATF3-GST but not with GST alone. Occasionally, I also observed another polypeptide, around 50 kDa. However, when examined in a competition experiment, excess free ATF3 could not compete away the binding of these polypeptides off the ATF3-GST column. Instead, it enhanced the binding. This enhancement was not observed when excess ATF1 was used in the competition experiment. To explain this observation, I hypothesize that ATF3 may dimerize with ATF3-GST and help the binding of those polypeptides. If this is the case, it indicates that these polypeptides prefer to interact with ATF3 homodimer. Therefore, these polypeptides should not be another protein containing the leucine zipper domain that dimerize with ATF3. Further analysis is necessary to determine the specificity of the interaction. For example, we can construct additional affinity columns containing different domains of ATF3 or other ATF proteins and examine whether they can interact with those polypeptides. If the interaction with ATF3 is specific, we may be able to scale up the ATF3-GST column and purify enough of these polypeptides for protein sequencing and identification.

Although these potential ATF3-interacting proteins remain to be characterized, we may be able to test whether they are the co-repressors by using the in vitro transcription assay. For example, we can elute these polypeptides from the ATF3-GST affinity column, and test whether they can further support ATF3 to repress transcription in vitro. Alternatively, using the ATF3-GST column, we can deplete these polypeptides from HeLa nuclear extract and examine whether the depleted HeLa nuclear extract can still support ATF3 to repress transcription in vitro.
Chapter VI

Future plans

To continue the research on the transcriptional function of ATF3, I propose the following directions:

1. To further test the "co-repressor" model

   a. To analyze the "co-repressor interacting domains": Deletion study indicates that two distinct domains of ATF3, amino acids 1-100 and 136-181, interact with the co-repressors. However, the evidence for the interaction is only indirect; it requires further study to substantiate the findings. As an example, one can examine these two "co-repressor interacting domains" by domain swap experiments. Although the full length ATF3 failed to transfer its inhibitory activity to the Gal4 DNA binding, it is possible that, when fused with a heterologous DNA binding domain, the "co-repressor interacting domains" may be able to repress transcription. Alternatively, one can examine the "co-repressor interacting domains" by mutagenesis analysis.

   b. To investigate the ATF3-GST column bound polypeptides: Using ATF3-GST affinity column, I observed three polypeptides (50 kDa, 80 kDa and 90 kDa) that can interact with ATF3. However, further analysis is required to examine the specificity of the interaction. If the interaction is specific, one can purify these polypeptides for protein sequencing. Based on the amino acid sequences, one can then isolate the corresponding cDNAs and test their function by co-transfection or in vitro transcription analyses. As an example, the gadd153/Chop10 promoter would be a good candidate promoter to test whether these polypeptides can support the transcriptional repression by ATF3 in vitro. If these polypeptides are not the putative co-repressors, one can also isolate more cDNA clones
encoding the ATF3 interacting proteins from libraries using yeast two-hybrid approach or protein-protein blot approach.

2. To analyze the post-translational modification of ATF3

Evidence from in vitro transcription assay indicates that post-translational modification may affect the activity of ATF3. At present, many different types of post-translational modification have been identified including phosphorylation, methylation, glycosylation, hydroxylation, isoprenylation, ubiquitination, acetylation, adenylylation, and ADP-ribosylation. Among them, phosphorylation has been shown to be the most frequently found post-translational modification that affects the activities of transcription factors. Previously, we and other laboratories have shown that ATF3 is a phosphoprotein. However, by analyzing the amino acid sequence, there is no consensus kinase phosphorylation site in ATF3. To test whether phosphorylation plays a role in regulating the activity of ATF3, initially, one can examine whether dephosphorylation by phosphatase treatment affects the transcriptional activity of ATF3 in vitro. Alternatively, one can identify the phosphorylated residues by two dimensional gel analysis and examine whether mutating these residues affects the activity of ATF3 by transfection or in vitro transcription analyses. If phosphorylation does not affect the activity of ATF3, one can examine whether other post-translational modification events affect the transcriptional activity of ATF3.
LIST OF REFERENCES


