STUDIES ON GENE EXPRESSION PROFILING IN JB6 CELLS SUSCEPTIBLE AND RESISTANT TO TUMOR PROMOTER INDUCED NEOPLASTIC TRANSFORMATION AND REGULATION OF GENE EXPRESSION AT THE AP-1 DNA BINDING SITE

A Dissertation

by

SHAIJA SAMUEL

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2004

Major Subject: Medical Sciences

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Approved as to style and content by:

Lori R. Bernstein (Chair of Committee)	Julius A. Gordon (Member)
Julian L. Leibowitz (Member)	David O. Peterson (Member)
Robert S. Chapkin (Member)	George E. Davis (Head of Department)

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ABSTRACT

Studies on Gene Expression Profiling in JB6 Cells Susceptible and Resistant to Tumor Promoter Induced Neoplastic Transformation and Regulation of Gene Expression at the AP-1 DNA Binding Site. (August 2004)

Shaija Samuel, B.S., Madras University;

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Chair of Advisory Committee: Dr. Lori R. Bernstein

Gene expression underlies all important biological processes in a cell and mis-regulated gene expression plays a causal or contributory role in several diseases including cancers. Towards identifying molecular determinants that confer susceptibility and resistance to tumor promoter induced neoplastic transformation, we analyzed the gene expression profile differences among tumor promoter TPA treated and untreated mouse epidermal JB6 cells by means of cDNA microarray analyses. The expression patterns for several genes were validated by real time PCR analyses. Seventy-four genes belonging to six functional categories were found to be differentially expressed. Data from this study implicate pathways which mediate cell adhesion, migration and interferon signalling, tumor suppressors, apoptotic proteins and transcription factors and includes twenty-six genes whose involvement has not been previously implicated in cancer.

In a second study we used a DNA affinity chromatography based assay to purify

two proteins that bound specifically to the AP-1 DNA binding site. Analyses of the purified proteins by mass spectrometric sequencing determined the identities of these proteins as nucleolin and Y-box binding protein 1 (YB-1). We tested the hypothesis that these proteins regulate transactivation at the AP-1 site. Overexpression of nucleolin and YB-1, both alone or in combination, repressed AP-1 dependent gene transactivation. To understand the mechanism of transrepression, we analyzed whether nucleolin and/or YB-1 affected the levels and/or disrupted the intracellular localization of the AP-1 protein subunits. Western blot analyses of all the AP-1 subunits revealed that the levels of AP-1 were unaffected. Cell fractionation confirmed that the AP-1 levels were not altered in the nuclear or cytoplasmic compartments. We further tested the hypothesis that nucleolin and YB-1 repressed AP-1 transactivation by competing with AP-1 proteins for the AP-1 site. The results from this experiment were inconclusive and the precise mechanism of repression is currently under investigation.

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TABLE OF CONTENTS

CHAPTER Page

LIST OF FIGURES

FIGURE Page

FIGURE Page

LIST OF TABLES

LIST OF ABBREVIATIONS

AP-1	Activator protein-1
APC	Adenomatous polyposis coli
β-gal	Beta galactosidase
BME	Basal medium Eagles
BM	Basement membrane
cDNA	Complementary DNA
CDP	Cytidyl diphosphate
CDS	Cytidyl diphosphate-diacylglycerol synthase
CMV	Cytomegalovirus
CRABPII	Cellular retinoic acid binding protein II
Ct	Threshold cycle
Cx43	Connexin 43
DBD	DNA binding domain
DMBA	7, 12-dimethyl-benzanthracene
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
Doc1	Deleted in oral cancer-1
DPBS	Dulbeccos phosphate buffered saline
ECM	Extracellular matrix
EGF	Epidermal growth factor

EMEM	Eagle's modified essential medium
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
Ests	Expressed sequence tags
FBS	Fetal bovine serum
FKN	Fractalkine
Fra1	fos related antigen 1
GALV	Gibbon ape leukemia virus
GAP	GTPase activating protein
GAPDH	Glyceraldehyde-3 phosphate dehydrogenase
GF	Growth factor
GMP	Guanosine mono phosphate
GTF	General transcription factor
GTP	Guanosine triphosphate
HA	Hemaglutanin
HAT	Histone acetylase
HDAC	Histone deacetylase
HMG	High mobility group protein
IFI	Interferon inducible protein
IEX	Immediate early response factor X
Kb	Kilobase
KDa	Kilodalton

KLF	Kruppel-related factor
LDL	Low density lipoprotein
Luc	Luciferase
MAPK	Mitogen-activated protein kinase
matrix-GLA	Matrix gamma carboxyglutamate
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MS/MS	Mass spectrometric mass spectrometry
Mut	Mutant
NAPSTER	Nucleotide Affinity Preincubation Specificity TEst of Recognition
NBRE	Nur77 binding response element
NCL	Nucleolin
Oct-1	Octamer-1
P+	Promotion-sensitive
P-	Promotion-resistant
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PKC	Protein kinase C
PM	Plasma membrane
PML	Promyelocytic leukemia

PMSF	Phenylmethylsulfonylfluoride
Prx2	Paired related homeobox gene 2
RA	Retinoic acid
RAR	Retinoic acid receptor
RNA pol II	RNA polymerase II
Rpt-1	Regulatory protein T lymphocyte 1
RXR	Retinoic X receptor
SDS	Sodium Dodecyl sulphate
SLPI	Secretory leucocyte protease inhibitor
TAD	Transactivating domain
TBP	TATA box binding protein
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase
TopoI	Topoisomerase I
TPA	12-*O*-tetradecanoyl phorbol-13-acetate
TRE	TPA response element
UAS	Upstream activating sequence
URS	Upstream repressing sequence
VLDL	Very low density lipoprotein
Wt	Wild-type
WHO	World Health Organization
YB-1	Y-box binding protein-1

CHAPTER I

INTRODUCTION

Overview

The human body is composed of trillions of cells, each programmed to grow, differentiate, divide, function, and die on a defined time scale in an orderly manner. Cancer develops when a cell undergoes mutations in its DNA and loses one or more of these biological restraints. According to the World Health Organization (WHO) statistics, more than 10 million people are diagnosed with cancer every year (WHO, 2004). With 6 million deaths every year, or 12% of total deaths, cancer has become one of the most devastating diseases worldwide (WHO, 2004). The last thirty years have seen an amazing growth in our understanding of cancer, resulting in significant breakthroughs in the prevention, detection and treatment of cancer. However, further research into the key events that mediate the process of malignant transformation is essential to elucidate the molecular basis of this multi-step process in order to improve cancer prevention and cancer treatment strategies.

Gene Expression and Cancer

Cancer encompasses a group of diseases characterized by uncontrolled and abnormal division of cells. Experimental and clinical studies carried out during the past century have established that many cancers develop through a multistage process

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This dissertation follows the style of *Oncogene.*

(Weinstein, 2000). Genetic alterations in oncogenes and/or tumor suppressor genes confer growth advantages to a cell resulting in formation of a benign tumor. Subsequent acquisition of genetic and epigenetic changes leads to progression of the cells to an invasive and metastatic phenotype. Hanahan and Weinberg have highlighted six essential alterations in cell physiology that underlie most cancers. These include: (1) Self-sufficiency in growth signals; (2) Insensitivity to growth inhibitory signals; (3) Evasion of programmed cell death; (4) Limitless replicative potential; (5) Sustained angiogenesis and; (6) Tissue invasion and metastasis (Hanahan and Weinberg, 2000). All of these biological endpoints are governed by the expression of specific sets of genes. As such, gene expression and its regulation play an important role in both physiology and pathology of cells.

Each cell in a eukaryotic organism contains the complete genomic template for the organism. Morphological and functional specificities of cells constituting a particular tissue or organ are determined by tightly controlled expression of a specific subset of genes within those cells. Cellular responses to various extracellular and intracellular cues are mediated through signaling pathways that ultimately result in regulated expression of specific genes. Regulation of gene expression is therefore one of the most important biological processes in the cell. Any disorders in the process of gene expression and subsequent alterations in functions within the cells can result in deviations from programmed cellular responses. A possible outcome is a tumor cell. Understanding mechanisms of gene regulation will not only help us to understand fundamental biological events such as proliferation, differentiation and apoptosis, but

also the causes of various diseases that result from deregulated gene expression, including cancers.

Regulation of Gene Expression

Regulation of eukaryotic gene expression can occur at several levels, including chromatin condensation, DNA methylation, transcriptional initiation, alternative splicing of RNA, mRNA stability, translational controls, several forms of post-translational modification, intracellular trafficking, and protein degradation (Lewin, 2000; Alberts et al., 2002). In the nucleus, genomic DNA is compacted via associations with nuclear proteins to form higher order structures which may restrict the accessibility of genes. Another level of control may be exerted by regulation of mRNA degradation rate (reviewed in Mitchell and Tollervey, 2000 and Guhaniyogi and Brewer, 2001). Increased stability of specific mRNA species leads to increases in the steady state levels of those mRNA transcripts in the cell. One of the most common and key forms of control is regulation at the level of transcription (Carey and Smale, 2000; Lemon and Tjian, 2000). Transcriptional regulation is a complex process, involving recruitment and interactions of several proteins with each other and with the DNA. The process is mediated both common proteins, called general transcription factors that act on all genes, and specific factors called regulatory transcription factors, that act on particular DNA sequences within the promoter of genes, under particular physiological conditions.

Initiation of transcription occurs when components of the basal transcription machinery, including RNA polymerase II (RNA PolII) and general transcription factors are recruited to the transcription initiation site (Orphanides and Reinberg, 2002,

Woychik and Hampsey, 2002). Most eukaryotic promoters for protein-coding genes share three common features: the transcription start site, the TATA box, and sequences bound by transcriptional regulators (see Lee and Young, 2000 for review). The core promoter element consists of the start site and the TATA box, and is sufficient for transcription initiation by the basal transcription machinery. The sequences that are bound by transcriptional regulators include Upstream Activating Sequences (UASs), enhancers, Upstream Repressing Sequences (URSs) and silencers (Lee and Young, 2000). The core promoter which is located approximately -40 base pairs (bp) upstream of the transcription start site often contains a TATA box consensus sequence. The general transcription factor (GTF) TFIID is believed to directly bind to the TATA box through its TATA box binding protein (TBP) subunit, followed by binding of the other GTFs including, TFIIB, TFIIF, TFIIE and TFIIH and RNA PolII (see Figure 1; Wray et al., 2003; Lemon and Tjian, 2000). The UAS and URS are located upstream of the promoter and are bound by activators and repressors respectively. Additional regulatory elements include enhancers and silencers that function in orientation- and position-independent fashion. The UAS elements are binding sites for sequence specific transcription factors and/or co-regulatory proteins that control the rate of gene expression in a tissue specific manner in response to specific stimuli. Regulatory transcription factors bind to the cis element in the promoter region and interact with the transcriptional machinery either directly, or by recruiting co-regulators (Narlikar et al., 2002). Regulatory transcription factors function to communicate with the core promoter machinery to enhance or decrease the rate of transcriptional initiation.

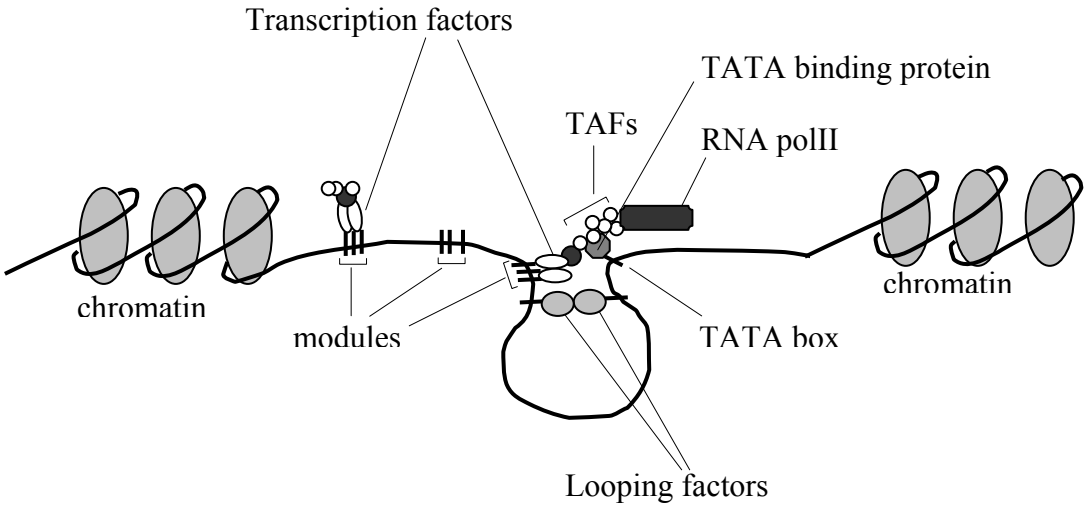

Figure 1 Schematic organization of a typical eukaryotic promoter. Transcriptional initiation at the promoter requires several different proteins that interact with each other in specific ways. These include the RNA polymerase II holoenzyme complex; TATA-binding protein (TBP); TAFs (TBP-associated factors) and regulatory transcription factors and transcription cofactors (Adapted from Wray et al., 2003).

Regulatory transcription factors are generally modular consisting of a DNA binding domain (DBD) and a transactivating domain (TAD; Mitchell and Tjian, 1989). The DNA binding domain dictates the enhancer sequence that the transcription factor can bind to and the transactivation domain determines its transactivation potential. Transcription factors can also interact with other proteins including transcription factors of the same or other families, co-regulators, and the general transcription factors (Narlikar et al., 2002). Co-regulatory proteins add another level to control of gene expression by either enhancing (e.g. co-activators) or repressing (e.g. co-repressors) gene transcription mainly through chromatin remodeling. The co-regulators may have either histone deacetylase activity (HDAC) associated with repression of transcription or histone acetylase activity (HAT) associated with pro-transcriptional role (McKinsey et al, 2001).

Research Outline and Objectives

Novel insights into the key events that mediate the process of malignant transformation can be obtained by studying gene expression and its regulation. Research presented in this dissertation addresses these two aspects of gene expression in the context of carcinogenesis. Given that the gene expression profile of a cell determines its phenotype, function and response to environmental stimuli, measuring the levels gene expression can provide insights into functions, regulatory mechanisms and biochemical pathways that underlie the cells phenotype. With this in mind, in the first part of this study we compared the global gene expression profiles of two cell lines which are either susceptible or resistant to tumor promoter induced neoplastic transformation, using

cDNA microarray analyses. As a complementary approach to understanding gene expression, we studied regulation of gene expression at a specific promoter element implicated in carcinogenesis, i.e. the AP-1 DNA binding site. The research is presented in the form of two studies in Chapters II and III. The following section briefly outlines the hypothesis and specific aims of each of the studies.

In Chapter II, TPA induced differential gene expression in tumor promotion sensitive and resistant JB6 cells were compared using cDNA microarray analyses. The following hypothesis provided the basis upon which the research presented in this part of the study was based. *We hypothesize that both intrinsic and TPA induced differential gene expression between the tumor promotion sensitive and resistant JB6 cells are determinants of susceptibility and resistance to tumor promoter induced neoplastic transformation.* This hypothesis was tested by pursuing the following specific aims:

1. Identify genes differentially expressed in untreated and TPA treated JB6 P+ and P- cells
2. Identify groups of genes sharing biological functions and expression patterns
3. Use expression patterns to define TPA modulated phenotypes of P+ and P- cells
4. Define biological pathways underlying susceptibility and resistance of JB6 cells.

In Chapter III, two novel transactivational repressors at the AP-1 DNA binding site were identified. The following hypothesis provided the basis upon which the

research presented in this part of the study was based. *We hypothesize that gene expression at the AP-1 DNA binding site is regulated by proteins that bind specifically to the AP-1 site.* This hypothesis was tested by pursuing the following specific aims:

1. Identify specific AP-1 DNA binding proteins.
2. Characterize the role of these proteins in AP-1 dependent gene expression.
3. Understand the mechanism of transcriptional regulation mediated by these proteins at the AP-1 site.

CHAPTER II

GENE EXPRESSION PROFILING IN JB6 CELLS SUSCEPTIBLE AND RESISTANT TO TUMOR PROMOTER INDUCED NEOPLASTIC TRANSFORMATION

Synopsis

Towards understanding the molecular mechanisms responsible for tumor promoter induced transformation, and identifying the molecular determinants that confer cancer susceptibility and resistance, we analyzed comparative gene expression profiles of tumor promoter TPA treated and untreated promotion susceptible (P+) and resistant (P-) mouse epidermal JB6 cells by means of cDNA microarray analyses. Seventy-four of the 9,500 gene elements in the array, belonging to six different functional categories, were differentially expressed as follows. (I) *ECM and basement membrane proteins (20 genes)*. While P+ expressed higher levels of fibrillar and basement membrane collagens and of proteases, and lower levels of protease inhibitors, P- cells preferentially expressed several genes encoding cell-cell, cell-ECM and cell-basement membrane adhesion molecules, and protease inhibitors. (II) *Cytoskeletal proteins (13 genes).* These include nine isoforms of actin and actin-associated proteins that are more highly expressed in P- cells. (III) *Enzymes and signal transduction proteins (12 genes).* Among these are several tumor suppressors

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Sections and figures in this chapter are reprinted with permission from *Adhesion, migration, transcriptional, interferon-inducible, and other signaling molecules newly implicated in cancer susceptibility and resistance of JB6 cells by cDNA microarray analyses*. Samuel S and Bernstein LR., Molecular Carcinogenesis, 39(1):34-60.

and apoptotic mediators including Ras GTPase activating protein (Ras-GAP), deleted in oral cancer-1 (Doc1) and connexin 43 (Cx43), all preferentially expressed in P- cells. (IV) *Interferon inducible proteins (3 sequences).* These include interferon inducible protein (IFI16), a transcriptional regulator expressed preferentially in P- cells. (V) *Transcription factors and DNA binding proteins (4 genes).* These include retinoic acid regulated proteins Nur77 and cellular retinoic acid-binding protein II (CRABPII), and paired related homeobox gene 2 (Prx2), all preferentially expressed in P- cells and RIN ZF preferentially expressed in P+ cells. (VI) *Sequences of unknown function*. Real time-PCR validated the expression patterns for 91% of genes analyzed. Phalloidin staining revealed that P- cells have more cell-cell contacts both in the presence and absence of TPA compared to P+ cells and wound healing assays revealed that TPA treated P- cells have impaired ability to migrate into wounds compared to P+ cells. Phalloidin staining also revealed that TPA treated P+ cells have more lamellipodia and filopodia than P- cells. Taken together these results support the hypothesis that gene expression patterns favoring an adhesive and tumor suppressive phenotype are implemented in P- cells, and those favoring motility and tumor promotion are implemented in P+ cells. Twenty five genes and one expressed sequence tag (est) are implicated for the first time in neoplastic transformation. Further investigations into the functions of the genes identified in this study should improve our understanding of the events that mediate neoplastic transformation.

Introduction

Cancer research over the past thirty years has revealed that carcinogenesis is a complex process, requiring cells to overcome numerous barriers that ensure their proper

functioning in the context of their locations in the human body. The diversity of cell types and the accumulation of a combination of multiple genetic and epigenetic events contribute to the wide heterogeneity of human cancers. However, despite the morphological and molecular heterogeneity between different types of cancers, there exist a few common features. The structures and/or expression of some functional classes of genes are invariably altered when a normal cell becomes transformed. These include receptors, enzymes, and/or genes involved in cell cycle, adhesion, motility, apoptosis and angiogenesis. Interactions between a cell and its environment play a major role in cell growth, proliferation, differentiation and death. The cellular microenvironment can influence gene expression, and adverse changes in the cellular microenvironment could trigger transformation processes.

The Extracellular Matrix

The extracellular matrix (ECM) is an important component of the extracellular environment and is typically composed of secreted proteins and polysaccharides collectively referred to as ECM proteins. These include the collagens, fibronectins, laminins, and proteoglycans that assemble into fibrils or other complex macromolecular arrays (Cooper, 2000). Cells attach either directly to components of the collagen-rich interstitial matrix or to the basement membrane, a more distinct sheath of the ECM that surrounds many kinds of tissues. The ECM can regulate cellular behavior by at least three mechanisms, including composition of the ECM, synergistic interactions between growth factors and matrix molecules and through cell surface receptors that mediate adhesion to ECM components (Adams and Watt, 1993). Contrary to general notions, the composition of

ECM is diverse and changing. ECM molecules not only interact with growth factors and serve as a store of growth factors by limiting their diffusion but can themselves serve as growth factors. ECM molecules act as important ligands for transmembrane receptors and therefore provide important outside-in signals (Bosman and Stamenkovic, 2003). They also play an important role in cell adhesion through receptor mediated interactions.

Role of Cell Adhesion

The cell adhesion receptors are usually transmembrane glycoproteins that mediate binding interactions at the extracellular (EC) surface with either other cell adhesion receptors on neighboring cells or with proteins of the ECM. They include members of the integrin, cadherin, immunoglobulin, selectin, and proteoglycan superfamilies (Cooper, 2000). At the intracellular surface of the plasma membrane, cell adhesion receptors associate with cytoplasmic plaque or peripheral membrane proteins and with cytoskeletal proteins (Critchley, 2000). Cooperation between adhesive systems and the actin cytoskeleton results in the formation of adhesive contacts between cells or between cells and the ECM. A common type of adhesive contact that cells make with the ECM is the focal adhesion contact (Yamada and Geiger, 1997; Sastry and Burridge, 2000). Normal cells require cell-cell and cell-ECM-mediated adhesion to survive; if detached, they undergo programmed cell death (apoptosis) in a process known as anoikis. By contrast, transformed cells exhibit two prime features of tumorigenicity, serum-independence and anchorage-independent growth. This ability to escape anoikis is a cardinal feature which allows transformed cells to detach from the primary site and metastasize. Cancer cells commonly show decreased intercellular adhesiveness when compared to their normal counterparts.

The acquisition of motile properties also correlates with a loss of their ability to recognize and adhere to their neighbors. Cell migration entails the coordination of a cycle of cytoskeletal-mediated extension of filopodia and lamellipodia, the formation of adhesive contacts at the leading edge of the cell, the breaking of adhesive contacts, and cytoskeletal-dependent retraction at the trailing edge of the migrating cell (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996). The cytoskeleton of a cell is composed of three types of cytoplasmic protein polymers including microtubules, intermediate filaments and actin filaments (Cooper, 2000). Actin filaments made up of actin monomers, are arranged as networks beneath plasma membrane called cell cortex, as tight bundles in filopodia, as criss-crossing networks in lamellipodia, as antiparallel structures in contractile rings and, as densely packed bundles in stress fibers (Welch and Mullins, 2002). Actin filaments not only play a critical role cell-cell adhesion, but are also involved in several dynamic cellular processes such as changes in cell shape, cell spreading, motility, cytokinesis and polarity (Pollard et al., 2000). Cells with decreased cell-cell contacts and interactions are more refractive to growth inhibitory signals.

Matrix Metalloproteinases and Their Inhibitors

Invading cancer cells overcome physical barriers such as the interstitial stroma and basement membrane by regulating the expression of adhesion molecules, actin cytoskeletal rearrangements and by increased expression or activity of degradative enzymes such as matrix metalloproteinases (MMPs) and cysteine proteases (cathepsins). Several studies have demonstrated that MMPs are upregulated in invasion and metastasis in a variety of cancers. MMPs are a family of zinc-dependent endopeptidases that can degrade ECM

components as well as cleave cell surface molecules to mediate tumor progression, invasion and metastasis (Kähäri and Saarialho-Kere, 1997). The MMP superfamily consists of collagenase, stromelysins, gelatinases, transmembrane MMPs and other MMPs (Coussens and Werb, 1996; Shapiro, 1998; Matrisian, 1999, Westermarck and Kahari, 1999). Increased expression of MMPs has been observed in lung carcinomas (Bolon et al., 1995), squamous cell carcinomas of the head and neck (Johansson et al., 1997), bronchial tumor (Galateau-Salle et al., 2000), and colorectal tumors (Murray et al., 1996). Furthermore, mouse models deficient in specific MMPs exhibit decreases in growth, angiogenesis and invasion in response to various carcinogens (McCawley and Matrisian, 2000). Studies are now elucidating specific mechanisms by which the expression and activity of individual MMPs contribute dynamically to tumor invasion and metastasis (Leeman et al., 2003). Evidence is also emerging for new significant biological roles for MMPs in tumor development and progression, including roles in apoptosis, cell proliferation, and cell differentiation (McCawley and Matrisian, 2000).

Studies in both human and animal models of colorectal cancer have demonstrated an important role for MMPs in the early stages of cancer development. MMP-7 (matrilysin) mRNA and activity have been shown to be present in colorectal adenomas (Yamamoto et al., 1994). In contrast to its absence in most normal tissues, matrilysin has been detected in a high percentage of pre-invasive lesions, and is expressed by the epithelial-derived tumor cells. Manipulating the level of this enzyme in vitro results in cell lines with enhanced tumorigenic potential, correspondingly ablating the gene in vivo leads to a significant reduction in tumor number in two

different animal models of intestinal tumorigenesis (Fingleton et al., 1999). Additionally, regulation of matrilysin gene expression appears to be under the control of genetic pathways which are activated very early in the tumor development sequence. The precise mechanism by which matrilysin activity contributes to early stages of tumor formation is not yet very clear.

Maintaining Homeostasis

Cells have several inherent strategic defense mechanisms to maintain homeostasis. One such mechanism is by secretion of tissue inhibitors of metalloproteinases (TIMPs), which bind to the highly conserved zinc binding site of active MMPs and specifically inhibit the activity of MMPs. The TIMP gene family consists of four structurally related members, TIMP-1, -2, -3, and -4. TIMPs-1, -2, and -4 are secreted in soluble form whereas TIMP-3 is associated with ECM (Gomez et al., 1997). Increased expression of TIMPs by either host or tumor cells results in reduced invasion and metastatic capacity of transformed cells (Kruger et al., 1998; Ahonen, et al., 1998).

Another protective mechanism is apoptosis or programmed cell death. Apoptosis is a genetically regulated, morphologically distinct form of cell death that can be initiated by many different physiological and pathological stimuli. Such intracellular programs are initiated in many instances during the normal life cycle and development of a multicellular organism in order to maintain homeostasis and to eliminate unwanted cells. Dysregulation of apoptosis has emerged as an important mechanism in carcinogenesis (Kukhta et al., 2003).

Multi-stage Origin of Cancers

Several types of human cancers are now believed to have a multi-stage origin (see Hanahan and Weinberg, 2000 for review) with each step conferring increased neoplastic characteristic to the cell. Multi-stage carcinogenesis generally involves both genetic and epigenetic events in the cell. While the genetic events, including structural alteration of critical gene(s) are mostly irreversible and occur rapidly, the epigenetic events are partially reversible, and occur more slowly over the course of several years. The genetic events are mediated by exposure to a sub-threshold dose of carcinogen and are not rate-limiting steps in tumor development. The epigenetic events, however, generally require repeated applications of a noncarcinogenic tumor promoting agent and are known to be the rate-limiting steps (see Digiovanni, 1992 for review) in tumor development. The molecular basis of such genetic and epigenetic events in tumor development has been the subject of extensive investigations.

Colorectal cancer is one of the best studied examples of the multi-stage nature of tumorigenesis in humans. It has been demonstrated that the development of colorectal carcinoma involves at least seven sequential alterations, including mutation of the adenomatous polyposis coli (*APC*) tumor-suppressor gene, mutation of Ki-*ras* and loss of function of *p53* gene (Kinzler and Vogelstein, 1996). Studies into the role and function of APC in colon carcinoma and melanoma development have led to the identification of another novel pathway for the tumor onset (Compagni and Christofori, 2000). While direct studies of human cancers such as these have greatly increased our understanding of tumor development, the difficulty in tumor tissue procurement and

diversity in genetic background of the subjects make it difficult to study tumorigenesis in humans. Additionally, the procurement and analyses of human cancer samples are further complicated by ethical and legal constraints. By contrast, animal systems such as the mouse offer several distinct advantages and have proved to be a valuable model systems for the study of multistep tumorigenesis (Wu and Pandolfi, 2001), although mice are often not predictive of effectiveness of cancer treatment modalities.

Mouse Skin Carcinogenesis and the JB6 Model

Mouse skin carcinogenesis constitutes one of the best studied models of multistage tumorigenesis. Skin tumors can be induced in mice by the sequential application of a carcinogen, followed by a non-carcinogenic promoter. Chemically induced mouse skin tumorigenesis proceeds through three stages of development: initiation, promotion and progression (Yuspa, 1998). In initiation, topical application of a single subcarcinogenic dose of a carcinogen, such as 7, 12-dimethyl-benzanthracene (DMBA) leads to initiation of tumor development resulting from irreversible DNA damage. The promotion is achieved by repeated application of skin promoters, most commonly the phorbol esters, such as 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA). Tumor promoters induce biochemical changes that promote the selective clonal expansion of initiated cells and leads to the formation of multiple squamous papillomas. The progression stage is generally a spontaneous process characterized by a high level of genetic instability (reviewed in Digiovanni, 1992).

Studies on the mouse skin carcinogenesis models have provided numerous leads into the potential tumorigenic roles of genes that have later been supported by other in-

vivo studies. Agonistic roles for cyclin D1 in the Ha-Ras pathway in tumorigenesis, p53 in the tumor-progression phase, c-*fos* gene in malignant conversion and epidermal growth factor (EGF) in SOS-dependent skin tumor development, as well as antagonistic role for promyelocytic leukemia (*PML*) tumor-suppressor gene the initiation phase of skin tumorigenesis and a dual role for TGF-β in tumorigenesis, have all been identified using mouse skin carcinogenesis models (see Wu and Pandolfi, 2001 for review). Recent progress in understanding the mechanism by which TPA induces tumor promotion comes from both *in vivo* studies using mouse skin model (Drinkwater et al., 1989; Digiovanni, 1989; Gould et al., 1989 and Malkinson, 1989), as well as from *in vitro* studies using the mouse epidermal JB6 system (Colburn et al., 1979; Colburn et al., 1980; Gindhart et al., 1985; Smith et al., 1986; Nakamura et al., 1988 and Bernstein and Colburn, 1989).

The JB6 model consists of distinct clonal genetic variants that were originally derived from untreated primary BALB/c mouse epidermal cells, that spontaneously gave rise to immortalized cells, that later stably acquired susceptibility (P+) or resistance (P-) to TPA induced anchorage independent colony formation and tumorigenicity in nude mice (Colburn et al., 1979; see Figure 2). The P+ cells undergo neoplastic transformation in response to TPA treatment while the P- cells do not. But, because these cells exhibit similar mitogenic responses to TPA (Colburn et al., 1981), they provide a means to specifically identify transformation-relevant differences in gene expression by excluding those related to cell proliferation. The key role of transcription factor AP-1 in the promotion of neoplastic transformation was first identified in the JB6 model (Bernstein and Colburn, 1989) and was later found to be applicable to mouse and human

keratinocyte progression models (Dong et al., 1997; Li, et al., 1998), and in mouse skin *in vivo* (Young et al., 1999). JB6 cells have also been used to identify several other key genes, expression events, and signaling pathways of major significance in cancer (described in detail in a later section).

Tumor Promoter TPA and Signal Transduction

TPA is one of the most potent known tumor promoters (Slaga et al., 1980). The effects of tumor promoters are pleiotropic, ranging from changes in cellular morphology, to alterations in cell cycle and apoptosis (Cmarik et al., 1999). Although tumor promoters such as phorbol esters and growth factors do not cause genetic changes in the DNA, they exert their influence by causing transient changes in gene expression through signal transduction pathways (Gindhart et al., 1985; Bernstein et al., 1991; Angel and DiGiovanni, 1999; Cmarik et al., 1999). A subset of these induced gene expression changes is thought to be relevant to tumorigenesis. Intracellular TPA signal transduction is initiated by its binding to protein kinase C (PKC; Ron and Kazanietz, 1999).

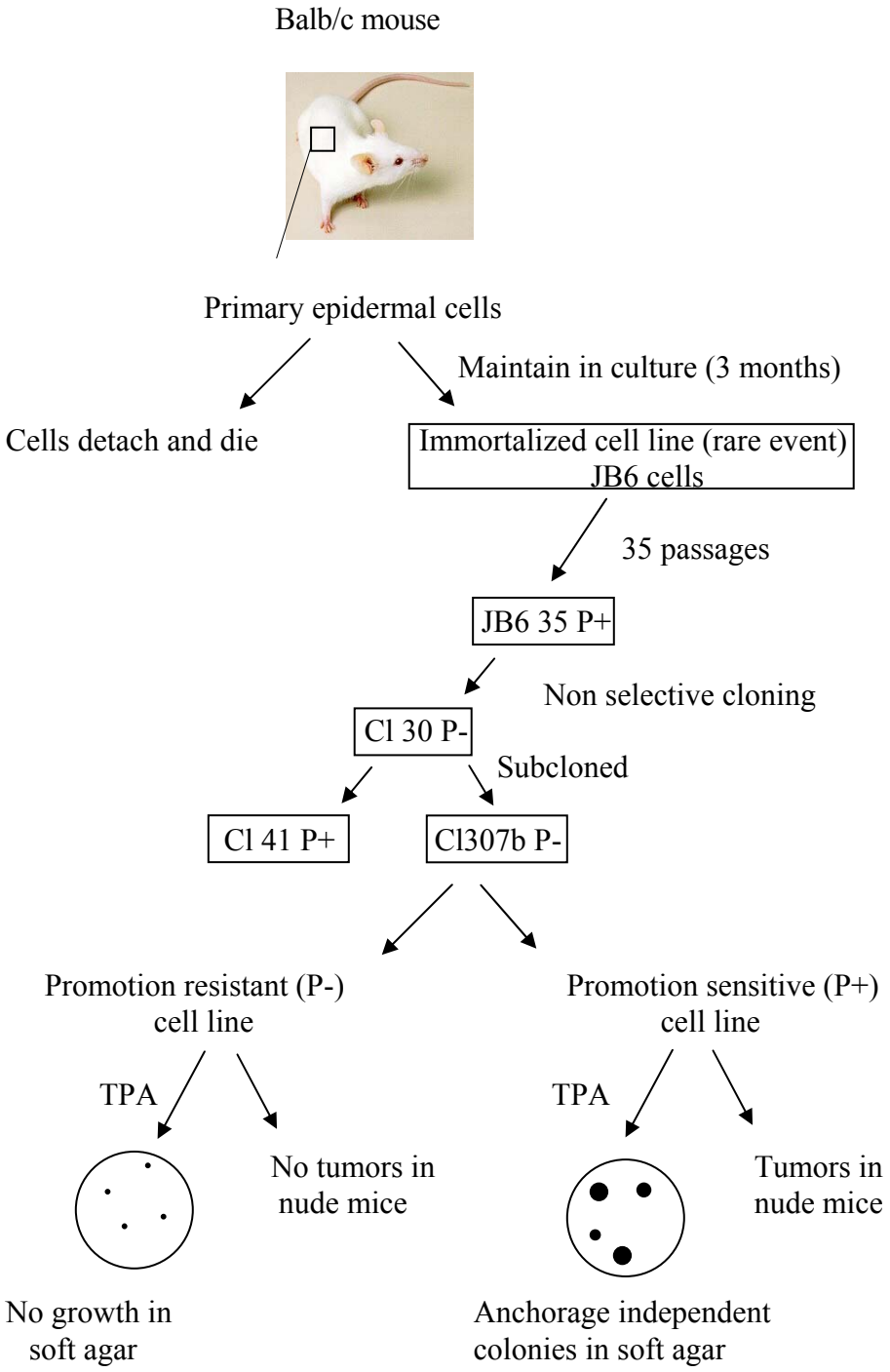


Figure 2 Derivation of JB6 Cl307b promotion susceptible (P+) and promotion resistant (P-) cells.

PKC is a family of serine/threonine kinases which are involved in intracellular signaling (Nishizuka, 1995). PKC serves as an intracellular receptor for TPA and its kinase activity is activated by binding to TPA (Ashendel, 1985; Castagna et al., 1982). Short term treatment of cells with TPA results in the translocation of PKC from the cytoplasm to the membrane where it interacts with phospholipid and becomes activated (Huang, 1989). Long term TPA treatment causes down regulation of PKC via the proteolysis of its hinge region (Fournier and Murray, 1987). Both activation and down regulation of the different PKC isoforms could play a role in tumor promotion process (Droms and Malkinson, 1991). The PKC family consists of at least 12 different isoforms that are classified into three groups; the conventional (cPKCs) that are activated by calcium and diacylglycerol (DAG), the novel (nPKCs) that are calcium-independent, but DAG-dependent and the atypical isoform (aPKCs) that are both calcium- and DAG-independent. These isoforms differ in terms of their cell type and subcellular distribution and the targets of their action. Only cPKCs and the nPKCs, but not aPKCs, can be activated by TPA (Mellor and Parker, 1998). Several events and molecules in the signaling cascade downstream of PKC have been identified. Activation of PKC results in rapid activation of extracellular signal-regulated kinases (ERKs, Hoshi et al., 1989, and Schonwasser et al., 1998). Functional ERK2 MAP kinase is required for TPA promotion of transformation, and for activity of activator protein-1 (AP-1) in P+ cells (Watts, 1998). However, induction of AP-1 by TPA occurs in PKC-depleted mouse suggesting the existence of additional TPA receptors (Kennard et al., 1995).

TPA treatment results in the activation of several genes including genes belonging to the AP-1 family that function downstream of the mitogen activated protein

kinase (MAPK) signaling cascade. The AP-1 family consists of members from the *jun* and *fos* oncogene families. AP-1 transcription factors exist as heterodimeric complexes containing one product each from the *jun* and *fos* families or homodimers containing two members of the *jun* family. AP-1 transcription factors transcriptionally activate genes that contain the AP-1 binding site or TPA-responsive element (TRE) in their promoter. Another transcription factor also downstream of MAPK that has been shown to play a role in TPA tumor promotion is NF-kB (Li et al., 1998). Inhibition of AP-1 and NF-kB activity in JB6 cells blocks the transformation response (Li et al., 1997). While a number of AP-1 and NF-kB regulated genes are known, only a few have been implicated in TPA induced transformation (Cmarik et al., 1999) and it is likely that genes downstream of other transcriptional regulators may also be involved.

Molecular Responses of JB6 Cells to TPA

The molecular responses contributing to differential TPA-induced transformation capacity of the JB6 P+ and P- variants have been studied extensively. Bernstein and Colburn demonstrated that P+ cells but not P- cells display tumor promoter TPA inducible AP-1 transactivation (Bernstein and Colburn, 1989). TPA also induced c-*jun* expression preferentially in P+ cells (Ben-Ari et al., 1992). Functional AP-1 activity is required for neoplastic transformation by TPA since expression of dominant negative c-*jun* in P+ cells inhibits TPA induced AP-1 transactivation and neoplastic transformation (Dong et al., 1994). Transgenic mice with transactivation mutant c-jun further demonstrated that AP-1 is required for tumor promotion (Young et al., 1999). Differential display of untreated P+, P- and transformed (Tx) cell mRNAs identified seven differentially expressed sequences

including the tissue inhibitor of the metalloproteinases (TIMP-3) gene, whose transcription undergoes progressive decline as a function of progression toward a neoplastic endpoint (Sun et al., 1994). Another study using differential display comparison of JB6 P+ and P- led to the identification of a gene product that blocks susceptibility to TPA tumor promotion. The cDNA, identical to the murine Pdcd4 gene, was found to be preferentially expressed in P- cells (Cmarik et al., 1999). Differential display comparison of P+ and P- cells treated with TPA also led to the cloning of the mouse pleckstrin gene which is induced in P- cells (Cmarik et al., 2000). Taken together, these differential display and other studies with JB6 cells suggest that the differential expression of a number of genes in P+ and P- cells, both constitutively and in response to TPA, may contribute to the transformation susceptibility and resistance of the JB6 variants.

cDNA Microarrays – A Tool for Gene Expression Analyses

The advent of microarray technology makes it possible to assess and compare global gene expression changes by simultaneously monitoring the relative expression levels of a large number of genes in a quantitative manner. It therefore allows for rapid and precise identification of critical genes that could potentially serve as diagnostic and prognostic markers in pathological states. Additionally, discovery of patterns of gene expression are informative about regulatory pathways involved. The challenge in a microarray experiment is 'making sense' from the overwhelming amount of data obtained and to understand, on a global level, the biology behind the differential expression of hundreds of genes.

Complementary DNA (cDNA) microarrays compare expression of genes in two

samples, by measuring the concentration of the cDNAs corresponding to the mRNA transcripts in two samples. A cDNA microarray consists of a glass or nylon slide containing a set of spots, each of which corresponds to a cDNA sequence from the genome, referred to as a probe. cDNAs are generated from the two samples by reverse transcription of the isolated messenger RNAs (mRNAs), fluorescently-labeled with two different dyes, and co-hybridized to the microarray chip. The fluorescently-labeled cDNAs from each sample competitively hybridize to the corresponding probe. The concentration of cDNA in each sample is then measured on the basis of the fluorescence level for the spot of the corresponding probe.

cDNA microarray technology has significantly contributed to the identification and characterization of global gene expression profiles in several types of cancers as substantiated by the existing literature in this field. A survey of recent literature on the use of cDNA microarray in gene expression profiling reveals its adaptability and applicability in a wide range of cancer types. Briefly, cDNA microarray based gene expression profiling have been performed to study of human medulloblastomas (Packer, 2003), T-cell leukemia/lymphoma (Tsukasaki et al., 2004), prostate cancer cells (Chesire et al., 2004), cervical cancer (Ahn et al., 2004) and papillary thyroid carcinoma (Yano et al., 2004), and many others.

A central challenge in the application of the available gene expression data to molecular diagnosis and treatment of cancer, is to define a set of molecular features that, taken together, distinguish a given cancer, or type of cancer, from all normal cells and tissues. Microarray analyses of a broad variety of adenocarcinomas have led to the

identification of 61 genes whose expression levels predict the site of origin of the primary tumor (Dennis et al., 2002). In gastric cancer of the intestinal subtype, 124 genes were commonly up or down regulated, and profiles of 12 of these were associated with lymph node metastasis (Hasegawa et al., 2002). In breast cancer a gene expression signature based on 70 genes was highly predictive of recurrence and survival in both node-negative and node-positive patients (van de Vijver et al., 2002). Gene expression profiling of esophageal adenocarcinomas has led to identification of characteristics that can be used to distinguish between Barrett's esophagus and esophageal adenocarcinoma (Xu et al., 2002; Barret et al., 2002). Molecular classification of cancers based on gene expression data have also been used in distinguishing myeloid from lymphoid cancer and subclasses within these two diseases (Golub et al., 1999; Armstrong et al., 2002; Schoch et al., 2002; Debernardi et al., 2003). These and several other such studies underscore the efficacy of cDNA microarray analyses in identification of global gene expression changes that contribute to a given cellular phenotype.

One of the biggest contributions of microarray technology in cancer biology, however, will be its application as a diagnostic tool for cancer patient profiling for prediction of progression of the disease. Several new clinical trials using DNA microarray chips are currently underway including those in breast cancer at the Netherlands Cancer Institute, Massachusetts General Hospital, M. D. Anderson Cancer Center and Baylor College of Medicine and, in multiple myeloma at the Dana-Farber Cancer Institute (Branca, 2003). The first patient profiling tests to diagnose the chance of developing metastases based on gene expression profiling were launched by a Dutch

company ‘Agendia’, in early march of this year (Garber, 2004). Larry Norton, an oncologist from the Memorial Sloan-Kettering Cancer Center, is said to have aptly described DNA array technology as, "This is the Wright Brothers' airplane. ... It's just the beginning of the era of molecular characterization of cancer" (Quoted from Branca, 2003).

Research Outline

In this study, we used cDNA microarray analysis to identify constitutive and TPA induced global differences in the gene expression profile between the P+ and P- cells that contribute to their susceptibility and resistance to TPA induced neoplastic transformation. Validation of the array data was accomplished through real-time PCR analysis of candidate genes. We then classified all the genes according to their biological functions as described in the biomedical literature in an attempt to link the expression pattern of gene classes to the phenotype of the two cells. Candidate cancer susceptibility and resistance relevant genes were grouped into six functional classes. Valuable clues emerged as to the processes that might underlie the coordinate expression of these genes contributing to the migratory phenotype of the TPA treated P+ cells and the adhesive phenotype of the TPA treated P- cells. A number of genes within the functional groups showed coordinated up- or down-regulation, which suggests the existence of distinct molecular pathways that are possibly deregulated during the process of neoplastic transformation.

Preliminary Note

In this gene expression study the endpoint being measured is mRNA. The term 'gene' has been used to refer to the identity of a gene or its expression product that is represented by a cDNA clone on the array. Identities of clones on the array were those provided by the company, Incyte Genomics. The identities of some of the clone which lacked known identities were later determined by us by aligning the clone sequence to the NCBI nucleotide database. For still many unknown genes and sequences, their identity is subject to change as the public database becomes updated. Furthermore, the current gene names are also subject to changes as the databases are continually updated. The genes that are newly implicated in neoplastic transformation this study have been identified by doing extensive literature searches using the names of the genes. It is possible that one or more of these genes have been identified previously in microarray studies and exist in other array databases.

Materials and Methods

Cell Culture and Derivation of P+ Cl307b Cell

The Cl307b, tumor promotion resistant (P-) cell line was purchased from ATCC (Manassas, VA). Cells were grown and passaged as described (Bernstein and Walker, 1999). The cells were routinely monitored for their tumor-promotion resistant phenotypes by analyzing the number of TPA induced colonies in soft agar assays. After 91 passages, the Cl307b P- cells spontaneously gave rise to cells that displayed a P+ phenotype in soft agar assay. The change in phenotype was confirmed through soft agar assays performed in duplicate and repeated three times. Frozen Cl307b P- cells (passage 85) which are close in

passage number to the now Cl307b P+ cells were revived and their phenotype was confirmed by soft agar assay. The two Cl307b P+ and P- cell lines were thus within six passages of one another and formed a matched pair of cell lines of highly similar genetic background.

Soft Agar Transformation Assay

This assay was carried out according to established protocol (Colburn et al., 1981). Briefly, bottom and top agar layers were prepared with agar mixture containing fetal bovine serum (FBS), dulbeccos phosphate buffered saline (DPBS), basal medium eagle's (BME) and Bacto agar (1.25%; Difco, Detroit, MI). The bottom layer consisted of 7 ml of 0.5% agar medium with DMSO or 10 ng/ml of TPA prepared in 60 mm petridishes. Cells at 10^4 cells per dish were suspended in the 1.5 ml of 0.33% top agar medium containing DMSO or TPA and layered on the prepared layer of bottom agar. The dishes were incubated at 37°C and the colonies were counted after 14 days.

Treatment of Cells and RNA Isolation

Cl307b P- and P+ cells were grown in T150 flasks to 80% of plateau density and treated with 10ng/ml TPA or with DMSO solvent control for 8hrs in fresh EMEM with 4% FBS as described (Bernstein and Walker, 1999). Total RNA was extracted from the treated cells by phenol guanidine isothiocyanate method using TRIZOL reagent (GibcoBRL Life technologies, Gaithersburg, MD). High quality RNA is essential to the success of a microarray experiment. The quality of the RNA samples was assessed by electrophoresis through denaturing agarose gels and staining with ethidium bromide. Intact bands corresponding to 18S and 28S ribosomal RNA constituents were visualized under UV light

in an Alpha Multi-imager light cabinet (Alpha Innotech Corporation, San Leandro, CA). Poly (A) $^{+}$ RNA was purified from total RNA using an Oligotex mRNA isolation kit (Qiagen Inc, Valencia, CA) and quantitated using the Ribogreen RNA quantitation kit (Molecular Probes, Eugene, Oregon) according to manufacturer's instructions.

Microarray Set-Up

800ng/sample of poly $(A)^{+}$ RNA from TPA treated and untreated P+ and P- cells were dispatched at 50ng/µl concentration in TE buffer to Incyte Genomics (Palo Alto, CA). cDNAs were generated using reverse transcription. The experimental set-up consisted of three sets of comparative hybridizations: (I) P- TPA vs. P+ TPA; (II) P- TPA vs. P- DMSO and (III) P+ TPA vs. P+ DMSO. Each pair of cDNA was respectively labeled with Cy3 (green) and Cy5 (red) flourophores, then co-hybridized for competitive binding to the mouse GEM^{TM} 2 cDNA microarray (Incyte Genomics, Palo alto, CA). The spots on the arrays corresponding to each of the hybridization sets were scanned independently for the two fluorescent colors and the ratios of the two fluorescence intensities provided a quantitative measurement of the relative gene expression level in the two samples. To offset differences in the fading properties of the Cy3 and Cy5 dyes, the poly $(A)^{+}$ RNA quality in terms of rRNA contamination and minor quantity differences between the samples, the fluorescent intensity corresponding to Cy5 (P2; Probe 2) was expressed as the balanced P2 signal which is a product of the P2 signal times balanced coefficient derived from the average Cy3 and Cy5 fluorescence intensities for each array. The relative gene expression level is thus expressed as balanced differential, which can be written as P1 signal (Cy3) / P2 balanced signal (Cy5).

Primer Design and Reverse Transcription

Specific primers for real time PCR analyses were designed corresponding to accession numbers obtained for genes from the microarray results using the Primer Express software (Perkin-Elmer Applied Biosystems, Foster City, CA). BLASTn searches were performed against the nonredundant set of genbank database sequences to confirm the likelihood of specific amplification of the chosen mRNA species without concomitant amplification of other gene products. Primers were custom synthesized by Macromolecular Resources (Colorado State University, Fort Collins, CO) and Proligo USA Corp (La Jolla, CA). Primer sequences are shown in Table 1. Amplification of the mRNA of interest was further verified by agarose gel electrophoresis of the PCR product. 100ng of poly (A)+ RNA/sample was reverse transcribed in a final volume of 100 µl containing 1X reverse transcriptase buffer (500mM each dNTP, 3mM $MgCl_2$, 75mM KCl and 50mM Tris-HCl, pH 8.3), 25units of RNase inhibitor (Promega, Madison, WI), 10mM DTT, 10units/µl of Superscript II RNAse H- reverse transcriptase (Life Technologies Inc., Gaithersburg, MD) and 5µM oligo d(T) primer. Samples were incubated at 42°C for 50 min and reverse transcriptase was inactivated by heating at 90°C for 5 min and cooling at 4°C for 5 min. The samples were then stored at -20°C.

Table 1 Sequences of primers used in real time PCR analysis.

GENE	5' - FORWARD - 3'	5' - REVERSE - 3'
HMG I-C	TTACCGCCCATCTCCAGAGT	TGTTGGTGCCCGTTTGC
ZYXIN	AGATGACAACGGCGGTTT	GCTCTAGCGGAGTGGCACTT
PROCOLLAGEN TYPE V	AAATGTCTTATGATAACAACCCCTACA	TTCTGTTAGCCTTTCTTGGTAGCA
PROCOLLAGEN TYPE I	CCTCCACCCCAATCTGGTT	TGGGTTGTTCGTCTGTTTCCA
NUR77	TCCTGGCAGACCTTCAACAGT	CTGCAGTGGCCTTTCCAGAT
TENASCIN C	CTATCGCAACTGGAAGGCCTAT	TTGCTCAGGTTATCCAGTCCAA
PG-M/ VERSICAN	AACTGCTTTCCTGATTGGCATT	CAGAGATCAGGTCCTGGTAGGTAAA
GAPDH	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGA
LYSYL OXIDASE	TTTTAATGAGTGAGCCACAATTTGA	GAGGCAAATATAGGCTCATCATCA
SLPI	CCTCGGGACTGGTCATCAGA	GAGCACCGTGAAAGGTAAAAGG
CATHEPSIN H	TCCCATTCCTCAGGTATAAGCC	CAGTTCCTCCCTTGGTCTGC
CATHEPSIN L	AGCCAAAGACCGGGACAAC	CCCATCAATTCACGACAGGAT
TIMP 1	TCATGGAAAGCCTCTGTGGAT	CGGCCCGTGATGAGAAACT
MATRIX GLA	GCTCCCTCTGGCCATCCT	TCCATGCTTTCGTGAGATTCG
DECORIN	TGGAAAGGCTTTACCTGTCTAAGAA	GACACGAAGTTCCTGGAGAGTTC
WDR1	ACATTGCCTGGACGGAAGAC	GCTCCAAACTTCTCCCTTCCTT
CALPONIN 2	CTTTCCCACGACTCTCAGACTTC	ACATCTGGCTTGCCTCAGTTC
P203/P205 (AI036073)	TGTGTGGAGACCACAGTTTCGT	TTGTGCTTGACAGGACAGTTGA
IFI – 16	TGGGTTGCAGCTGAAGTCTGT	TTGAGTGGCTTTCCTTCACCTT
PROCOLLAGEN TYPE IV	AGCTGGGAAGTTGCCTGTGT	GTTAGCCTCCTTCATCCTGCAT
CONNEXIN 43	GGCCTGATGACCTGGAGATTT	ACGTTCTGCAAGCACCCTTT
SLIT2	TCATTTGTGGACGAGGTTGAGA	CGCGCTTAGGAGGCACAT
CRABP II	TGGAGAGCTGATCCTGACAATG	AGGCACTCACTCTCGGACGTA
RAS GAP	TCAGTTCAGCATAAGTGGCCTACA	CAGGATAGCAGGGCAGATAGG
PRX2/S8	GGCCAAAGAGTTCAGCCTACA	AGCTGGACCCAATGCACAGT

Real-time PCR Analysis

All Real-time PCR reactions were performed using an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster city, CA). PCR was performed using SYBR Green PCR Core Reagent Kit (Perkins-Elmer Applied Biosystems, Foster City, CA) according to manufacturer's instructions. Each reaction (50µl) contained 25 µl of 2X Master SYBR Green I mix (containing Taq DNA polymerase, dNTP, $MgCl_2$ and SYBR Green I dye), 0.3 µmol/L primers and 2.5 µl of cDNA from the reverse transcription reaction detailed above. The thermal cycling conditions comprised an initial denaturing step at 95°C for 10min and amplification program of 50 cycles at 95°C for 15s and 65°C for 1 min. The end point used in the real-time PCR quantitation, threshold cycle (C_t), is defined as the PCR cycle number at which the fluorescence signal generated passes a fixed threshold over baseline. Experiments were performed in duplicates or triplicates for each gene analyzed.

Wound Healing Assay

P+ Cl307b and P- Cl307b cells were plated into 6 well tissue culture dishes (Nunc, Inc. Napersville, IL) at 1.5 x 10^4 cells per well in 4% fetal bovine serum (FBS) in Eagle's minimal essential medium (EMEM) on day 1 of the experiment. Media was then changed to 4% FBS in EMEM with 10ng/ml TPA or DMSO solvent control. Within 15 minutes of media change, cultures were wounded by scratching the monolayer of cells with a P1000 eppendorf pipet tip, and media was again changed to fresh EMEM with or without TPA. Cells were allowed to migrate into the wound for up to 48 hours. Wounds were fixed in 2% (w/v) paraformaldehyde in PBS, stained with 1% crystal

violet and photographed within the course the recovery period with an Olympus SC 32 type 12 camera in an Olympus CK2 microscope at 4X magnification.

Phalloidin Staining

P+ and P- Cl307b cells at 90% confluence growing in 60mm Nunc tissue culture dishes (Nunc Inc., Napersville, IL) were treated with TPA (10ng/ml) or DMSO solvent control for 8 hours duration then stained with phalloidin as described (Stephens and Banting, 2000) and photographed with a Nikon FX-35A camera in a Nikon Labophot fluorescent microscope (Nikon, Tokyo, Japan) at 10X, 20X and 40X magnifications.

Results

To identify candidate genes that mediate transformation susceptibility and resistance and early events in TPA tumor promoter-induced transformation, we analyzed the comparative gene expression profiles of tumor promoter TPA treated and untreated P+ and P- JB6 cells by comparative cDNA microarray analyses. To minimize the likelihood of gene expression differences attributable to non-specific genetic differences, the cell lines used in this comparative gene expression profiling were a closely matched pair. The P+ cells arose spontaneously from the P- cell line and were within six passages of the transition. Transformation assays for anchorage independent growth demonstrated that the P+ cells formed significantly more number of colonies in response to TPA treatment than the P- cells. The mean number of colonies per 10,000 cells was 739 for P+ cells and 82 for P- cells. The average colony size for the P+ cells was also significantly larger compared to that for P- cells as shown in (Shown in Figure 3).

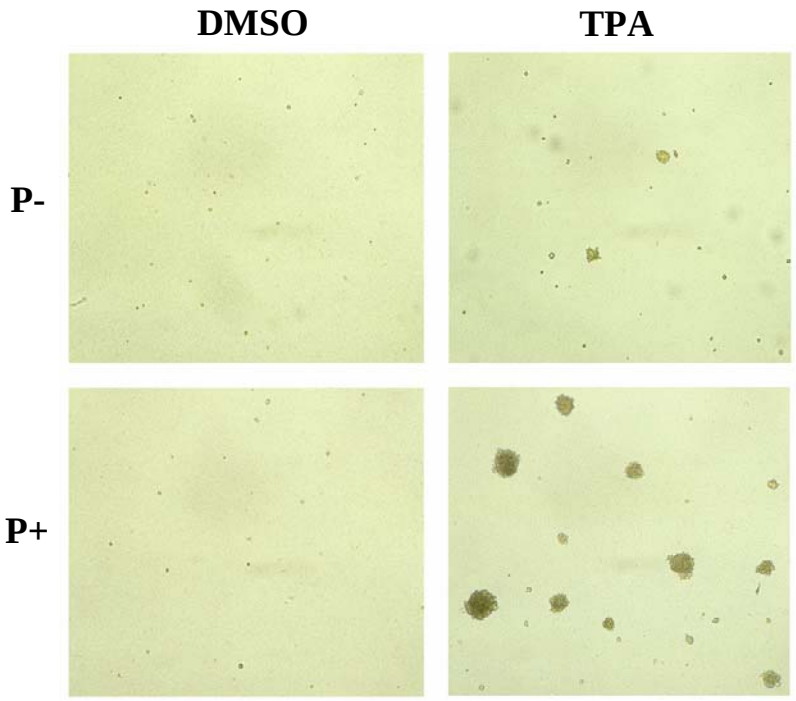


Figure 3 TPA treated P+ cells form significantly larger and greater number of anchorage independent colonies than P- cells. Cl307b P- and P+ JB6 cells at 10^4 cells per dish were suspended in the 1.5 ml of 0.33% top agar medium containing DMSO (solvent control) or TPA (10 ng/ml) and layered on the prepared layer of bottom agar. The dishes were incubated at 37˚C and the colonies were counted after 14 days and photographed with an Olympus microscope at 20X magnification.

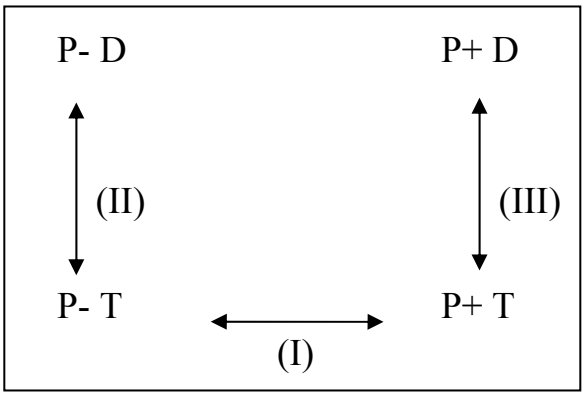


Figure 4 Comparative cDNA microarray strategy. The experimental setup consisted of three comparative arrays: (I) TPA treated P- cells and TPA treated P+ cells; (II) TPA and DMSO treated P- cells and (III) TPA and DMSO treated P+ cells.

To specifically identify both endogenous and TPA induced differences in the gene expression profiles between the two cell lines, three comparative microarray hybridizations were performed. To identify genes whose expression levels are modulated by TPA in the resistant (P-) cells, Cy3 and Cy5 fluorescent labeled probes prepared from P- cDNA treated with TPA or DMSO were co-hybridized for competitive binding to the GEM^{TM} 2 cDNA microarray. This constituted the first "leg" of the experimental setup (Figure 4). The second leg, designed to identify genes whose expression levels are modulated in response to TPA in the susceptible (P+) cells, consisted of Cy3 and Cy5 labeled cDNA probes prepared from P+ cells treated with TPA or DMSO. The third leg was designed to identify those TPA modulated genes whose expression levels are differential between the P- and the P+ cells and consisted of co-hybridization of Cy3 and Cy5 labeled probes generated from TPA treated P- and P+ cDNAs.

In the initial analyses, balanced differential expression data corresponding to every element (i.e., genes and ests) on the array was evaluated. The raw fluorescence hybridization data illustrating differential expression of several prototypical genes are shown in Figure 5. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) serves as a control showing little or no significant differential expression in any of the three legs of the experiment. The detectable levels of differential expression in the Incyte array are differential expression ratios greater than or equal to 1.7. Therefore 1.7-fold differential expression was chosen as the minimum differential and genes exhibiting fold changes of less than 1.7-fold were excluded from further analyses.

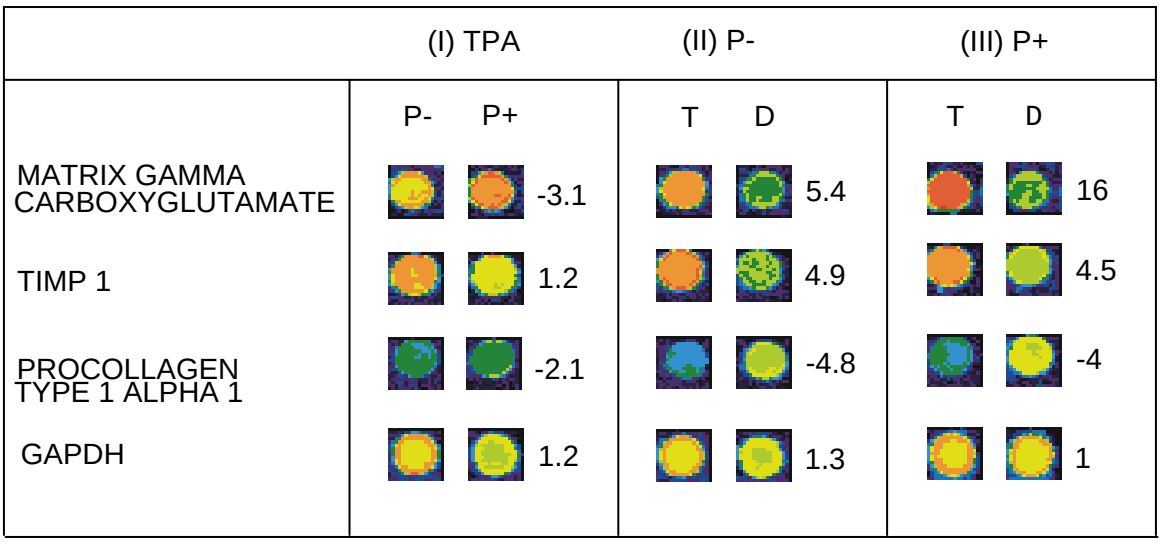


Figure 5 Microarray scan data for select prototypical genes showing Cy3 and Cy5 fluorescence intensities. The spots on the arrays for each leg of the experiment (I, II and III) were scanned independently for the red and green fluorescent colors and the ratios of the two fluorescent intensities provided a quantitative measurement of the relative gene expression level in the two samples. Yellow spots represent a range of combinations of the red and green contributions.

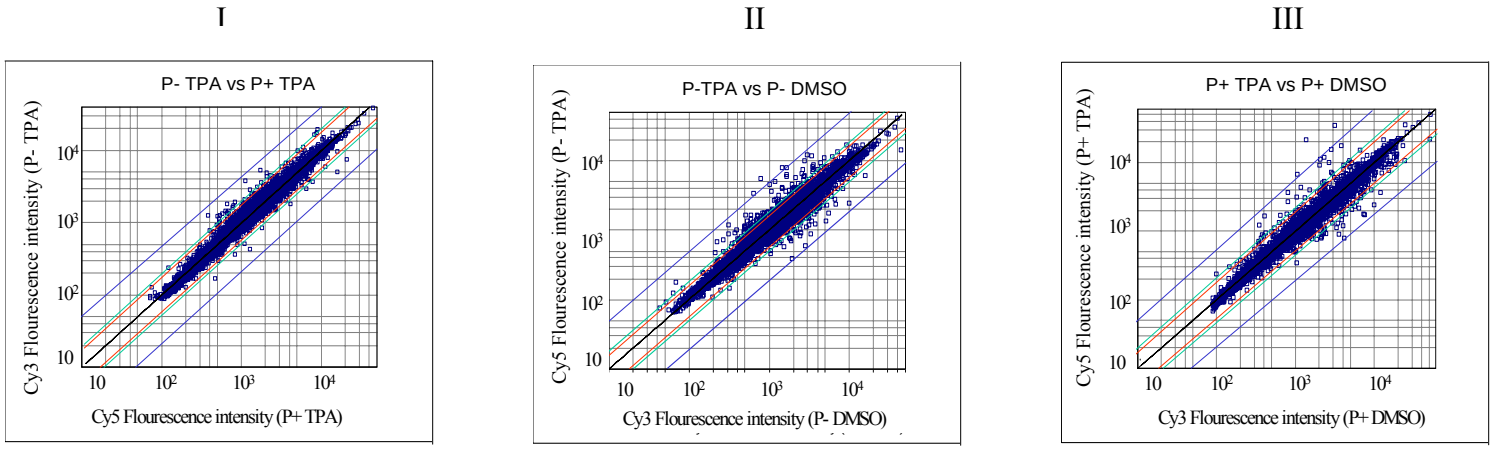


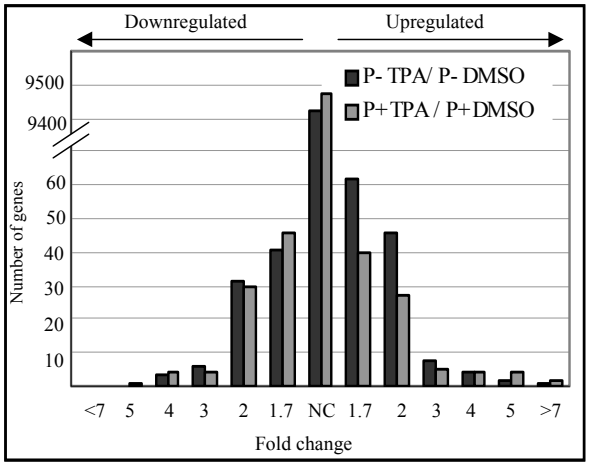
Figure 6 TPA up regulates more genes in P- cells. Scatterplots showing the modulation of gene expression in P- and P+ cells following TPA treatment. cDNA was generated from P+ and P- cells treated with TPA or DMSO and labeled with Cy3 and Cy5 fluorescent dyes for microarray hybridization. Samples were hybridized to the Mouse GEM^{TM} 2 array (Incyte Genomics, Palo Alto, CA) as described in Materials and Methods. Data were analyzed using the SPSS software (SPSS Inc. Chicago, IL) and plotted as balanced differential revealing up- and down- regulated expression of genetic elements on the array. The x and y-axes represent Cy3 and Cy5 fluorescence intensity values in log scale. Each small blue square in the plot represents one gene element on the array. Data points above diagonal black lines were for upregulated genes; below diagonal black lines were for downregulated genes. All spots between the red diagonal lines exhibit 1.7-fold or lower differential expression ratios; those between the red and green diagonal lines have between 1.7-fold to 2-fold; those between the green and dark blue diagonal lines have between 2-fold and 5-fold ratios; those outside the dark blue lines exceed 5-fold ratios. (I, II and III) Ratios of the distribution of fluorescence intensities between cDNAs prepared from (I) TPA treated P- cells and TPA treated P+ cells; (II) TPA and DMSO treated P- cells; (III) TPA and DMSO treated P+ cells.

TPA Induces More Genes in P- than P+ cells by Comparative cDNA Microarray

The relative fluorescence intensities of Cy3 and Cy5 for the 9500 elements are represented as scatterplots in Figure 6 showing the global gene expression profile in the three microarray hybridizations. As shown in the Figure 6, majority of the 9500 represented elements are not differentially expressed as a function of TPA treatment or phenotype (compare I and II, and III). However, the spread of the data points in the last two scatterplots demonstrate that there are more TPA modulated genes in the P- cells than the P+ cells. These data are further illustrated by Figure 7 which shows histograms comparing the distribution of fold change in differential expression ratios for all the elements in the arrays. TPA treatment resulted in the modulation of expression of 204 genes in P- cells and 168 genes in the P+ cells. As shown in Figure 7B, 123 genetic elements are upregulated in P- cells while only 82 are upregulated in the P+ cells. The number of genetic elements down regulated by TPA is almost equal in the P- and P+ cells (81 and 86 respectively). Seventy-four of the 9500 genes (0.78%) were differentially expressed between the two lines following TPA treatment, 52 genes were induced by TPA in P- cells and 22 genes were induced the P+ cells.

The differential expression ratios of all the genes and expressed sequence tags (ests) that show more than 1.7-fold TPA modulated differential expression in P- and P+ cells (First leg of the hybridizations) are summarized in Table 2. Both constitutive and TPA-induced differential expressions are considered to be significant, since either differential can have impact upon promotion susceptibility or resistance. Since comparison of expression levels in TPA treated P+ vs. TPA treated P- cells will yield a composite of the constitutive

A



B

Upregulated by TPA			Down regulated by TPA		
	P-	**P+**		**P-**	**P+**
1.7 – 2 fold	62	40	1.7 – 2 fold	41	47
2 – 5 fold	58	36	2 – 5 fold	40	38
> 5 fold	3	6	> 5 fold	0	1
Total	123	82	**Total**	81	86

C

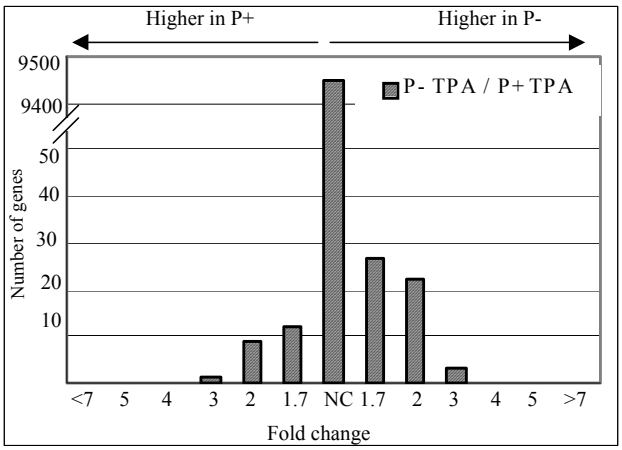


Figure 7 Distribution of fold change in gene expression: TPA modulates more genes in P- cells than P+ cells. A. Distribution of TPA induced fold change in legs II and III of the array. Each category includes all genes that have at least (and up to the next higher value) the fold change indicated on the x-axis. 'NC', no change. B. Number of genes up-regulated and down-regulated by TPA in P+ and P- cells. C. Distribution of fold change in leg I of the array.

Table 2 Genes up- or down-regulated in P+ and P- cells in comparative cDNA microarray.

GENES/ESTs	Accession Number	Leg 1[a] P-T/P+T	Leg 2[a] P-T/P-D	Leg 3[a] P+D/P+T
P- > P+				
PG-M/Versican	AA755868	3.4	-1	1.3
Fractalkine/neurotactin	W16003	3.4	3.3	-1.9
Recombining binding protein suppressor of hairless	AI390138	3.1	3.5	-1.7
Complement factor H-related protein	AA570965	2.8	-1.4	1.3
Interferon inducible p203/p205	AI036073	2.4	1.3	1.2
Neuritin	AA237727	2.4	2.2	-1.5
Similar to microsomal dipeptidase precursor	AA444538	2.4	2.3	-1.3
Decorin	AA755007	2.3	-1.2	-1.3
SLIT 2	AA691931	2.3	-1.7	-1.2
LOC328494 gene	AA856323	2.3	2.5	-1.6
Interferon inducible family	AA472976	2.2	1.4	1.1
Interferon inducible IFI-16	AA238257	2.2	1.5	-1.1
Secretory leucocyte protease inhibitor (SLPI)	AI020539	2.2	1.9	-1
WDR1	AA624634	2.2	1.8	1
Nur77/N10	AA209882	2.1	2.5	-1.2
Zyxin	AA617310	2.1	-1.4	2.7
HSP 25	W34262	2.1	1.8	-1.1
VLDL receptor	AA020307	2.1	1.4	2.5
Calponin 2	W34124	2	-1.4	1.9
Skeletal muscle alpha actin	AA770902	2	1.5	1.7
Vascular smooth muscle alpha actin	AA624460	2	1.4	2.3
Semaphorin 3a/D/collapsin 1	AI894009	2	-1.1	1.3
UDP-glucoronosyltransferase 1	AA822117	2	1.1	-1
Ras GAP	AA432613	2	3.4	-3
Phosphoserine phosphatase	AA066981	2	1.3	1.8
Tenascin C	W12942	1.9	4.3	-1.8
Smooth muscle enteric gamma actin	AA756136	1.9	1.5	1.8
Transgelin 2 / SM22 beta	AI643202	1.9	2.2	1
Connexin 43	AA738914	1.9	1.3	-1.4
Procollagen, type VI, alpha 1	W16221	1.9	-1.4	2
Nidogen / entactin	AA606605	1.9	1.7	-1.7
Cellular retinoic acid binding protein II (CRABPII)	AA051397	1.9	-1.1	1.2
Regulatory protein, T lymphocyte 1	AI614435	1.9	-1.8	2
Cardiac alpha actin	AI325745	1.8	1.5	1.6
Deleted in oral cancer 1 (Doc1)	AA638778	1.8	1.6	-1.3
Voltage dependent calcium channel, alpha2/delta	AA734015	1.8	1.1	-1.3
Variable group of 2-cell-stage gene family	AA415841	1.8	1.2	1.2
Monocyte to macrophage differentiation-associated gene	AA072083	1.8	1.3	-1.3
cGMP-dependent protein kinase type II	AA771678	1.7	4.5	-3.8
Destrin	AI390104	1.7	1.5	1.3
Immediate early response 3 / IEX 1	AA833402	1.7	2.3	-1.5

Table 2 continued

GENES/ESTs	Accession Number	Leg 1[a] P-T/P+T	Leg 2[a] P-T/P-D	Leg 3[a] P+D/P+T
Selenoprotein P	AA066225	1.7	-1.7	1.1
Ninjurin 1	AI036381	1.7	1.3	1.2
Thymic epithelial cell surface antigen	AA619756	1.7	1.4	1.1
Bone morphogenetic protein 5 (BMP5)	AA242542	1.7	2.4	-1.7
Kinesin family member 21A	AI390275	1.7	1.3	-1.1
Prx2/ S8	W97877	1.7	-1.2	1.3
Neprilysin/CD10/NEP	AA450725	1.7	-2.6	1.5
RIKEN clone 1600012F09 gene	AA789636	1.7	1.5	-1
Est	AA413015	1.7	1.1	1.2
Tissue inhibitor of metalloproteinase 1 (TIMP1)	AA184223	1.2	4.9	-4.5
Cathepsin L	AA619763	1	3.9	-3.7
P+ > P-				
Matrixgamma carboxyglutamate (matrix GLA)	W88093	-3.1	5.4	-16
Cathepsin H	W17422	-2.9	1	-1.7
RIKEN clone 5730559C18	AA689893	-2.8	0.45	-2.1
CDP-diacylglycerol synthase	AA067625	-2.8	4	-11
Troponin T2, cardiac	AA671284	-2.2	1.2	-2.3
Procollagen TypeI, alpha 2	AA798297	-2.2	-4	3.3
RIKEN clone 4932442K08	W62706	-2.2	-1.6	-1.3
Procollagen Type I, alpha 1	AI425767	-2.1	-4.8	4
Lysyl Oxidase	W83882	-2.1	1.1	2.7
Calcyclin (S100A6)	W09198	-2.1	-1.2	-1.3
RIKEN cDNA 2810418N01 gene	AA543968	-1.9	-1.4	-1.4
Clone B930019K04	AA760161	-1.8	1.9	-2.3
Clone RP23-10B20	AA575501	-1.8	-1.1	-1
RIN ZF zinc finger DNA binding protein	AA619834	-1.7	1	1
Procollagen, type IV, alpha 1	AA760135	-1.7	2	-2.3
60S ribosomal protein L38	AA718476	-1.7	-1.3	-1.5
Calreticulin	AA562976	-1.7	-1.4	-1.3
Homolog of 60S RIBOSOMAL PROTEIN L39	AA600661	-1.7	-1.4	-1.5
RIKEN cDNA 9130005N14 gene	AA718842	-1.7	-1.5	-1
RIKEN clone 5330401006	W80245	-1.7	-1.9	-1.1
LOC 209096 gene	AA220093	-1.7	-1.3	-1.3
Procollagen TypeV, alpha 1	AA792297	-1.1	-1.9	2.4
High mobility group protein I-C	AA097062	-1.2	2.7	-5.5

[a] The balanced differential expression data from the three microarray sets were analyzed (Columns 3-5). The table shows the ratios of mRNA expression for genes in the three legs of the array experiment. Column 1, gene names; column 2, accession numbers; column 3, leg I of the array (P- TPA/P+ TPA); column 4, leg II of the array (P-TPA/P-DMSO); column 5, leg III of the array (P+TPA/P+ DMSO).

and induced contributions to the final level, we focused on these genes for further analysis. A total of 74 genes and ests are differentially expressed in TPA treated P- vs. P+ cells (Leg I). Two genes showing high differential expression ratio in the P- TPA / P- DMSO hybridization have also been included in this list.

Real-time PCR Validates Differential Expression Ratios of 25 Candidate Tumor Promotion-Relevant Genes

Real-time PCR was carried out to validate the changes in gene expression observed by microarray using an independent method. Real-time PCR analysis was repeated at least twice for each gene analyzed. The first real-time PCR was performed using RNA samples identical to those used in the microarray experiment. The duplicate and triplicate real-time PCR experiments were performed with new samples of RNA prepared from the same P- and P+ cells. The values obtained from the different experiments were averaged and the mean values were then compared to the values obtained from the cDNA microarray analyses. Even though there was a slight variability in the magnitude of the response between the two methods for several genes, the patterns of response were essentially in concordance. The real-time PCR results for 91% (68 of the 75 profiles; see Figure 8) of the 25 genes analyzed correlated well with the various legs of the cDNA microarray data. Genes exhibiting discordance include; tissue inhibitor of metalloproteinase (TIMP1) and cathepsin H that showed significant differential expression by real time PCR but not in the array, and secretory leukocyte protease inhibitor (SLPI) that showed significant induction in the array but not in real time PCR.

While the three legs of the microarray experiment did not directly provide

A

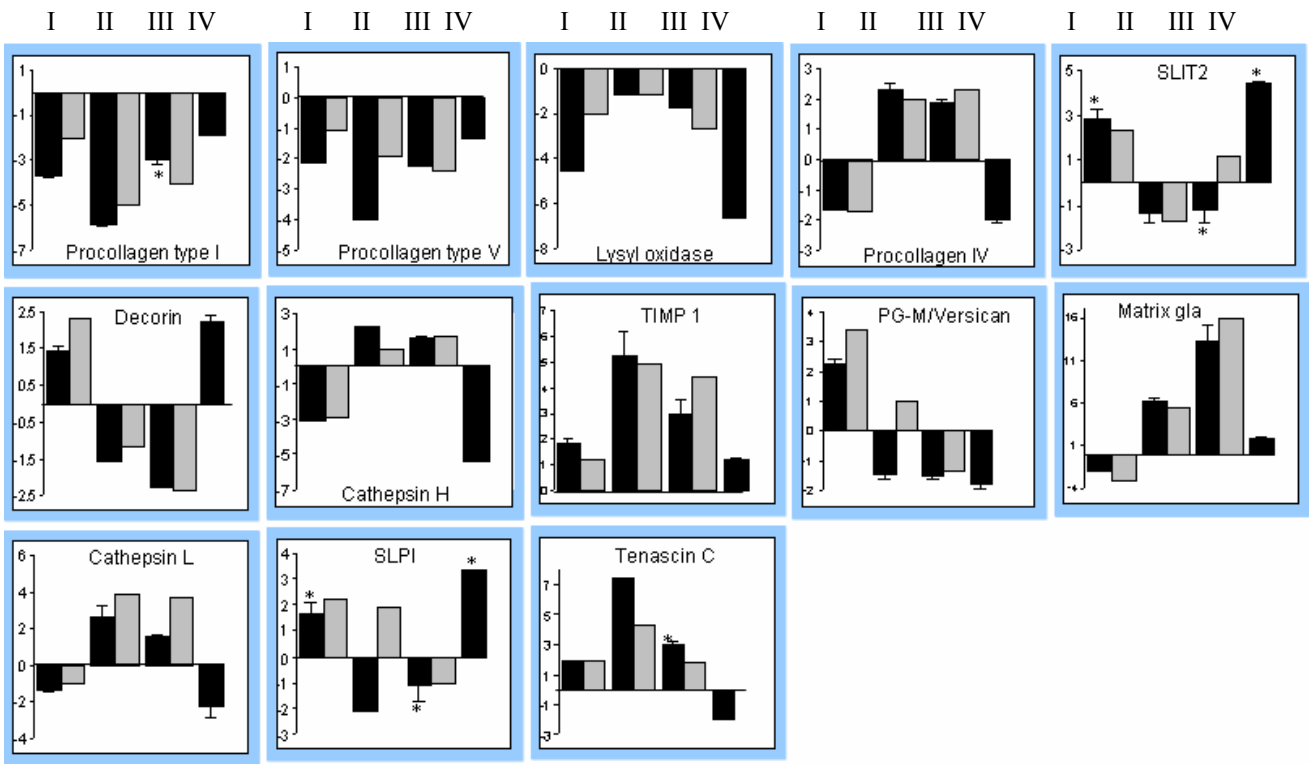


Figure 8 Real-time PCR analyses validate relative mRNA expression data obtained from cDNA microarray. Roman numbers on top panel represent one leg of the experiment. I, P- TPA vs. P+ TPA; II, P- TPA vs. P- DMSO; III, P+ TPA vs. P+ DMSO; IV, P- DMSO vs. P+ DMSO. A, Blue border, Class I genes (ECM and basement membrane proteins); B, Black, Class II (Cytoskeletal proteins); C, Pink, Class III (Enzymes and signal transduction proteins); D, Grey, Class IV (Interferon inducible proteins); E, Green, Class V (Transcription factors and DNA binding protein). Standard deviation for duplicates are indicated with asterisks; standard error of mean for triplicate have error bar and no asterisks. Black bar graphs, real time PCR; Grey bar graphs, microarray data. Black horizontal reference line in each graph indicates no change in expression Values below black line, down regulation. Values above black line, Up regulation.

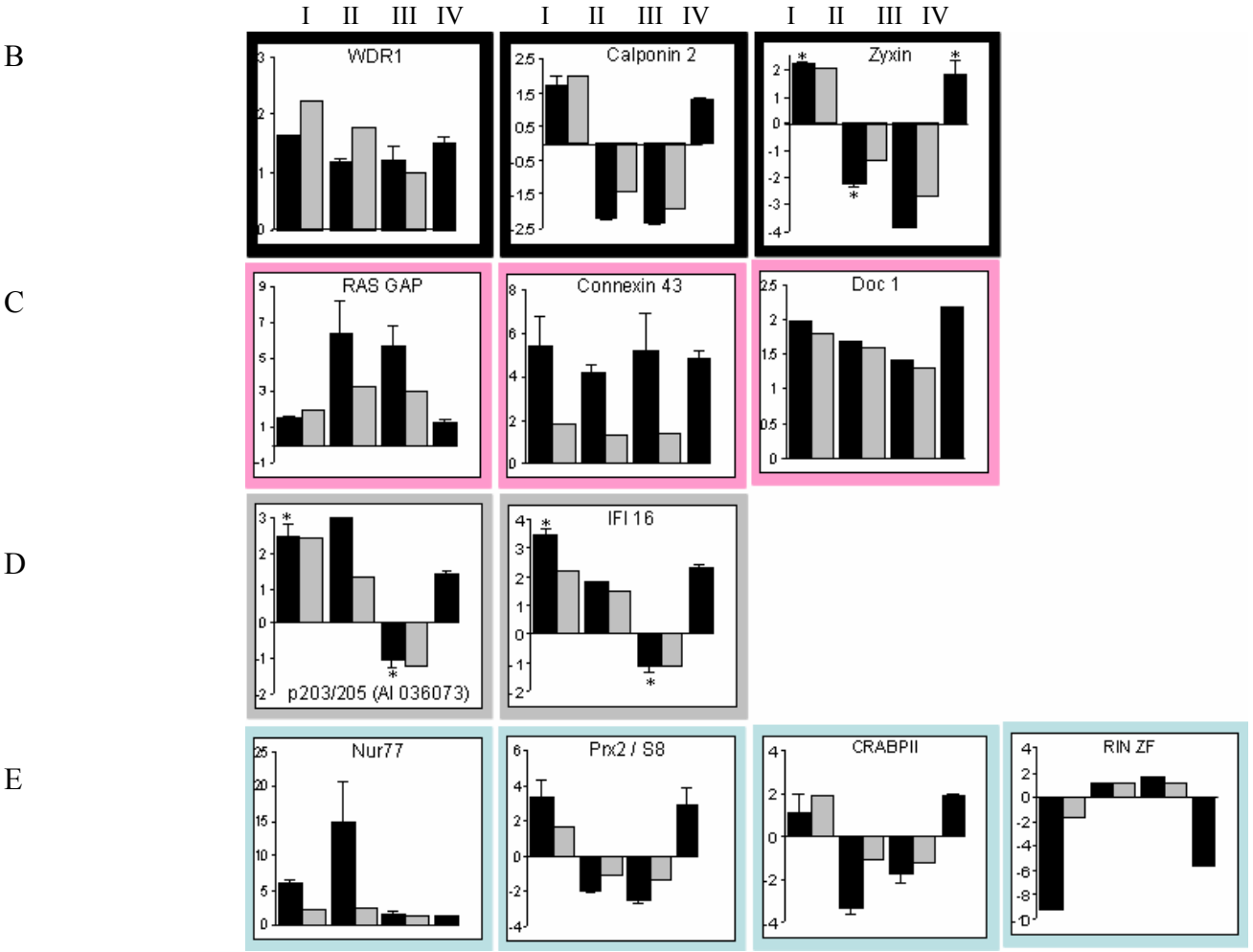


Figure 8 continued

Table 3 Functional categorization of genes differentially expressed in TPA treated P- and P+ cells into six classes.

Molecule[a]	**P+ vs. P-**[b]
CLASS I: Extracellular Matrix/ Basement membrane structural and regulatory proteins	
ECM collagen related	
Procollagen I, α1	**P+** > P-
Procollagen I, α2	**P+** > P-
Procollagen V, α2	**P+** > P-
Lysyl oxidase	**P+** > P-
Procollagen VI, α1	P- > **P+**
Basement membrane structural protein	
Nidogen/entactin	P- > **P+**
Procollagen IV, α1	**P+** > P-
Proteases and inhibitors	
Proteases	
Cathepsin H	**P+** > P-
Neprilysin/CD10/NEP	**P+** > P-
Inhibitors	
TIMP1	P- > **P+**
SLPI	P- > **P+**
Adhesion and migration factors	
ECM proteoglycans	
Decorin	P- > **P+**
PG-M/versican	P- > **P+**
Tenascin C	P- > **P+**
Axon guidance specific adhesion factors	
SLIT2	P- > **P+**
Semaphorin D	P- > **P+**
Fractalkine	P- > **P+**
Neuritin	P- > **P+**
Ninjurin	P- > **P+**
Calcium regulatory ECM protein	
Matrix GLA	**P+** > P-

Table 3 continued

Molecule	P+ vs. P-
CLASS II: Cytoskeletal proteins	
Calcium regulated cytoskeletal proteins	
Calponin 2	P- > **P+**
Calcyclin	P- > **P+**
Troponin T2	**P+** > P-
Actin and other cytoskeletal proteins	
Smooth muscle enteric actin	P- > **P+**
Vascular smooth muscle cell actin	P- > **P+**
Cardiac actin	P- > **P+**
Skeletal muscle actin	P- > **P+**
Transgelin	P- > **P+**
HSP25	P- > **P+**
Zyxin	P- > **P+**
WDRI	P- > **P+**
Destrin	**P+** > P-
Macrophilin	**P+** > P-
CLASS III: Enzymes and signal transduction proteins	
Enzymes and associated proteins	
Ras GTPase activating protein	P- > **P+**
Bone morphogenetic protein	P- > **P+**
Phosphoserine phosphatase	P- > **P+**
UDP gluconosyl transferase-1	P- > **P+**
cGMP dependent protein kinase type II	P- > **P+**
CDP diacylglycerol synthase	**P+** > P-
Receptors	
VLDL receptor	P- > **P+**
Calcium regulatory proteins	P- > **P+**
Voltage dependent calcium channel alpha2/delta	P- > **P+**
Calreticulin	**P+** > P-
Cell cycle and apoptotic mediators	
Deleted in oral cancer-1	P- > **P+**
IEX-1	P- > **P+**
Intercellular junctional protein	
Connexin 43 GAP junction protein	P- > **P+**

Table 3 continued

Molecule	P+ vs. P-
CLASS IV: Interferon inducible genes	
IFI 203/205	P- > **P+**
IFI 16	P- > **P+**
CLASS V: Transcriptional regulators	
RIN ZF zinc finger DNA binding protein	**P+** > P-
Nur77/N10	P- > **P+**
Retinoic acid binding protein II (CRABPII)	P- > **P+**
Prx2/S8	**P+** > P-
CLASS VI: Genes of unknown function	
Ribosomal proteins and homolog	
60S ribosomal protein L38	**P+** > P-
Homolog to ribosomal protein L39	**P+** > P-
Other genes	
Kinesin family member 21A	P- > **P+**
Complement factor H related protein	P- > **P+**
Recombining binding protein suppressor of hairless	P- > **P+**
Variable group of two cell stage family	P- > **P+**
Selenoprotein P, plasma 1	P- > **P+**
Thymic epithelial cell surface antigen	P- > **P+**
Regulatory protein T lymphocyte (Rpt-1)	P- > **P+**
Monocyte to macrophage differentiation associated gene	P- > **P+**
Similar to microsomal dipeptidase	P- > **P+**
RIKEN cDNA 1600012F09 gene	P- > **P+**
LOC328494 gene	P- > **P+**
RIKEN cDNA 9130005N14	**P+** > P-
RIKEN cDNA 5730559C18	**P+** > P-
RIKEN cDNA 4932442K08	**P+** > P-
RIKEN cDNA 2810418N01	**P+** > P-
Clone B930019K04	**P+** > P-
RIKEN clone 5330401006	**P+** > P-
LOC209096	**P+** > P-
Clone RP23-10B20	**P+** > P-
Ests of unknown genes	
EST AA413015	P- > **P+**

[a] Column 1, Names of genes; [b] Column 2, Relative expression in TPA treated P- and P+ cells: genes for which P-/P+ ≥ 1.7say P- > P+; genes for which P+/P- ≥ 1.7 say P+ > P-.

differential expression ratios of TPA untreated P+ versus P- cells, the real-time PCR analysis directly provided values corresponding to the endogenous differential expression ratios of P- DMSO vs. P+ DMSO and these were used for verification of the ratios derived from the three legs of the cDNA microarray analysis. The endogenous expression ratios derived from the three legs of the microarray hybridization analysis are in agreement with those obtained from the real-time PCR analyses.

Candidate Genes Relevant to TPA Induced Transformation Susceptibility and Resistance Belong to Six Functional Categories

The genes showing significant differential expression are categorized into six different functional groups according to their biological functions as shown in Table 3, and are; Class I, Extracellular matrix (ECM) /basement membrane structural and regulatory proteins (20 genes); Class II, Cytoskeletal proteins (13 genes); Class III, Enzymes and signal transduction proteins (12 genes); Class IV, Interferon inducible genes (IFI; 2 genes); Class V, transcription factors/DNA binding proteins (4 genes); Class VI, Genes of unknown function (21 genes and 1 EST).

Increased Cytoskeletal Actins and Cell-Cell Interaction in P- Cells

Nine isoforms of actin and actin-binding proteins are preferentially expressed in P- cells. Actins and actin binding proteins are key cytoskeletal components involved in formation of cell cortex and stress fibers. The formation of stress fibers favor a adhesive phenotype. These data along with the expression pattern of several other groups of genes (including higher expression of several adhesion-related genes in the ECM category and, protease inhibitors) in the P- cells support the notion that P- cells have a

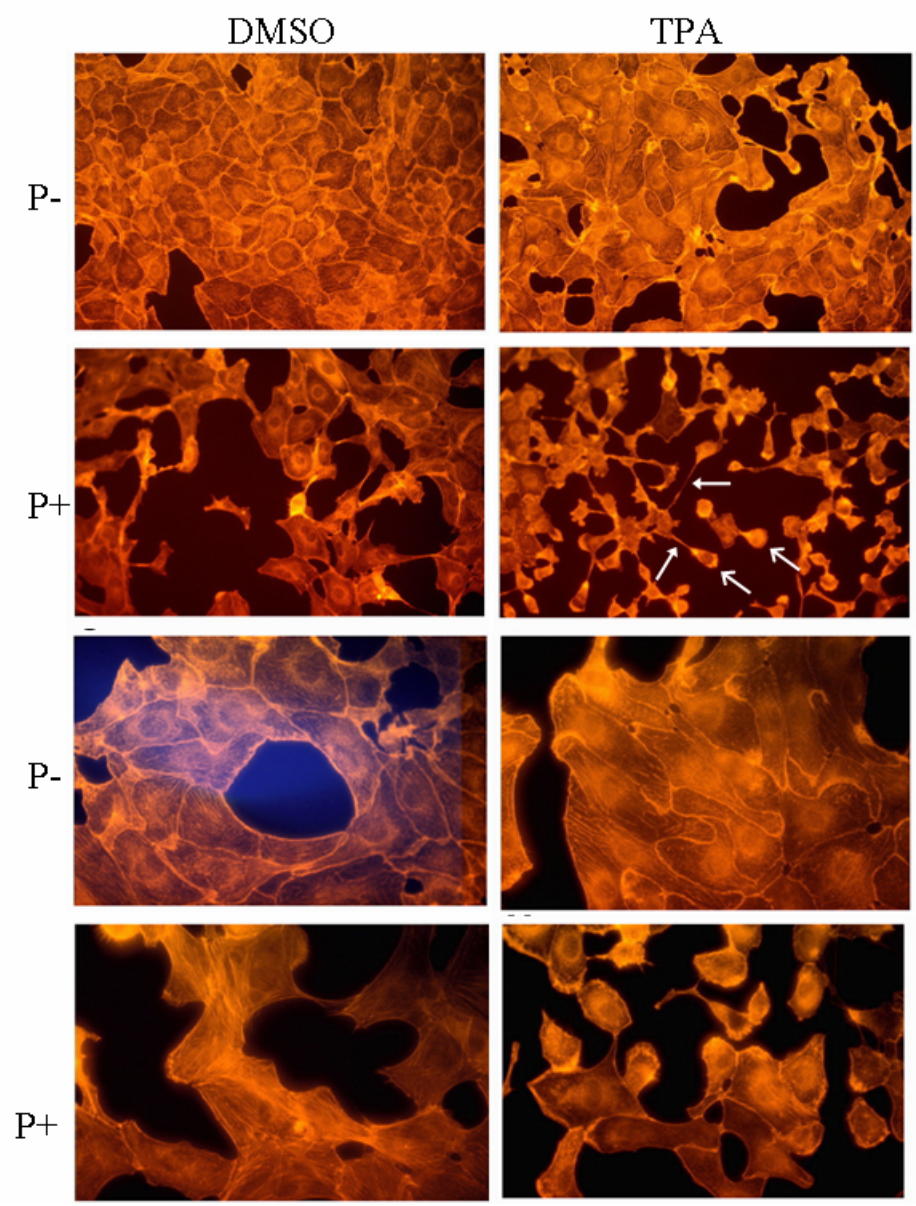


Figure 9 TPA treated P- cells contain more cortical actin staining, and cell-cell interaction compared to P+ cells. P+ and P- cells plated at equal densities were treated with TPA or DMSO solvent control for 8 h, stained as described in 'Materials and methods' and photographed. Left column, DMSO treated cells; right column, TPA-treated cells; Top row; P- cells, 20X magnification; second row, P+ cells, 20X magnification; Third row, P- cells, 40X magnification; Fourth row, P+ cells, 40X magnification. → , Lamellipodia; → , Filopodia

more adhesive phenotype. Cytoskeletal arrangement and cell-cell interactions in P+ and P- cells were therefore studied by phalloidin staining. Phalloidin staining in Figure 9 shows that P- cells exhibit significantly more cortical actin compared to P+ cells. Additionally, P- cells also exhibit more cell-cell adhesion, both in the presence and absence of TPA, compared to P+ cells. TPA-treated P+ cells exhibit limited cell-cell adhesion and contained significantly more lamellipodia and filopodia than P- cells. While almost 100% of TPA untreated P- and P+ cells contain stress fibers, most of the cells of both phenotypes lack stress fibers following 8 hours of TPA treatment.

Increased Wound Healing in TPA Treated P+ Cells

Phalloidin data illustrated increased lamellipodia and filopodia in TPA treated P+ cells. Because lamellipodia and filopodia implement cell migration and because of the expression pattern of several other groups of genes that mediate migration (including higher expression of proteases and the cytoskeletal proteins, Calcyclin and Troponin T2) in the P+ cells, we hypothesized that TPA treated P+ have a more migratory phenotype than P- cells. To test this hypothesis, P+ and P- migration rates were compared in wound healing assays (Figure 10). P- and P+ cells exhibited similar rates of wound closure in the absence of TPA, with partial closure by 12 hours, and with the majority of the wound closed by 48 hours in both cell types. However, the P- and P+ cells displayed markedly different migratory responses following TPA treatment. While TPA significantly inhibited wound closure in P- cells, TPA enhanced healing in P+ cells such that wounds had closed completely after 48 hours. These data demonstrate that TPA-treated P+ cells migrate significantly faster than P- cells.

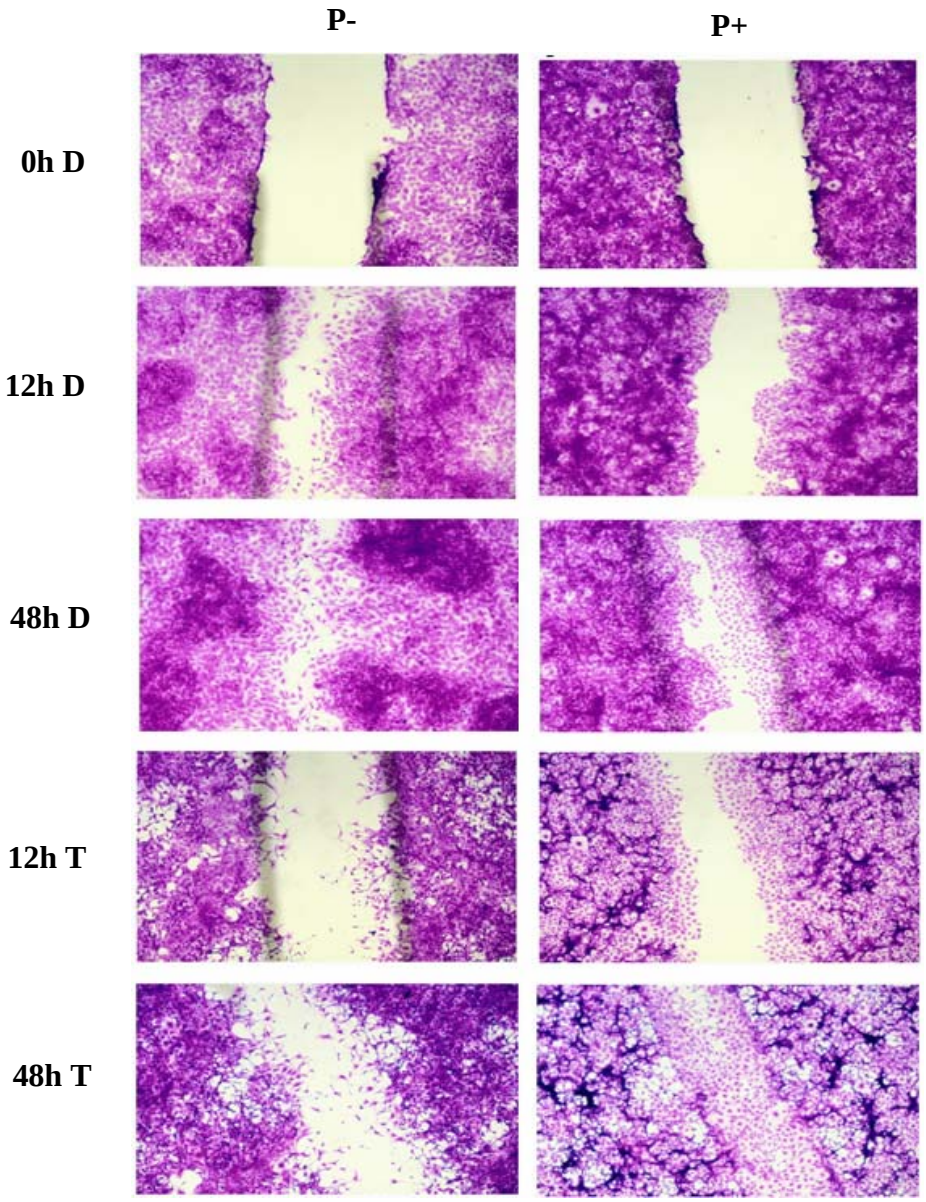


Figure 10 Increased cell migration and wound healing in TPA treated P+ cells compared to P- cells. P+ and P- cell cultures were wounded, treated with TPA for times ranging from 0 to 48 h, stained as described in 'Materials and methods' and photographed at 4X magnification. Left column, P- cells; right column, P+ cells; top row, 0h, DMSO; second row, 12h, DMSO; third row, 48h, DMSO; fourth row, 12h, TPA; Fifth row, 48h, TPA.

Table 4 Twenty-six genes and ESTs that have not been previously implicated in carcinogenesis are differentially expressed in TPA treated promotion-susceptible and resistant cells.

Adhesion (Neuronal guidance)
Neuritin (P- > **P+**)[a]

Transcription factors
Prx2/S8 homeobox transcription factor (P- > **P+**)
RIN-ZF putative transcription factor (**P+** > P-)

Ribosomal Protein
Homolog to ribosomal protein L39 (**P+** > P-)

Interferon inducible proteins (IFI family)
IFI 16 (P- > **P+**)
IFI p203/p205 (P- > **P+**)

Cytoskeletal function/actin binding
WDR1 (P- > **P+**)
Transgelin 2/SM22β (P- > **P+**)

Signal Transduction
Cytidyl diphosphate (CDP)-diacylglycerol synthase (CDS) (**P+** > P-)

Uncharacterized
Kinesin Family member 21A (P- > **P+**)
Thymic epithelial cell surface antigen (P- > **P+**)
Recombining protein suppressor of hairless (P- > **P+**)
Variable group of two cell-stage family (P- > **P+**)
Monocyte to macrophage differentiation associated protein (P- > **P+**)
RIKEN clone 9130005N14 gene (**P+** > P-)
Similar to putative to microsomal dipeptidase precursor (P- > **P+**)
RIKEN cDNA 1600012F09 gene (P- > **P+**)
LOC328494 gene (P- > **P+**)
CloneRP23-10B20 (P- > **P+**)
RIKEN clone 5730559C18 (**P+** > P-)
RIKEN clone 4932442K08 (**P+** > P-)
RIKEN clone 2810418N01 gene (**P+** > P-)
RIKEN clone 5330401006 (**P+** > P-)
Clone B930019K04 (**P+** > P-)
LOC209096 gene (**P+** > P-)
EST AA413015 (P- > **P+**)

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[a] The genes with known functions have been categorized into their respective classes. Relative expression in TPA treated P+ or P- cells are designated with '>' or '<' signs in parenthesis.

Nine Known and Sixteen Unknown Genes are Newly Implicated in TPA Induced Transformation

Among these differentially expressed genes are 26 sequences implicated in cancer for the first time in this study (shown in Table 4). These include 25 genes and an EST. Of the 25 genes, 13 sequences have no known function. Genes with known functions include transcription factor Prx2/S8, interferon inducible genes IFI16 and IFI-p203/p205, CDS and adhesion molecule neuritin.

Discussion

The study described in this chapter has led to the identification of several functional categories of genes that are potential candidates in susceptibility and resistance to tumor promoter induced neoplastic transformation. Furthermore, the study has implicated several genes for the first time in the process of neoplastic transformation. Collectively, our results provide indications of a more adhesive, pro-apoptotic, pro-tumor suppressive effect, consistent with a protective response in the P- cells against TPA induced neoplastic transformation and more motile, anti-tumor suppressive effect in P+ cells consistent with susceptibility to TPA induced neoplastic transformation. The following model (Figure 11) illustrates an overview of the major categories of differentially expressed genes in the P- and P+ JB6 cells that contribute to their susceptibility and resistance.

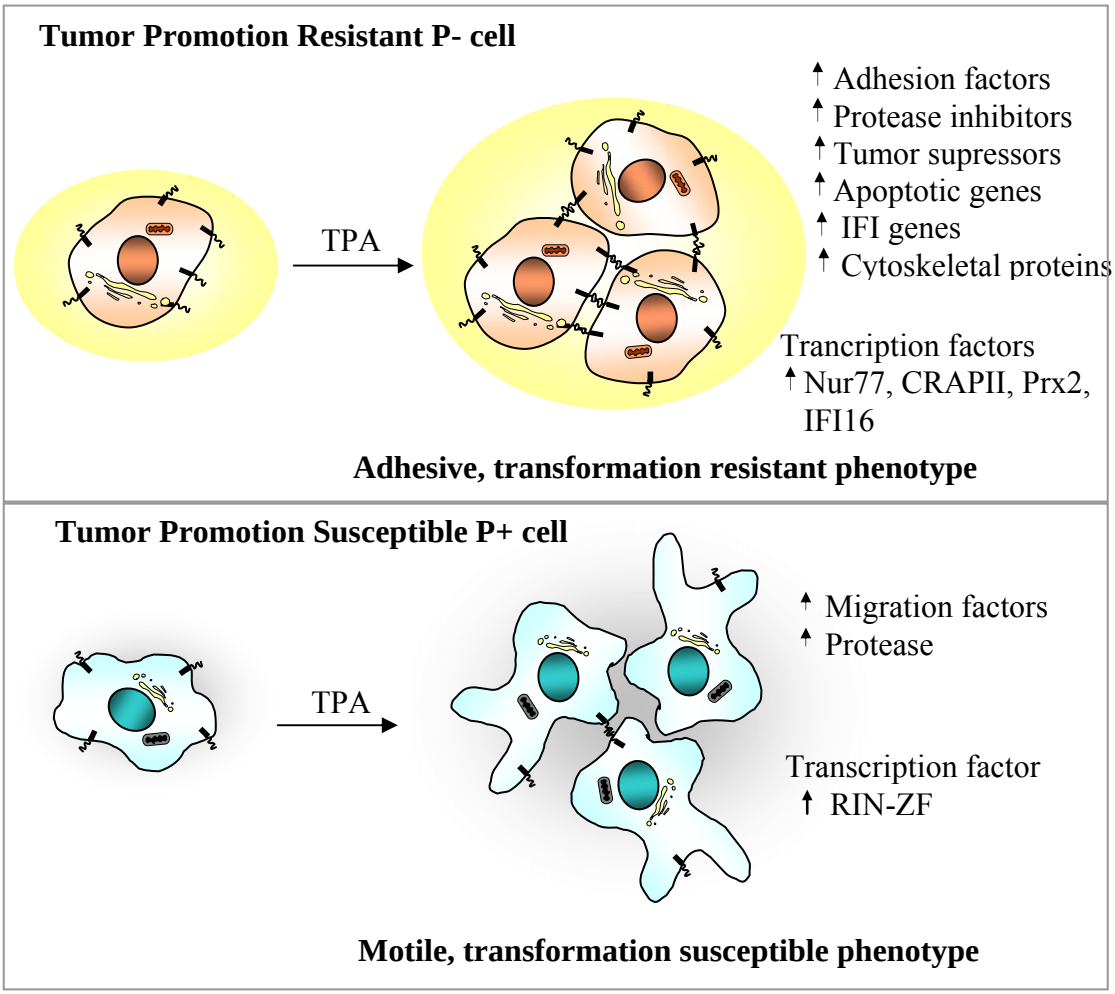


Figure 11 Model illustrating an overview of the major categories of differentially expressed genes in the P- and P+ JB6 cells that contribute to their susceptibility and resistance.↑ , increase in gene expression.

In this study, we used gene expression profiling by high density cDNA microarray and real-time PCR analyses to obtain exact information about the identities of candidate genes that provide potentially important insights into the events and pathways in tumor promoter induced neoplastic transformation. Since differences in gene expression profiles could potentially arise from differences in genomic background of the P+ and P- cells due to a large difference in passage numbers of the two cell lines, we chose a matched pair of P+ and P- cell line that had very similar genetic backgrounds. The P+ cell line used in this study had spontaneously arisen from a P- line and the two cell lines were within six passages of the P- to P+ transition. The 8 hour timepoint of TPA treatment selected for this study was early relative to manifestation of transformation effect which takes four days and therefore it is more likely that the gene expression changes observed are causative of transformation rather than resulting from the transformation event.

Comparison of the global gene expression profile of the P- and P+ cell lines reveals that majority of the genes are not differentially expressed in the two lines either constitutively or as a function of TPA treatment. These results are consistent with previous reports by Cmarik et al. using differential display according to which only a small percentage of genes are differentially expressed between the two JB6 cell lines (Cmarik et al., 2000). A comparison of the TPA modulated genes in P- and P+ cells reveals that there are more TPA modulated genes in the P- cells when compared to the P+ cells. This result is also in agreement with the differential display data of Cmarik et al. (Cmarik et al., 2000).

It is interesting to note that the TPA modulated pattern of gene expression observed in the P+ and P- cells does not correlate with the activities in P+ and P- cells of AP-1 and

NF-kB, two major transcription factors implicated in tumor promotion. Both AP-1 and NF-kB activation occurs in P+ cells but not in P- cells and have been shown to be necessary for susceptibility of P+ cells to TPA induced transformation (Bernstein and Colburn, 1989; Dong et al., 1994; Li et al., 1997). If these two transcription factors were the sole transcriptional regulators responsible for tumor promoter induced transformation, we would expect to find more TPA modulated genes in P+ cells than in P- cells. On the contrary, differential display studies by Cmarik et al. (Cmarik et al., 1999) and the current study demonstrate that significantly more genes are modulated by TPA in P- cells, suggesting the existence of additional pathway/s possibly controlled by other transcription factor/s that are important for regulating signal transduction in P- cells. Little work has been done to date to identify transcriptional activators in P- gene expression that may control resistance to transformation. Identification of these transcription factors may provide key insights into this process and advance our understanding of resistance to tumor promoter induced transformation. This study has identified several transcription factors, described later in this section, which are more highly expressed in P- cells. These constitute good candidates for further studies.

Differences in both basal (TPA untreated) and TPA induced mRNA expression levels between P+ and P- cells can provide significant clues to understanding tumor promotion susceptibility and resistance. Of the three legs of the cDNA microarray experimental design that we performed, comparison of expression level of TPA treated P- vs. TPA treated P+ cells (i.e., the first leg of the array) yields a composite of the basal and induced contributions to the final expression levels and therefore provides the most insight

into transformation relevant differences in P+ and P- gene expression. We therefore focused on the genes that showed higher differential expression in the first leg of the array.

Candidate genes identified in this study can be broadly classified into six categories based on their physiological functions. The first class is comprised of 20 members broadly classified as 'Extracellular matrix/ basement membrane structural and regulatory proteins. This group is further categorized into 5 subgroups based on the specificity of the function of the members. This includes: ECM collagen related, basement membrane structural proteins, proteases and inhibitors, adhesion and migration factors and calcium regulatory ECM proteins. This group includes several isoforms of procollagen that are more highly expressed in P+ cells, including α1 and α2 subunits of collagen I and collagen V. Expression of all three genes was down regulated by TPA in both cell types. The TPA dependent decrease in levels of the two procollagen species is in agreement with a previous report by Dion et al (Dion et al., 1982). Additionally, lysyl oxidase is also preferentially expressed in P+ cells. Lysyl oxidase is crucial collagen cross-linking, and disruption of the crosslinking process can result in severe structural collagen changes (Hong et al., 2004).

Among the other genes in this group, differential expression of those encoding the cysteine proteases cathepsin H and transmembrane ectoprotease neprilysin is noteworthy. Both the proteases are expressed at higher levels in P+ cells both constitutively and following TPA treatment. This expression pattern seems consistent with the expected pattern in final stages of tumor progression i.e., during invasion and metastasis, as proteases have been previously shown to be involved in tumor progression either by direct

degradation of extracellular matrix or by activation of other proteases, such as urokinase-type plasminogen activator (reviewed in Kos and Lah, 1998; Sloane, 1990; Lah and Kos, 1998). Cathepsins may be involved either directly in the degradation of components of ECM, such as laminin, fibronectin, and collagen, or through the modulation of protease-sensitive regulatory networks, involving other proteases as well as non-proteases, such as annexin II, found at the cellular surface of cancer cells (Turk et al., 2004). In general, the roles of these proteases in tumor invasion and metastasis are well characterized. Recent studies on the expression of cathepsin H in basal cell carcinomas (BCC) revealed that cathepsin H activities, immunoreactivities and mRNA were higher in BCC cells than in normal skin cells (Frohlich et al., 2004). Joyce et al., reported upregulation of several cathepsins during tumor progression and indicated cathepsins as being effectors of invasive growth and angiogenesis during multistage tumorigenesis (Joyce et al., 2004). The expression of these two proteases in our system, which is a model for the *early* stages of tumorigenesis, is intriguing.

Based on our observation of higher protease expression in P+ cells, we hypothesize that these proteases may play a role susceptibility to tumor promoter induced transformation possibly by increased destruction of collagen and proteoglycan components of the extracellular matrix, which could trigger the release of sequestered growth factors such as epidermal growth factor (EGF) and transforming growth factor beta (TGFβ) that enhance the growth rate of the cells, and have also been demonstrated to have transformation promoting activity in P+ cells (Taipale and Kaeski-Oja., 1997; Wilder and Rizzino, 1991). This hypothesis is consistent with increasing evidence demonstrating that proteases are

involved in earlier events of tumorigenesis. Increased expression of cathepsin B, another member of the cysteine protease family, has been reported in certain premalignant lesions (Koblinski and Sloane, 2000) and it is postulated to increase cell growth and, thereby tumorigenesis, by activating growth factors or liberating them from the ECM where they are sequestered. In studies by Colandrea et al., TPA induced tumor promotion in mouse skin was found be enhanced by transgenic overexpression of interstitial collagenases (Colandrea et al., 2000). Stromelysin-1 (MMP-3) has been shown to function as a tumor promoter in mouse mammary tumorigenesis (Sternlicht et al, 1999 and 2000; also see Sternlicht and Werb, 2001 for review). Although degradation of ECM has largely been attributed to MMPs, the importance of other classes of proteases including cathepsins in ECM degradation is now becoming clearer. The failure of broad-spectrum MMP inhibitors in clinical trials has opened the door for other proteases to be considered as relevant drug targets in anticancer therapies (Turk et al., 2004).

In reciprocal relation to the proteases, the two protease inhibitors TIMP 1 and SLPI, show higher levels of constitutive and TPA modulated expression in P- cells. TIMP 1 is a key metalloproteinase inhibitor that can inhibit active forms of all MMPs studied to date, with the exception of MMP-19 (Baker et al., 2002). TIMPs play an important role in tumor invasion and metastasis (Belloc et al., 1995 and Azuma et al., 1993) by functioning as negative regulators. In tissue culture, TIMP-1 can be induced in keratinocytes (Petersen et al., 1989) and fibroblasts in response to treatment with tumor promoting agents (Murphy and Werb, 1985). TIMP1 has been shown to block TPA-induced neoplastic transformation in culture (Shoji et al., 1997). The biological significance of higher expression of TIMP 1

in P- cells is unclear in light of conflicting reports on the role of TIMP 1 in tumorigenesis with an antitumor activity reported by some investigators (Gomez et al., 1997) and cell growth stimulation activity reported by others. While there are no definite explanations for these opposite functions of TIMP 1, it is possible that TIMP 1 functions both as an inhibitor of MMP and as a growth- promoting factors in different contexts. P- cells may express higher level of TIMP 1 in an effort to control MMP activity and preserve the ECM integrity.

In the context of the antitumor activity attributed to TIMP1, it is interesting to note the reciprocal pattern of expression of the proteases and protease inhibitors in P- and P+ cells with proteases being expressed at higher levels in P+ cells and the inhibitors being expressed at higher levels in P- cells. Such a tilt in the balance between levels of proteases and protease inhibitors may be a significant factor in determining the susceptibility or resistance of cells early on in the process of neoplastic transformation. SLPI is a potent serine protease inhibitor and is a member of the rapidly evolving seminal transcribed (REST) gene family (Lai et al., 2004). It inhibits elastase, cathepsin, trypsin and chymotrypsin (Zitnik et al., 1997). SLPI also downregulates MMP biosynthesis thus indirectly downmodulating collagen hydrolysis (Zhang et al., 1997).

Cell-cell and cell-matrix attachments are mediated through interactions between extracellular matrix (ECM) components, integrins, focal adhesion-linked molecules, and the actin cytoskeleton. Decreased cell-cell and cell-matrix adhesiveness is associated with loss of contact inhibition of proliferation that allows cells to escape from contact mediated growth control signals (Bast et al., 2000). This results in uncontrolled proliferation and the dramatic morphological changes that occur at the cell surface and in the cytoskeleton of

transformed cells and is crucial for both early and late stages of tumorigenesis (Wijnhoven et al., 2000). Based on this, we hypothesize that decreased constitutive and TPA modulated expression of cell adhesion molecules in the P+ cells contribute to their tumor promotion susceptibility and increased anchorage independent growth in soft agar assays. Consistent with our hypothesis, multiple genes that mediate cell adhesion show higher expression levels in P- cells than P+ cells, this includes the three ECM proteoglycans (PGs): decorin, tenascin C and PG-M/versican.

Decorin is essential for collagen fibril formation and promotes the stability of the fibers (Stander, 1999). Decorin suppresses neoplastic transformation by blocking multiple pathways that stimulate proliferation (Stander et al., 1999), including the EGF and TGFβ pathways. Decorin binds to TGFβ thereby inhibiting its activity and also downregulates EGF receptors on the cell surface (Santra et al., 2000; Csordas et al., 2000). Decorin is also a suppressor of malignant phenotype by inhibiting the migration on ECM (Merle et al., 1999; Kinsella et al., 2000). Moreover, decorin expression is down-regulated in hepatocellular carcinomas (Miyasaka *et al*., 2001) and in about 70% of ovarian carcinomas and ovarian cancer cells (Shridhar *et al*., 2001) as compared to their normal counterparts. Reed et al., demonstrated that transient transgene expression decorin causes a significant growth inhibition of colon carcinoma and squamous carcinoma tumor xenografts (Reed et al., 2002). Tenascin C can reorganize the actin cytoskeleton and modulate focal contacts (Jones and Jones, 2000). Tenascin C has been shown to modulate cell adhesion both positively and negatively (Sheetz-Jones and Jones, 2000). PG-M/versican modulates cell-matrix adhesion and promotes cell-cell adhesion (Yang et al.,

1999).

In addition to these ECM proteoglycans, several axon guidance molecules that mediate both cell-ECM and cell-cell adhesion are higher in the P- cells. These include fractalkine, ninjurin 1, Semaphorin D (SemaD) and SLIT2. Fractalkine and ninjurin are membrane bound cell-cell adhesion molecules (Lucas, 2001). Soluble forms of fractalkine and ninjurin have been identified that promote migration. SemaD and SLIT2 are secreted ECM adhesion molecules. SLIT functions in cooperation with sema D and both proteins are inhibitors of cell migration (Goshima et al., 2002). Higher expression of these multiple cell adhesion molecules in the P- cells may contribute to their resistance to neoplastic transformation and decreased anchorage independent growth in soft agar assays.

The second group comprises of 13 'cytoskeletal proteins'. In the first subgroup, 'Calcium regulated cytoskeletal proteins,' Calcyclin and Troponin T2 are higher in P+ cells. Both proteins have been shown to promote the actomyosin bridge cycle, which is exhibited during cell migration, in the presence of increased intracellular Ca^{2+} (El-mezgueldi, 1996; Fujii et al., 1994; Taylor, 1986). Additionally, calcyclin expression is correlated with metastatic migratory behavior (see Stradal and Gimona, 1999 and references therein). A third member in this subgroup, Calponin 2, known to inhibit the cross-bridge cycle is expressed at higher levels in the P- cells. In the context of the expression pattern of these genes, it is conceivable that the P+ cells more actively implement the contractile cross-bridge cycle than P- cells, thereby promoting a more motile phenotype that we observe in P+ cells. The migratory phenotype of P+ cells is supported by the presence of significantly more lamellipodia and filopodia as revealed

by phalloidin staining of the cells. Lamellipodia and filopodia are known to implement cell migration and as could be expected, the P+ cells also demonstrated markedly higher TPA induced migratory response in wound healing assays. Induction of cellular motility by phorbol ester induced PKC activation has been described previously (Montesano and Orci, 1985; Murphy et al., 1995; Hirakata et al., 1993).

In the second subgroup of 'actin and other cytoskeletal proteins', nine members have higher TPA induced levels in the P- cells. In the context of the anchorage dependent growth property of the P- cells, we hypothesized that P- cells have more stress fibers than P+ cells. The increased expression of the genes in this subgroup supports this hypothesis as many of them have been previously demonstrated to have important roles in the formation of stress fibers. Among these are four isoforms of actin; cardiac α actin, skeletal muscle α actin, vascular smooth muscle α actin and smooth muscle enteric γ actin and actin-associated proteins; SM22β, heat shock protein 25, WDR1 and destrin. Formation of stress fiber favors an adhesive phenotype (Alberts et al., 2002) and is associated with inhibition of anchorage independent growth (Masuda et al., 1996). Dynamic function of actin cytoskeleton is controlled by rates of polymerization, depolymerization, and assembly of F-actin filaments and stress fibers. These are in turn dependent upon levels of actin expression, and upon the expression and binding of various actin binding proteins that regulate actin function. The higher expression of multiple actin isoforms and actin-associated proteins in conjunction with higher expression of several cell-adhesion molecules in P- cells may thus promote a more adhesive phenotype. This notion is further supported by phalloidin staining data

which demonstrates that P- cells have significantly had more cortical actin and also more cell-cell contacts than P+ cells.

Phalloidin staining also revealed that both P+ and P- cells had abundant stress fibers in the absence of TPA. Four hours of TPA treatment resulted in a drastic reduction in the number of P+ and P-cells with stress fibers. After eight hours of TPA treatment majority of cells still lacked stress fibers, however a significantly higher percentage of TPA-treated P- cells had reconstituted them compared to P+ cells. Scoring of phalloidin stained cells for the presence stress fibers was performed by Dr. Lori R. Bernstein. Dr. Bernstein observed that whereas 35% of P- cells contained stress fibers after 8 hours of TPA treatment (58/163 cells scored for presence or absence of stress fibers), only 11% of P+ cells contained them (15/139 cells scored). These data indicate that TPA induces stress fiber decomposition by 4 hours, and that by 8 hours P- but not P+ cells have partially reconstituted their stress fibers. Takahashi et al. previously reported that TPA induced irreversible decomposition of actin stress fibers in P+ cells, whereas in P- cells the effects of TPA treatment were reversible within a 10 day period, being first detectable within 5 days (Takahashi et al., 1986).

It is interesting that several genes that mediate motility and metastasis are expressed at a higher level in the P+ cells. While the prevailing theory regarding metastasis supports the notion that only a tiny portion of a primary tumor contains cells with metastatic potential, Weinberg et al. argue that some of the initial mutations that transform a normal cell into a cancerous one can also cause metastasis (Couzin, 2003). In support of Weinberg's theory, recent work by Golub et al. demonstrates a 17-gene

signature in primary tumors predictive of metastasis indicating that a 'tumor's destiny is carved out early on' (Couzin, 2003). In this context it is intriguing that we observe expression of several markers of motility and metastasis in our model of early stage tumorigenesis, before neoplastic transformation has even occurred.

In the third group of 'Enzymes and signal transduction proteins' group, all five constituent members of first subgroup of enzymes and associated proteins are higher in P- cells compared to the P+ cells. This includes p120/Ras-GTPase activating protein (Ras GAP), BMP-5, phosphoserine phosphatase, UDP glucoronosyl transferase-1 and cGMP dependent protein kinase type II. TPA treatment results in activation of Ras via PKC-dependent (Hirai et al, 1994) and independent mechanisms. Ras induces cell proliferation and neoplastic transformation (Hay and Zuk, 1995). Physiological function of Ras includes the disassembly of actin stress fibers (Uberall et al., 1999; Maruta et al., 1999). We hypothesize that the P- cells by virtue of increased Ras GAP (which negatively regulates ras by enhancing its intrinsic GTPase activity) expression inhibit Ras activity and thereby prevent the disassembly of actin stress fibers. This mechanism further favors the role of actin stress fibers in the promotion resistance of P- cells.

TPA treated P- cells also preferentially express higher levels of nine genes that encode tumor suppressors and/or apoptotic mediators. Notable among these are the genes, deleted in oral cancer-1 (Doc 1), connexin 43 (Cx43), Ras-GAP, immediate early response factor X-1 (IEX-1), Nur77, IFI16, decorin and SLIT2. Doc 1 is a tumor suppressor gene for oral cancer (Daigo et al., 1997) and suppresses anchorage independent growth in soft agar when overexpressed in oral keratinocytes (Todd et al.,

1995). Connexin 43 is a major gap junctional protein that mediates intercellular communication and passage of solutes and second messengers. Gap junctional proteins play crucial roles in relaying contact inhibitory signals to the cells. Cx43 has been shown to inhibit anchorage independent growth and tumorigenesis (Hirschi et al., 1996; Su et al., 2000; King et al., 2002). Decreased constitutive and TPA-induced levels of Cx43 in P+ cells may allow them to override contact inhibition and anchorage-dependence, and become tumorigenic. Both IEX-1 and Nur77 are proapoptotic in stressed cells and IFI16, decorin and SLIT2 have all been shown to inhibit growth of tumor cells (Dallol et al., 2003a and 2003b). The preferential expressions of all these molecules in P- cells indicate that multiple pathways implementing growth arrest are effective in these cells possibly contributing to their transformation resistance.

In the fourth group of interferon inducible genes, the increased constitutive and TPA induced expression of IFI16 and IAP 203/205 homolog in P- cells is noteworthy. The interferon (IFN) family of cytokines is known for its growth-inhibitory activity, which plays an important role in IFN-mediated antitumor activity. These functions are performed by proteins encoded by interferon inducible genes that are induced by IFN (see Johnstone and Trapani, 1999 for review). IFIs belongs to the 'gene 200 cluster' (HIN-200 family / IfI 200 genes) which consists of several genes located in the q21-q23 domain of murine chromosome 1 (Johnstone and Trapani, 1999). HIN-200 proteins are primarily nuclear proteins involved in the transcriptional regulation of genes important for cell cycle control, differentiation, and apoptosis (Asefa et al., 2004). Members in this family include IFIs 202, p203, p204, p205, D3, MNDA, AIM2, IFI-16, and I-8U. These

proteins have in common one or two contiguous conserved 200 amino acid domains (A and B) that bear a LXCXE motif known to be a potential binding site for the Rb-family proteins (reviewed in Johnstone and Trapani, 1999).

Recent reports have shown that IFI16 is expressed in epithelial cells in addition to lymphoid cells (Gariglio *et al.*, 2002; Wei *et al.*, 2003). Ding et al., recently reported the identification of IFIX, a new member of the IFI family and demonstrated that IFIX is expressed in normal breast tissues and nontransformed breast epithelial cell lines (Ding et al., 2004). These observations suggest that IFIs may play a role in maintaining the normal growth of epithelial cells and the downregulation of IFIs expression may contribute to the uncontrolled cell growth and lead to tumorigenesis. Interestingly, both IFI16 and IAP 203/205 homolog show similar pattern of regulation in our system and they have not been previously observed in or associated with promotion resistance or cancer.

The antitumor activity of several HIN-200 proteins has been demonstrated. p202 suppresses tumor growth, reduces tumorigenicity, induces apoptosis, and suppresses metastasis and tumor angiogenesis of human cancer cells (Wen *et al.*, 2000, 2001; Ding *et al.*, 2002). Overexpression of IAP 202 decreases growth rate of normal and cancer cells (Lembo et al.1995, Yan et al., 1999). Furthermore, cells overexpressing p202 show reduced anchorage independent growth in soft agar (Yan et al., 1999). Overexpression of IAP 204 has been shown inhibit cell proliferation (Lembo et al., 1998) and, it was later demonstrated that this inhibition is mediated via the pRb regulatory system (Hertel et al., 2000). In the context of known anti-proliferation functions of various members of this family, it is conceivable that the increased expression of these genes in the P- cells may contribute to

their transformation resistant phenotype.

In the fifth group of transcription factors and DNA binding protein group, three of the four members, including Nur77/N10, cellular retinoic acid binding protein II (CRABPII) and Prx2/S8 homeobox transcription factor are more highly expressed in TPA treated P- cells, while RIN/ZF zinc finger DNA binding protein are more highly expressed in P+ cells. Nur77 and CRABPII are regulators of the retinoic acid (RA) pathways. RAs inhibit cell proliferation, papilloma formation in mouse skin, and neoplastic transformation in JB6 cells and are potent anticancer agents in several human cancerous and precancerous conditions (Budhu and Noi, 2002; Verma and Boutwell, 1977; Li et al., 1996 and Altucci and Gronemeyer, 2001). Nur77 encodes an orphan member of the steroid/thyroid/retinoid nuclear receptor superfamily (Lin et al., 2004) and modulates gene expression linked to cell proliferation and apoptosis (Winoto and Littman, 2002; Zhang, 2002). TPA selectively induced Nur77 expression in P- cells and the induced level was 6-fold higher in P- cells than in P+ cells. These results are consistent with previous studies using northern blot analysis of Nur77 mRNA in P+ and P- cells by Cmarik et al (Cmarik et al., 1994). Nur77 also transactivates transcription in a RA-independent manner. Similarly, CRABPII can also interact with the RAR-RXR-RARE complex independently or coupled to bound ligand thereby inducing transcription. The enhanced expression of Nur77 and CRABPII in P- cells may thus mediate tumor promotion resistance via transcriptional activation of genes that confer resistance.

Like the Nur77 and CRABPII transcription factors, the Prx2/S8 homeobox transcription factor is expressed higher in P- cells. Prx2/S8 is mesenchymal specific

factor that modulates ECM synthesis in vessel wall nonmuscular cells and fibroblasts (Kongsuwan et al., 1988). Prx2 binds to a consensus homeobox DNA sequence and contains trans-activating homeo- and Prx domain and a transrepressing OAR domain (de Jong and Meijlink, 1993; Noris and Kern, 2001). Prx2 may also function to activate the expression of genes that mediate resistance to TPA induced transformation. A fourth gene in this group, RIN ZF, is more highly expressed in P+ cells. RIN ZF was originally identified as a zinc finger transcription factor that binds to an SP-1 response element and RIN ZF coexpression with SP1 inhibits activating effects of SP1 mediated through SP-1 response element (Tillotson, 1999). These findings suggest that RIN ZF may regulate gene expression by interfering with SP-1 transactivation. However, no other literature is available about RIN ZF and it represents a novel protein that may potentially control the expression of genes involved in tumor promotion susceptibility. Functional characterization of this protein would be a novel contribution to the understanding of the process of neoplastic transformation.

Limitations of Present Study

- While our study uncovered many genes that are differentially modulated by TPA in the P- and P+ cells, our study was limited to an 8 hour TPA treatment period. We cannot rule out the possibility that the time point selected may not have been optimum and that some of the relevant changes in expression have been missed.
- Differences in mRNA levels detected may not always be reflected at protein level. Confirmation of expression at protein level may thus be necessary before embarking on detailed analysis of any differentially expressed genes identified

through this study.

- It is also possible that activities of these differentially expressed gene products may be modulated post-translationally for which it may be necessary to employ proteomics based approaches of analyses.
- Microarray systems are limited to detection of what is on the array, e.g., the source of the cDNA clones. Genes with low mRNA abundance may not be equally represented on the array. Conversely, genes that show very little change in expression in the array may have a significant effect physiologically. Furthermore, the combined effect of subtle changes in many genes may have a significant net physiological effect.

Suggestions for Future Work

Based on existing literature, the differentially expressed genes identified in this study can be classified into four groups:

1. Known genes, well characterized, known role in neoplastic transformation
2. Known genes, well characterized, implicated for the first time in neoplastic transformation
3. Known genes, not well characterized
4. Unknown gene

This microarray analysis identified many known genes, of which some had previously been implicated in the process of neoplastic transformation. For many of the other genes identified in this study however, this represents the first report implicating their potential role in carcinogenesis. This includes genes such as neuritin, transgelin 2 IFI16 and

IFI 203/205. These genes serve as very interesting candidates for further investigations. In addition to known genes, several genes with unknown functions have been identified. This includes genes such as RIN ZF, kinesin family member 21A and thymic epithelial cell surface antigen. The characterization and assignment of function to genes in the third and forth groups is most challenging and likely to contribute to novel and important leads into the process of neoplastic transformation. Further analysis of these genes to establish their specific role in the transformation event may provide information about other unknown pathways involved.

It is noteworthy that three transcription factors are highly expressed in the P- cells. Whether one or more of these transcription factors regulate groups of genes showing coordinated pattern of expression in these cells, remains to be determined. Members belonging to several transcription factor subfamilies have been shown to be key regulators in cancers. RIN ZF zinc finger DNA binding protein is a putative transcription factor found to be differentially expressed in our system and shows higher expression in P+ cells. RIN ZF, a largely uncharacterized gene represents a novel protein that may control the expression of genes involved in tumor promotion susceptibility. Functional characterization of this protein would be a novel contribution not only to understanding of the process of neoplastic transformation but also to the transcription factor field in general. Further studies, following verification of differential expression pattern in P- and P+ cells by realtime PCR and westerns may involve construction of sense and antisense expression vectors. Overexpression of the RIN ZF sense construct in P- and/or inhibition of RIN ZF in P+ cells through antisense constructs or siRNA

followed by transformation assays may reveal whether this protein is necessary and/or sufficient for transformation.

In an identical approach, the role for IFI16 in transformation may also be assessed. The expression of IFI16 and two other interferon inducible proteins was higher and also TPA inducible in P- cells but not in P+ cells. IFI16 has been previously shown to be a negative regulator of cell proliferation and also to possess transactivation functions. In this context, it will be interesting to study the tumor suppressor role of IFI16 in mediating transformation resistance in P- cells. Furthermore, another clone homologous to IAP 203/205 is selectively upregulated in P- cells and is also an interesting candidate for future investigations to better understand the role of interferon pathway in carcinogenesis.

Identification of novel genes is of significance not just in the context of neoplastic transformation but also in understanding their roles in other biological processes in the cell. One est identified in our study shows a 2-fold TPA induced expression in P+ cells compared to the P- cells. Interestingly, the expression is upregulated by TPA in P+ cells and downregulated by TPA in P- cells. This est represents yet another novel protein that may control the expression of genes involved in tumor promotion susceptibility. The unknown identity of this candidate represents some challenges in characterization, however follow-up experiments can proceed even in the absence of accurate identities since sequence from the arrays provide the necessary information. The complete clone may be picked out from a mouse library by 5' RACE using primers based on the sequence from the array. Sequence information of the clone

can then be obtained and compared to those in databases for identities to known gene families. Functional roles can be determined by generating fusion proteins and using them for identification of interacting partners. Functional characterization may also involve overexpression and antisense studies described earlier in this section.

Assuming that cascades of gene expression events operate as a function of time to produce the hallmark TPA responses observed in these cells, further studies of time-course analysis of expression patterns of selected candidate genes in response to TPA are needed to better understand this process in its entirety.

CHAPTER III

REGULATION OF GENE EXPRESSION AT THE AP-1 DNA BINDING SITE: IDENTIFICATION OF TWO TRANSACTIVATIONAL REPRESSORS

Synopsis

The AP-1 DNA binding site, present in the promoters of a number of genes, is a critical regulatory element that contributes to the transcriptional regulation of gene expression. Transcriptional regulation at the AP-1 site controls several important physiological and pathological processes, including neoplastic transformation. We isolated several AP-1 DNA binding proteins by a combination of DNA affinity chromatography followed by mass spectrometric analysis to identify novel proteins that bind the AP-1 site, with the hypothesis that these proteins may regulate transcriptional activation at the AP-1 site. Here we report that the proteins nucleolin and Y box binding protein 1 bind specifically to AP-1 DNA binding sequence. Reporter gene assays demonstrated that nucleolin and YB-1 repress AP-1 dependent gene expression in response to TPA treatment and c-fos overexpression. Analysis of intracellular levels and intra-compartmental localization of the AP-1 proteins in cells overexpressing nucleolin and/or YB-1 revealed that repressive effect of nucleolin and YB-1 is not due to reduced availability of AP-1 proteins. The precise mechanism of nucleolin and YB-1 mediated AP-1 transrepression is currently under investigation. Specific binding of nucleolin and YB-1 at the AP-1 site may be a key component in the integration of different signaling

pathways enabling cells to best respond to a given stimuli.

Introduction

This section of the dissertation describes the characterization of nucleolin and YB-1 as two AP-1 DNA binding transrepressors. The nucleolin project was originally started by Dr. Lori Bernstein and Dr. Vinay Kumar in an effort to isolate and characterize MAPK-related proteins that bound to the AP-1 DNA binding site. They observed a 97 kDa protein which bound specifically to the AP-1 site in NAPSTER (Kumar and Bernstein, 2001). The identification the p97 protein as nucleolin was done by Dr. Jean-Claude Twizere in the Bernstein laboratory. During the course of p97 isolation by Dr. Twizere using the NAPSTER assay, I observed two additional coomassie stainable bands at 47 and 49 kDa that also bound specifically to the AP-1 DNA. I initiated the identification and characterization of p47 and p49 proteins. Following the separation of his protein of interest, the large scale NAPSTER assay samples generated by Dr. Twizere were further processed by me for the purification of these two proteins. The 49 KDa protein was identified as YB-1 and the 47 KDa protein is believed to be a degradation product of YB-1 protein. Because of the theoretical and experimental overlaps between the nucleolin and YB-1 projects, some data generated by Dr. Twizere are presented herewith with his kind permission and are cited wherever used. The major focus of this section is however on isolation and characterization of YB-1 protein as an AP-1 DNA binding transrepressor.

Overview

Eukaryotic gene promoters are composed of multiple cis-acting sequence elements.

Gene expression is regulated through combinatorial interactions among a wide variety of transcription factors that recognize these cis-acting promoter elements through both protein-DNA and protein-protein interactions. Activator protein-1 (AP-1) family is one such key family of transcription factors. Although the AP-1 factors were identified 17 years ago (Angel et al., 1987; Lee et al., 1987), transcriptional responses at the AP-1 DNA binding site and the biological and physiological relevance of these responses are still being elucidated. We set out to identify proteins that regulate AP-1 dependent gene expression by binding to the AP-1 site, with the aim to better understand the molecular mechanisms underlying regulation of AP-1 dependent gene expression.

The AP-1 Transcription Factors

The AP-1 transcription factors belong to the bZIP family of proteins and have been demonstrated to regulate many biological functions including cell differentiation, proliferation, apoptosis and oncogenic transformation (Angel and Karin, 1991; Jochum et al., 2001; Mechta-Grigoriou et al., 2001). A variety of external stimuli, such as growth factors, tumor promoters, hormones, and analogs of the cAMP second messenger, have been shown to induce the levels and binding of AP-1 proteins (Angel and Karin, 1991; Shaulian and Karin, 2001; Mechta-Grigoriou et al., 2001). AP-1 complexes are comprised homo- (Jun-Jun) or hetero- (Jun-Fos) dimers of proteins from the Fos (c-Fos, Fos B, Fra-1 and Fra-2) and Jun families (Jun B, c-Jun and Jun D; Angel et al., 1987, 1988; Bohmann et al., 1987; Lee et al., 1987; Chiu et al., 1988; Sassone-Corsi et al, 1988) that share the same structural domains

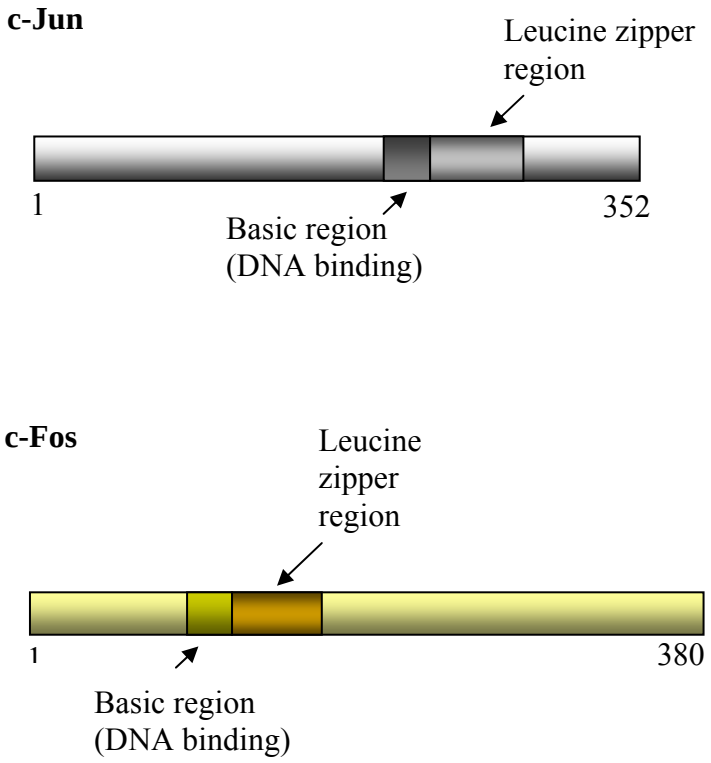
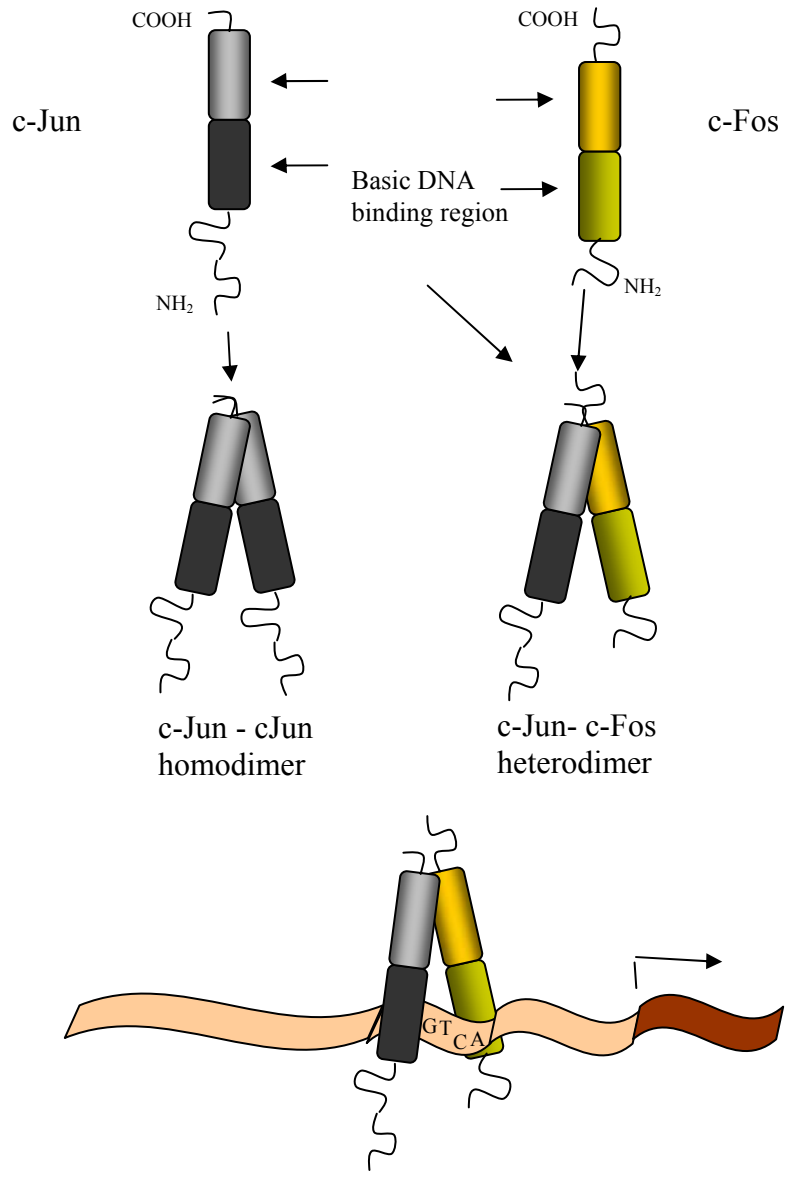


Figure 12 Schematic illustration of the structural domains of c-Jun and c-Fos proteins. Detailed description provided in the text. Adapted from the web address http://www.blc.arizona.edu/marty/411/Modules/homeo.html

for dimerization and DNA binding: a basic region and a leucine zipper region (Figure 12). The leucine zipper enables the formation of homo- and heterodimeric AP-1 complexes which are essential for DNA binding, and enhances their nuclear translocation (Chida et al., 1999; Figure 13). The basic region contains the actual DNA binding surface. AP-1 complexes regulate transcription by binding to the DNA sequence, 5' - TGA(C/G)TCA – 3' known as an AP-1/TRE element or AP-1 site (Lee et al., 1987; Angel et al., 1987; Angel and Karin,1991). The TRE element was first identified in the collagenase promoter as an element necessary for gene induction by phorbol ester 12-O tetradecanoylphorbol-14 acetate (TPA) (Lee et al., 1987; Angel et al., 1987; Angel et al., 1988). AP-1 sites in promoters of several genes have been characterized including collagenase (Frisch and Morisaki, 1990), metallothionein IIA (Angel et al., 1986), stromelysin (Kerr et al., 1990), transforming growth factor β (Kim et al., 1989) and interleukin 2 (Muegge et al., 1989).

AP-1 complexes have been reported to regulate transcription in response to several other stimuli including stress, UV irradiation, growth factors and genotoxic agents (Angel and Karin, 1991; Angel et al., 1988; Derijard et al., 1994; Devary, et al., 1991; Hibi et al., 1993; Shaulian and Karin, 2001). AP-1 mediated transcription at the AP-1 site is dependent on a variety of factors including the composition of AP-1 dimer (De Cesare et al., 1995; Chinenov and Kerppola, 2001), phosphorylation (Angel and Karin, 1991; Gruda et al., 1994), binding (Angel et al., 1987; Auwerx et al., 1990) potential of AP-1 complexes, AP-1 binding site context (Ryseck and Bravo, 1991; Vogt,



Binding of AP-1 dimer to TPA response element (TGAGTCA)

Figure 13 Schematic illustration of formation of AP-1 dimers and binding to the TRE sequence. NH_2, Amino terminal; COOH, Carboxy terminal. Adapted from the web address http://www.blc.arizona.edu/marty/411/Modules/homeo.html

2001), other proteins that interact with AP-1 proteins (Vogt, 2001; Chinenov and Kerppola, 2001; De Cesare et al., 1995) and/or other proteins that bind at the AP-1 site (Farrell, et al., 1989; Masquilier and Sassone-Corsi, 1992).

Nucleolin

Nucleolin (NCL) is a conserved and abundant protein present predominantly in the nucleolus but also present diffusely throughout the nucleus especially in proliferating cells (Zhou et al., 1997, Schwab et al, 1998). Its major function is the regulation of ribosomal RNA transcription and processing and ribosome biogenesis (Serin et al., 1997; Ginisty et al., 1998). However, NCL is believed to be multifunctional protein (reviewed in Srivastava and Pollard, 1999). NCL is also involved in several other cellular processes such as transcription (Ying et al., 2000), attachment of genomic DNA to nuclear matrix and chromatin decondensation (Erard et al., 1990), cell proliferation and growth, and in embryogenesis and nucleogenesis (Ginisty et al., 1999; Srivastava and Pollard, 2000). Interestingly, NCL is a substrate for several kinases including protein kinase C (Zhou et al., 1997). Both, transcriptional activator and repressor roles have been demonstrated for NCL. NCL has been shown to be an activator of the E6 and E7 oncogene transcription in HPV-18 (Grinstein et al., 2002) and to transactivate expression of the c-myc P1 and the Epstein-Barr virus F promoters (Brys and Maizels, 1994; Bulfone-Paus et al., 1995). NCL has also been implicated as a repressor of transcription of the alpha-1 acid glycoprotein promoter via the B motif (Yang et al., 1994). NCL has also been shown to represses transcription by inhibiting the transcriptional machinery and/or via interactions with promoter sequences in rDNA (Roger et al., 2002). NCL

binds to several nucleotide sequences in RNA, single-stranded DNA and double stranded DNA.

YB-1

The YB-1 protein represents a multi-gene family called the Y-box binding proteins defined by their ability to bind to the DNA sequence CTGATTGG (C/T)(C/T)AA; called inverted CCAAT box or Y-box (see Wolffe et al., 1992 for review). YB-1 family members have been identified in a number of eukaryotic and prokaryotic organisms and found to be conserved in sequence throughout evolution from bacteria to vertebrates (Li et al., 1992). YB-1 is translocated from the cytoplasm into the nucleus in response to various cellular inducers (Holm et al., 2002; Higashi et al., 2003; and Sun et al., 2000). The YB-1 protein contains three domains: a variable N-terminal domain, a central cold shock domain (CSD) and a hydrophilic C-terminal tail domain (Figure 14). The N-terminal domain contributes to variability between the various YB-1 family members. The CSD serves in nucleic acid recognition and binding, and is highly conserved across species. The C-terminal domain is hydrophilic and consists of alternate segments of basic and acidic residues (Fukada and Tonks, 2003). This domain is involved in protein-protein interactions and interestingly has also been shown to play an important role in DNA binding (Chen et al., 1995; Nambiar et al., 1998).

Y-box binding proteins can also bind to many other DNA sequences that do not contain an inverted CCAAT box (Mertens et al., 1997; Li et al., 1997), as well as to single stranded DNA and RNA (Wolffe et al, 1992; Hasegawa et al., 1991) contributing to the pleiotropic cellular functions (Ohga et al., 1996) that Y-box binding proteins have

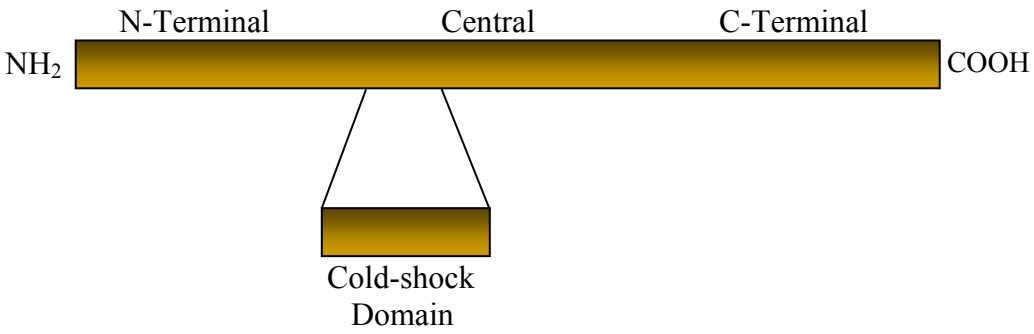


Figure 14 Schematic illustration of the YB-1 protein domains. Detailed description provided in the text.

been demonstrated to possess. YB-1 proteins have been implicated in direct transcriptional activation and repression of gene expression and the transcriptional regulation mechanism by YB-1 has been studied using several different genes (Matsumoto and Wolffe, 1998; Ogha et al., 1998; Higashi et al., 2003, Lasham et al., 2000).

The presence of NCL and YB-1, together, has been observed in several multiprotein complexes. Roles for NCL and YB-1 in mRNA stabilization have been demonstrated for renin and IL-2 transcripts. NCL and YB-1 bind to the 3' untranslated region of the renin mRNA (Skalweit et al., 2003; Persson et al., 2003), and to a Jnk Response Element (JRE) within the interleukin-2 (IL-2) mRNA sequence (Chen et al., 2000). NCL and YB-1 are present in a multicomponent complex composed of at least 60 protein subunits along with multiple ribosomal components (Yanagida at al., 2001). NCL and YB-1 have also been found together in a fragile X mental retardation protein-associated ribonucleoprotein particle composed of 40 proteins (FMRP-associated mRNP particle; Ceman et al., 2000). In a more recent study, NCL and YB-1 were identified in a multiprotein complex by pull-down experiments using YB-1 fusion protein and a role in DNA repair was implied (Gaudreault et al., 2004). Although direct interaction between NCL and YB-1 has not been demonstrated, their presence in the same multiprotein complex suggests a possible cooperative function in cellular processes.

In the present study we used the NAPSTER assay to identify p97 and p49, two new AP-1 DNA binding proteins. We then determined that p97 and p49 were NCL and YB-1 respectively. We performed reporter gene assays in order to elucidate the trans-

regulatory effect of NCL and YB-1 on gene regulation at the AP-1 site. The present study has shown for the first time that NCL and YB-1 repress transactivation at the AP-1 site. NCL and YB-1 do not affect the expression or intracellular localization of any of the AP-1 subunits. Our data are consistent with the hypothesis that the NCL and YB-1 mediate repression by directly binding to the AP-1 site. The regulation of AP-1 dependent gene expression may possibly be subject to cooperative control by NCL, YB-1 and AP-1 family of proteins.

Materials and Methods

Cell Culture

Human colon HT29 adenocarcinoma cells and human cervical carcinoma HeLa cells were obtained from American Type Culture Collection (ATCC; Manassas, VA). HT29 cells were cultured as described earlier (Kumar and Bernstein, 2001). HeLa cells were grown in minimal essential media (MEM; Gibco-InVitrogen) supplemented with 10% fetal bovine serum (FBS).

Antibodies

The αNCL, αHemaglutinin (HA), αOct-1 and all the αAP-1 antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA); αYB-1 was a rabbit polyclonal antibody to a 15 amino acid "C1" peptide corresponding to residues 299-313 in the C-terminal of human YB-1 protein. Initial experiments using αYB-1 were performed with whole rabbit αYB-1 antisera kindly provided by Dr. K. Kohno (University of Occupational and Environmental Health School of Medicine, Kitakyushu, Japan). Subsequent experiments were performed with immunoaffinity purified αYB-1 antibody custom

prepared against the C1 peptide antigen by Bethyl Laboratories (Woodlands, TX). αFlag M2 was a mouse monoclonal antibody from Sigma (St. Louis, MO). αGlyceraldehyde phosphate dehydrogenase (GAPDH) was a mouse monoclonal from Research Diagnostics Inc. (RDI, Flanders, NJ). Peroxidase conjugated αrabbit and αmouse secondary antibodies were from Calbiochem (San Diego, CA).

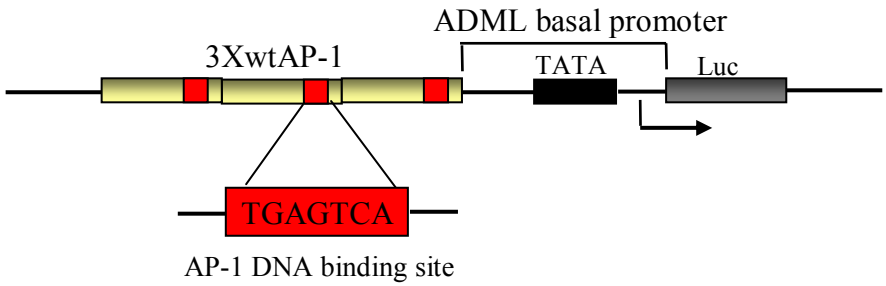
Plasmid Constructs

The Flag epitope tagged YB-1 expression construct, pcDNA3-Flag-YB1, harboring a full-length human YB-1 gene was kindly supplied by Dr. K. Kohno (University of Occupational and Environmental Health Kitakyushu, Japan). Hemagglutinin (HA) tagged NCL expression construct, pNtag4, harboring full length NCL gene was a gift of Dr. N. Maizels (University of Washington). The constitutive pSVBgal plasmid harboring a full-length beta galactosidase gene (expressing beta galactosidase) was obtained from Promega (Madison, WI). pGL3AdML-Luc used as a template for generation of GALV AP-1 reporter constructs were a kind gift of Dr. Gary Kunkel (Texas A&M University). pcDNAc-Fos and pcDNAJunD expression constructs harboring full length human c-Fos and JunD genes and 4X GCN AP-1 luciferase reporter construct were a gift of N. Colburn (NCI-FCRDC, Frederick, MD).

Wild Type and Mutant GALV AP-1 Luciferase Reporter Constructs

These luciferase reporter constructs were generated by Dr. Twizere in the Bernstein lab. Oligonucleotides ("oligos") harboring three tandem copies of the wild type or mutant AP-1 DNA binding site from the gibbon ape leukemia virus long terminal

3XwtAP-1-Luc



3XmutAP-1-Luc

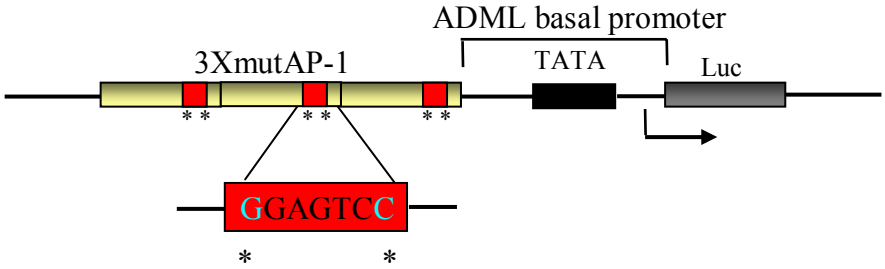


Figure 15 Schematic diagram of wild type and mutant AP-1 luciferase reporter constructs. Wild type and mutant constructs contain three tandem GALV-LTR AP-1 sequences (shown in brown), each containing minimal AP-1 DNA binding sites (shown in red), ligated upstream of the adenovirus major late basal promoter (AdML basal promoter). Promoter sequences are upstream of the firefly luciferase reporter gene ("Luc," grey rectangle). Upper diagram: wild type 3XGALV-AP1-luc construct; lower diagram, mutant GALV-mut-AP1-luc construct harboring three tandem mutant AP-1 sites. Mutations are shown with asterisks below mutated sequences. Base sequences of wild type and mutant AP-1 DNAs are shown in red boxes.

repeat (GALV-LTR; Kumar and Bernstein, 2001) flanked by 5'-XhoI and 3'-BamH1 restriction sites were custom synthesized (Integrated DNA Technologies, Inc.; IDT; Coralville, IN). The sequences of the oligos (sense strand) are; Wild type: 5'-agccagagaaatagatgagtcaacagc-3'; Mutant: 5' agccagagaaatagaggagtctacagc-3' (mutations in bold). The oligos were subcloned into the plasmid vector pGL3AdML-Luc at XhoI and BamH1 restrictions sites by replacing the 5X UASG sequences with the 3XGALVAP-1 sequences to generate wild type 3XAP-1-GALV-luc and mutant 3XAP-1mutGALV-luc luciferase reporter constructs (Figure 15).

Preparation of Nuclear Extract

Nuclear extracts (NE) from TPA treated HT29 cells were prepared as described (Bernstein and Walker, 1999). Briefly, HT29 cells were plated at 6 x 10^6 cells per 150 mm dish in DMEM containing 10% serum and grown for 4 days. Cells were treated with TPA (10ng/ml) for 90 minutes prior to NE preparation. Cells were washed 3X with PBS and scraped using a rubber spatula into 5ml of PBS (per dish) containing the protease inhibitors (PI), leupeptin, aprotinin and PMSF. Pooled cell suspension was washed 3X with PBS containing PI. The packed cell volume (PCV) was estimated. The pellet was resuspended in 5X the PCV in Buffer A (10 mM Hepes, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 4 mM BME, 0.1 mM Phenyl Methyl Sulfonyl Fluoride (PMSF), 10 µg/ml Leupeptin, 10 µg/ml Aprotenin) and washed 2X. The pellet was resuspended again in 5V of Buffer A and allowed to swell on ice for 10 minutes and then spun down. The pellet was further resuspended in 3V of Buffer A and the cells were lysed with about 25 strokes of a Teflon pestle driven by a hand-held power drill. The nuclear pellet

was spun out and resuspended in 5V of Buffer B (20 mM Hepes, pH 7.5, 0.2 mM EDTA, 20% glycerol, 4 mM BME, 0.5 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml Aprotenin). 4 M ammonium sulfate was added to yield a final concentration of 0.3 M, and the suspension was allowed to incubate at 4 °C with twirling for 30 min and then centrifuged at 60,000 rpm for 20 minutes. The supernatant (NE) was quickly recovered prior to re-swelling of the nuclear pellet and frozen in small aliquots at -80°C.

NE from TPA-treated HeLa cells was prepared by the same method after plating 3 x 10^6 HeLa cells per 150 mm dish and growing cells in MEM containing 10% fetal bovine serum for four days. Protein concentrations of extracts were estimated using the Bradford colorimetric method (Biorad, Hercules, CA).

Preparation of DNA Affinity Beads and NAPSTER AP-1 DNA Binding Assays

Streptavidin coated beads were obtained from Roche (Cat. #1529188) or from Pierce (Cat. #20349) and were conjugated to biotinylated double-stranded oligonucleotides (Macromolecular Resources, Boulder, CO) containing wild type AP-1 core sequence (sequence as used in reporter construct above) from the GALV-LTR as described (Kumar and Bernstein, 2001a) according to manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN).

Batchwise microscale AP-1 DNA affinity chromatographic NAPSTER assays were performed as described (Kumar and Bernstein, 2001a). NE was dialyzed into Buffer Z (25 mM HEPES pH 7.8, 0.1 M KCl, 12.5 mM $MgCl_2$, 1 mM dithiothreitol, 20% v/v glycerol, 0.1% v/v Nonidet P-40, 0.1 µM $ZnCl_2$, 5 mM NaF, 1 mM sodium orthovanadate) containing PI for 50 min at 4°C. Dialyzed NE was then incubated with

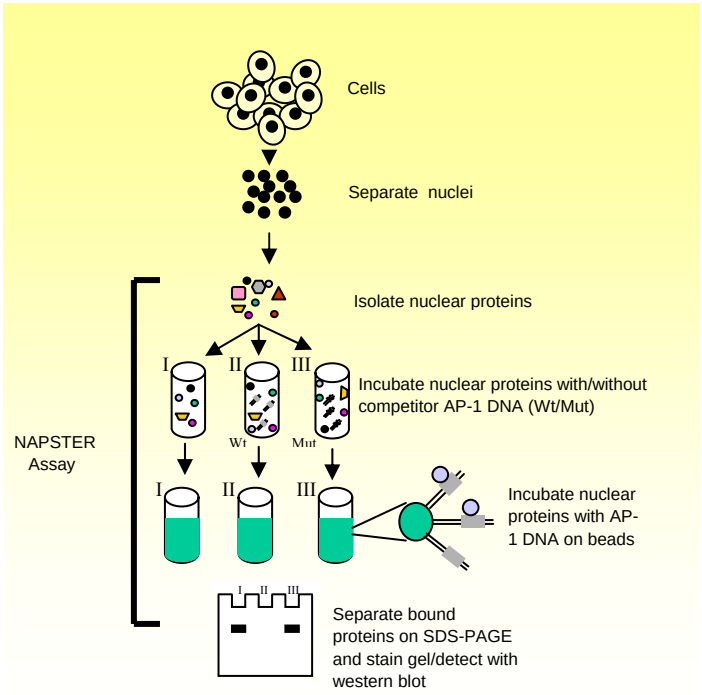


Figure 16 Schematic illustration of the NAPSTER assay. Nuclear extract proteins from human adenocarcinomal HT29 cells were preincubated with excess wild type or mutant AP-1 oligos in solution and then subjected to DNA affinity chromatography with AP-1 DNA coupled to an immobilized streptavidin matrix. Purified proteins were visualized by SDS-PAGE followed by coomassie staining or by western blot with specific antibodies.

6 µg/ml poly (dI/dC) for 10 min on ice. NE was then split into three equal samples and subjected to NAPSTER analyses with wild type GALV-AP-1 DNA beads (Figure 16). Sample I consisted of direct batchwise AP-1 DNA affinity chromatography of the NE proteins. Samples II and III were pre-incubated with excess wild type or with excess mutant GALV AP-1 DNA in solution for 15 min on ice. The AP-1 DNA affinity chromatography was performed as per Lee et al. (Lee et al., 1987) with several modifications (Kumar and Bernstein, 2001a and 2001b) for 75 min at 4°C on a rotating clip wheel. Beads were quickly spun down at 4°C, the supernatant was removed and then washed again 3X with Buffer Z. Bound proteins were eluted by boiling the beads in SDS sample buffer, and loaded in SDS-PAGE.

Purification of p47 and p49 Proteins

HT29 NE containing 60 mg of protein was dialyzed against buffer Z as described (Kumar and Bernstein, 2001a) and then subjected to DNA affinity chromatography on a column containing 2.5 mg of mutant AP-1 DNA beads at 4°C. The flow-through from the column was incubated with 120 µg of poly dI/dC for 10 minutes on ice and then split into three portions: sample I, the starting material for NAPSTER purification, and samples II, and III, as NAPSTER competitors for detection of specific p47 and p49 protein bands. Sample I contained 54 mg of protein and samples II and III contained 3 mg each of NE protein. 375 mg of wild type or mutant competitor oligo in solution was then added to samples II and III respectively and incubated for 15 minutes at 4°C. Streptavidin beads containing 2.7 mg of bound wt AP-1 DNA was added to sample I and beads containing 150 micrograms of AP-1 DNA were added to samples II and III and

incubated as described (Kumar and Bernstein, 2001a). The supernatant was withdrawn, and the beads were washed 5 times in buffer Z.

The proteins bound to beads were eluted twice with buffer Z containing 1M KCL after twirling for 30 minutes at 4°C on a rotating clip wheel. Eluted proteins were precipitated in a final concentration of 10% trichloroacetic acid (v/v; TCA) on ice for 30 minutes, and then spun at 9,500 x g at 4°C in a microfuge for 15 minutes. Pelleted samples were then dissolved in 100 µl of distilled water, reprecipitated in 1 ml of absolute acetone (EM Science, Darmstadt, Germany) at room temperature for 30 minutes, and spun at 9,500 x g at 4°C for 15 minutes. The supernatant was removed and the pellet was air dried for 30 minutes at room temperature, dissolved in SDS sample buffer, boiled, and loaded onto a 10% gel in a large format gel apparatus (BioRad PROTEAN® II xi, BioRad Hercules, CA). Samples were then run at 30 mA overnight such that the 30 kDa molecular weight marker was run to the bottom of the gel (Kumar and Bernstein, 2001a). The gel was then stained with Coomassie brilliant blue and the bands corresponding to p49 and p47 were excised. One fourth of each of the p47 and p49 samples isolated from sample I were run on a second SDS-PAGE gel and subjected to immunoblotting analyses with PAN AP-1 Jun/Fos antibodies, and the remaining material analyzed by nanospray MS/MS sequencing.

Mass Spectrometric Mass Spectrometry Fragmentation Sequencing Analysis of p47 and p49

The excised gel bands corresponding to p47 and p49 prepared as described above were submitted for mass spectrometric mass spectrometry fragmentation

sequencing analyses (MS/MS sequencing) at the W.M. Keck Foundation Biotechnology Resource Laboratory (http://info.med.yale.edu/wmkeck/prochem) at Yale University. Proteins for each sample were trypsinized in-gel for 18 hours, eluted, and then subjected to nanospray MS/MS analysis. The resulting spectra were searched manually and by using the Mascot peptide mass fingerprint algorithm for protein identification (http://www.matrix-science.com/search_form_select.html).

Immunoblotting Analyses

Western transfer and immunoblotting analyses were performed as described (Walker and Bernstein, 1999). Western blot targets were visualized using enzyme chemiluminescence (ECL, Amersham). Antibodies were used at the following concentrations: αJunD: 0.5 µg/ml; αYB-1 (0.5µg/ml); αFlag (0.49µg/ml); αGAPDH (0.4ng/ml).

Fractionation of Cytosol and Nucleoplasm

HeLa cells were plated at 3 x 10^6 cells per 150 mm culture dish in MEM containing 10% serum and allowed to grow for two days. Cells were transiently transfected with 15 µg/dish of pcDNA 3.1 (vector control) or with either pcDNAFlag-YB1 or pNtagHA-NCL or both, using Lipofectamine 2000 (1 µl/µg of DNA; Invitrogen, Carlsbad, CA). The transfection medium was replaced 5 hours following transfection, with fresh MEM containing 10% serum and TPA (100ng/ml). Twenty-four hours post-transfection, cells were washed 3X in PBS and processed essentially as described for nuclear extract preparation in an earlier section with the following modification. Following cell lysis and separation of nuclear pellet by centrifugation, the supernatant

which constitutes the cytosolic fraction was recovered and frozen in small aliquots at -80°C. The nuclear pellet was processed further as described and the nuclear extract was recovered and also frozen in small aliquots at -80°C.

Densitometric Analyses

Protein densitometric analysis of immunoblotted proteins were performed on a Macintosh computer using the public-domain NIH Image 1.62 image analysis program (http://rsb.info.nih.gov/nih-image/).

Luciferase Reporter Gene Assay

HeLa cells were plated at 3 x 10^5 cells per well in 6 well Nunc dishes (Nunc Inc., Napersville, IL). Twenty-four hours after plating, the cells were transiently cotransfected with 2 µg of either GALV-AP1-Luc or GALV-mutAP1-Luc reporter plasmids along with 1 µg of pcDNAFlagYB1 or 1 µg pNtag4 HA-NCL and 200 ng of pSVBgal, using Lipofectamine 2000 (1 µl/µg of DNA; Invitrogen, Carlsbad, CA). Cells that received no transfected pcDNAFlagYB1 were transfected with 1 µg pcDNA3.1 (+) vector control plasmid (Invitrogen, Carlsbad, CA). 10 ng of c-Fos or 50 ng of JunD AP-1 expression constructs were also co-transfected with the other constructs for some experiments. Twenty-four hours post-transfection, cells were washed 3X in PBS and cell lysates were prepared by adding 200 µl of lysis buffer/well (Roche Luciferase Reporter Gene Assay Kit). Cells were then scraped with a rubber policeman and incubated at room temperature for 15 minutes. Cellular debris was removed by spinning the lysates at 9,500 x g for 10 seconds at room temperature. Luciferase assays were performed using a Luciferase Reporter Gene Assay Kit (Roche, Indianapolis, IN)

according to the manufacturer's instructions, using a Packard Lumicount Luminometer (Packard Instruments Co. Downers Grove, IL). β- galactosidase activity assays for normalization of transfection efficiency were performed with 25 µl of lysate using a β-Galactosidase Enzyme Assay Kit from Promega (Madison, WI), according to manufacturer's instructions.

Confocal Microscopy

HeLa cells were plated at 1 x 10^5 cells per ml in a LabTek II chamber slide (NalgeNunc International Corporation, Naperville, IL). Twelve hours post-culture, cells were treated with TPA (100ng/ml) for 30 minutes, then fixed in 3.7% formaldehyde (Mallinckrodt-Baker Inc., Paris, Kentucky) and permeabilized with 0.1% Nonidet P40 (NP-40; Roche, Indianapolis, IN). The permeabilized cells were then incubated with antibodies against YB-1 or nucleolin (1:1000 dilution for both antibodies) in dPBS at room temperature for 60 minutes, followed by incubation with fluorescein- or Texas Red-coupled anti-rabbit or anti-mouse immunoglobulin conjugates (Molecular Probes, Eugene, OR) for 60 minutes, according to the method of Lefebvre et al (Lefebvre et al, 2002). Stained cells were fixed with mounting medium (Prolong Antifade kit, Molecular Probes), and visualized using a Biorad Radiance 2000 MP confocal microscope. Images were generated according to procedures described at http://www.cvm.tamu.edu/ial/.

Results

The NAPSTER Assay for Identification of New DNA Binding Proteins

In order to identify new proteins that bind specifically to the AP-1 DNA

sequence, we used the affinity chromatography based assay termed the Nucleotide Affinity Preincubation Specificity TEst of Recognition Assay ("NAPSTER" Assay; Kumar and Bernstein, 2001a and b) designed previously in our laboratory. NAPSTER assay consists of three sample sets processed in parallel as follows. In sample "I", NE proteins are directly subjected to AP-1 DNA affinity chromatography. In samples "II" and "III", NE proteins are preincubated with excess wild type or mutant AP-1 oligo (in solution) prior to AP-1 DNA affinity chromatography. DNA affinity chromatography is then performed, by means adapted from Lee et al., with numerous modifications (Lee et al., 1987; Kumar and Bernstein, 2001a & b). Following NAPSTER isolation, proteins are subjected to SDS-PAGE and Western blotting with antibodies against the proteins of interest.

Validating the 'Proof of Principle' of NAPSTER Assay

As a control for the efficacy of the NAPSTER assay, we tested the specificity of binding of AP-1 subunits to the GALV AP-1 DNA using NAPSTER assay. We performed the NAPSTER assay with NE derived from HT29 human colon carcinoma cells and GALV AP-1 DNA coupled to streptavidin beads for samples I, II and III. In samples II and III of the assay, the NE proteins were preincubated with 2.5 fold excess of wild type or mutant GALV AP-1 oligos in solution respectively, prior to DNA affinity chromatography with wild type GALV AP-1 oligo on beads. Samples from NAPSTER isolation were subjected to SDS-PAGE followed by western blotting with antibodies against the seven AP-1 subunits. As shown in Figure 17, we reproducibly observed specific binding of multiple AP-1 subunits to the AP-1 DNA sequence. These

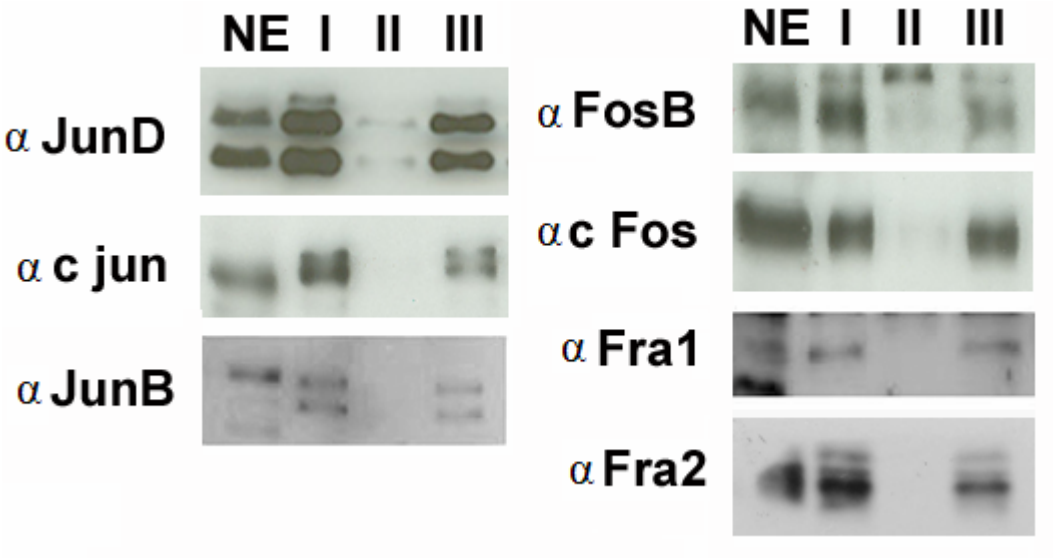


Figure 17 Specific binding of Jun and Fos AP-1 subunits to AP-1 DNA. NAPSTER analysis for all AP-1 subunits with the exception of FRA-1 were performed with 300 µg of HT29 NE protein per sample, and 20 µg of AP-1 DNA on beads. NAPSTERs for Fra-1 were performed with 600 µg of NE protein and 40 µg of beads. Samples I, II and III were divided into six parts and loaded onto SDS-PAGE gels and subjected to immunoblotting with antibodies against the various AP-1 subunits listed next to each blot.

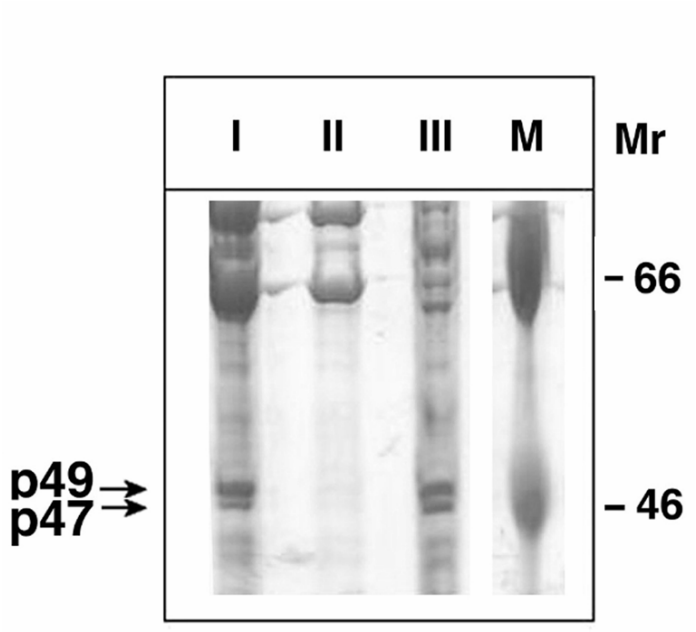


Figure 18 p47 and p49 proteins bind specifically to AP-1 DNA in NAPSTER assay. NAPSTER samples I, II and III were generated from 15 mg of NE proteins as described in Materials and Methods. Coomassie stained bands containing p47 and p49 are indicated by arrows. Mr, Molecular weight marker. Figure reproduced with permission from Dr. Twizere.

data demonstrate that NAPSTER assay efficiently detects specific AP-1 DNA binding proteins.

Nucleolin Binds to AP-1 DNA Specifically

Dr. Twizere isolated a 97 KDa band that bound specifically to AP-1 DNA in NAPSTER assay and identified that p97 was nucleolin using MS/MS sequencing (data not shown). The identity was verified by western analyses of NAPSTER samples with an antibody specific for nucleolin. A single band at 97 KDa was observed in samples I and III, but not II following immunoblotting with α-NCL antibody (data not shown).

p47 and p49 Are Novel AP-1 DNA Binding Proteins

NAPSTER assay was performed as described in Materials and Methods with NE derived from HT29 colon carcinoma cells and GALV AP-1 DNA. Coomassie staining of the gel following the NAPSTER assay revealed two protein bands, at 47 and 49 kDa that were present in lanes corresponding to samples I and III (Figure 18) and not in sample II. These data demonstrate that p47 and p49 are specific AP-1 DNA binding proteins.

Based on the molecular weights and specificity of binding of p47 and p49 proteins to the GALV AP-1 DNA, we initially hypothesized that p47 and p49 proteins were AP-1 family members. To test this hypothesis the p47 and p49 bands were excised from the Coomassie stained gel and run on a second SDS-PAGE gel followed by western blotting with a mixture of antibodies against all seven AP-1 subunits (Figure 19). Although the AP-1 antibodies recognized multiple AP-1 proteins in the NE neither p47 nor p49 proteins were recognized by any of the AP-1 antibodies included in the

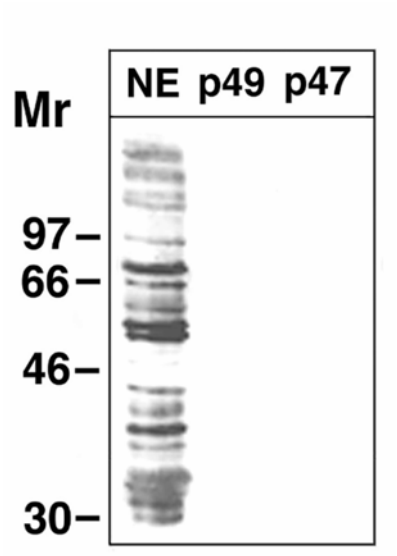


Figure 19 p47 and p49 are not AP-1 proteins. p47 and p49 proteins were purified from sample I of NAPSTER assay starting with 15 mg of NE protein as described in Materials and Methods. Purified proteins were loaded in four lanes in a 10% SDS-PAGE gel to resolve the two protein bands. The bands corresponding to p47 and p49 from one lane were excised from the gel and loaded onto a second gel alongside 30 μg of HT29 NE extract protein (NE, lane 1). The samples were immunoblotted with a mixture of all seven AP-1 antibodies. Mr, Molecular weight markers.

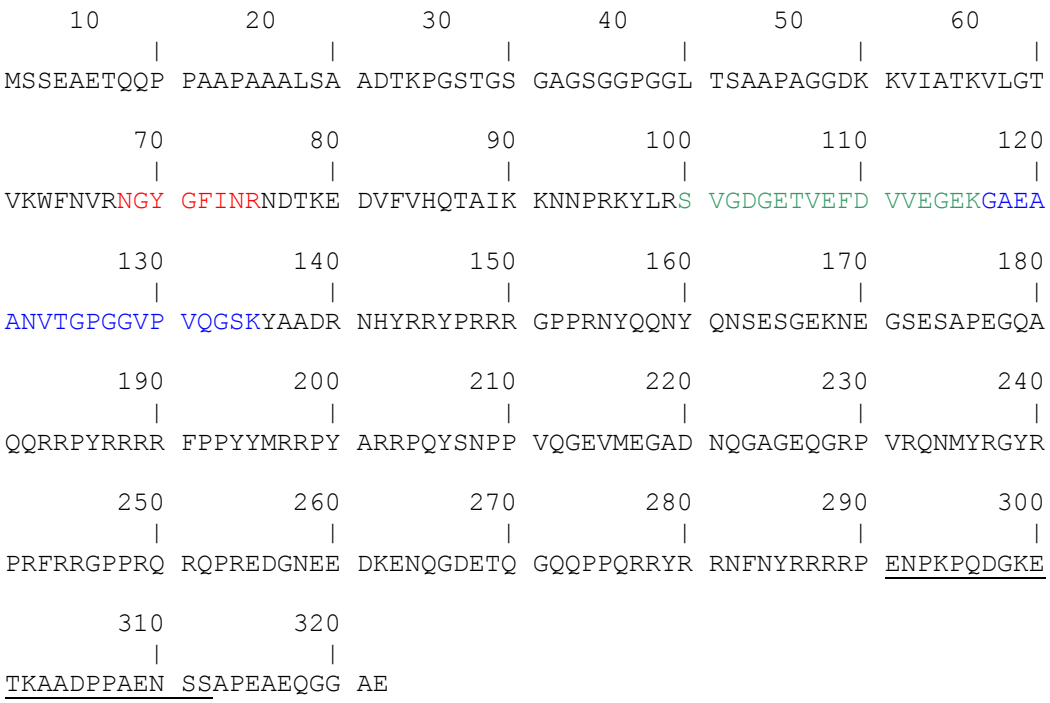


Figure 20 Location of the peptides identified for p47 and p49 bands by MS/MS sequencing in the human YB-1 protein sequence. Peptide sequences from MS/MS data were compared to the human YB-1 protein sequence. p47 peptides sequences that match the YB-1 protein sequence are shown in red and green colors and those for p49 are shown in blue and green colors respectively. The peptide sequence shown in green color was common to both p47 and p49 protein bands. Location of the C1 peptide used for YB-1 antibody production is also shown (underlined).

antibody mix despite having loaded coomassie stained quantities of the proteins (Figure 19, compare lanes 2 and 3 to lane 1). Preincubation of the AP-1 antibodies with specific and nonspecific peptide competitions prior to immunoblotting revealed that the antibodies specifically recognized their corresponding protein bands of the predicted molecular weights (data not shown). These data demonstrate that p47 and p49 are not AP-1 proteins.

Purification and MS/MS Identification of p49 and p47 Proteins as Y-box Binding Protein 1

Purified p47 and p49 protein bands were subjected to MS/MS sequencing. The resulting ion fragmentation spectra were then searched against the nr protein database manually and using the MASCOT peptide mass fingerprint algorithm for protein identification. Positive identifications are made on the basis of two or more MS/MS spectra matches to the same protein entry in the database. As shown in Figure 20, two peptide sequences derived from p49 protein matched the human Y-box binding protein 1 (YB-1). Two peptides from p47 protein band also matched YB-1 protein (Figure 20). These data suggest that p47 and p49 are YB-1.

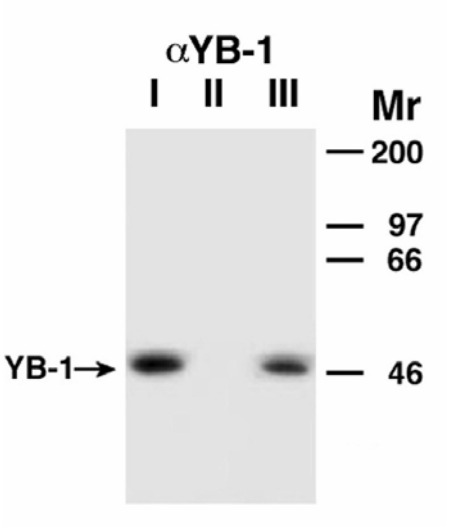
Validation of MS/MS Peptide Identification of YB-1 by Western Blot Analysis

In order to validate the MS/MS identification of YB-1, we performed western blot analysis on samples I, II and III following the NAPSTER assay with αYB-1 antibody. As shown in Figure 21A, YB-1 antibody recognized a specific band at 49 kDa in NE samples and in samples I and III, but not in sample II. The identification of YB-1 in samples I and III, but not in II, provides evidence for the specific binding of YB-1

protein to AP-1 DNA sequence. Preincubation of the YB-1 antibody with specific and nonspecific peptide competitions prior to immunoblotting revealed that the antibody recognized the protein band of the right molecular weights specifically (Figure 21B).

As indicated earlier, MS/MS fragmentation sequencing analyses of two peptides derived from p47 also matched YB-1. We believe that the p47 band observed in the coomassie stained gel is a proteolytic digestion product of YB-1 resulting from extensive processing of the protein samples prior to MS/MS sequencing. While YB-1 routinely visualized by NAPSTER protocol is isolated by a single-step involving wild type DNA affinity chromatography, p47 and p49 purified for coomassie staining and MS/MS sequencing were isolated by a multi-step procedure involving four additional steps including mutant DNA affinity chromatography, KCl elution, TCA precipitation, and acetone precipitation, which may have predisposed the protein to proteolysis. In some routine NAPSTER experiments (3 out of 40), we have visualized a doublet (corresponding to p47 and p49) by immunostaining with αYB-1. This further corroborated our hypothesis that p47 purified for MS/MS analyses is a proteolytic product of p49.

A



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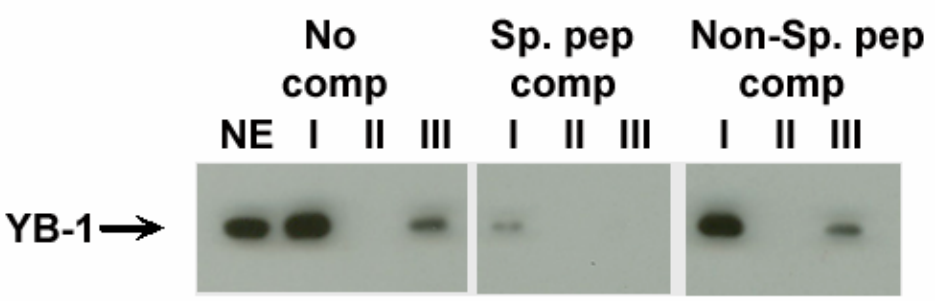


Figure 21 p49 is YB-1. A. 300 μg of HT 29 NE protein for each of samples I, II and III was subjected to NAPSTER analysis, SDS PAGE and then immunoblotted with □YB-1. B. Recognition of YB-1 protein by □YB-1 antibody is specific. 2700 μg of HT29 NE was subjected NAPSTER AP-1 DNA affinity chromatography (Sample I, II and III) and divided into three equal portions that were loaded in SDS PAGE and immunoblotted with □YB-1 antibody either directly ("No comp") or following preincubation with YB-1 peptide antigen ("Sp. pep comp") or with non specific JunD peptide ("non-Sp. pep comp") as described (Kumar and Bernstein, 2001).

YB-1 has been reported to bind to single-stranded DNA (Mertens et al., 1997). The double stranded AP-1 DNA beads are generally prepared by annealing equimolar ratios of the complementary strands prior to coupling to the affinity beads. To rule out the possibility of contaminating quantities of beads with single-stranded AP-1 DNA, the beads used in this experiment (Figure 22) were coupled to double-stranded AP-1 DNA made by annealing the biotinylated strand with at least five-fold molar excess of the complementary strand. This data demonstrates that YB-1 binds specifically to double stranded AP-1 DNA. In all the experiments henceforth, AP-1 beads were coupled to double stranded AP-1 DNA made by annealing the biotinylated strand with at least 1.5-fold excess of the complementary strand.

YB-1 from HeLa Cells also Bind Specifically to AP-1 DNA

To test whether AP-1 DNA binding of YB-1 could be observed in a different cell line and to test our hypothesis that YB-1 from HeLa NE would bind specifically to AP-1 DNA in NAPSTER, we performed NAPSTER with NE from HeLa cells as described in Materials and Methods. We observed specific binding of HeLa YB-1 to AP-1 DNA (Figure 22). As a control, specific binding of JunD from HeLa cells was also visualized. We chose HeLa cells because they are more amenable to further studies involving transfections compared to HT29 cells, which have very low transfection efficiency. Specific binding of nucleolin from HeLa cells to AP-1 DNA was also observed (data not shown). These data demonstrate that HeLa cells can be used for further characterization of AP-1 DNA binding of these proteins.

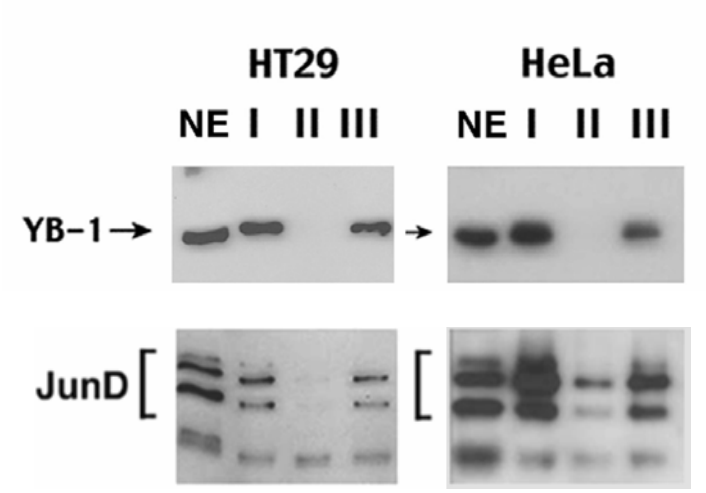


Figure 22 YB-1 from HeLa cells also binds specifically to AP-1 DNA in the NAPSTER assay. 300 μg of NE protein from both HT29 and HeLa cells were subjected to NAPSTER analyses for each of samples I, II, and III, followed by SDS-PAGE alongside 30 μg of NE protein and immunoblotting analyses with αYB-1 antibody (upper panels) and with αjunD antibody (lower panels). NE, nuclear extract.

YB-1 and Nucleolin Colocalize in the Nucleus

We observed specific binding of both YB-1 and nucleolin to AP-1 DNA. In order for this observation to be physiologically relevant, both YB-1 and nucleolin would need to localize to the nucleus *in vivo*. We hypothesized that endogenous YB-1 and nucleolin are present in the nucleus and our prediction was that both YB-1 and NCL would be detected in the nucleus. To test this hypothesis, cultured HeLa cells were subjected to immunocytochemical staining using an αYB-1 and αNCL antibodies. As shown in Figure 23, YB-1 immunostaining was detected in both the cytoplasm and the nucleus. Nucleolin immunostaining was detected mainly in the nucleus with more intense staining in the nucleoli. There was hardly any nucleolin detectable in the cytoplasm. These data demonstrate that both YB-1 and nucleolin are present in the nucleus.

YB-1 and Nucleolin Do Not Co-Immunoprecipitate

Since we observed colocalization of YB-1 and NCL proteins in the nucleus, and specific binding of both proteins to AP-1 DNA, we hypothesized that YB-1 and NCL may interact with each other through protein-protein interactions. If YB-1 and NCL interact with each other, then our prediction was that YB-1 and NCL could be reciprocally coimmunoprecipitated. Although, it should be noted that failure to detect coimmunoprecipitation of either protein would not rule out the possibility that they do interact, for the reasons that the conformation of transfected proteins, immunoprecipitation conditions, and the antibodies used may not be optimum for

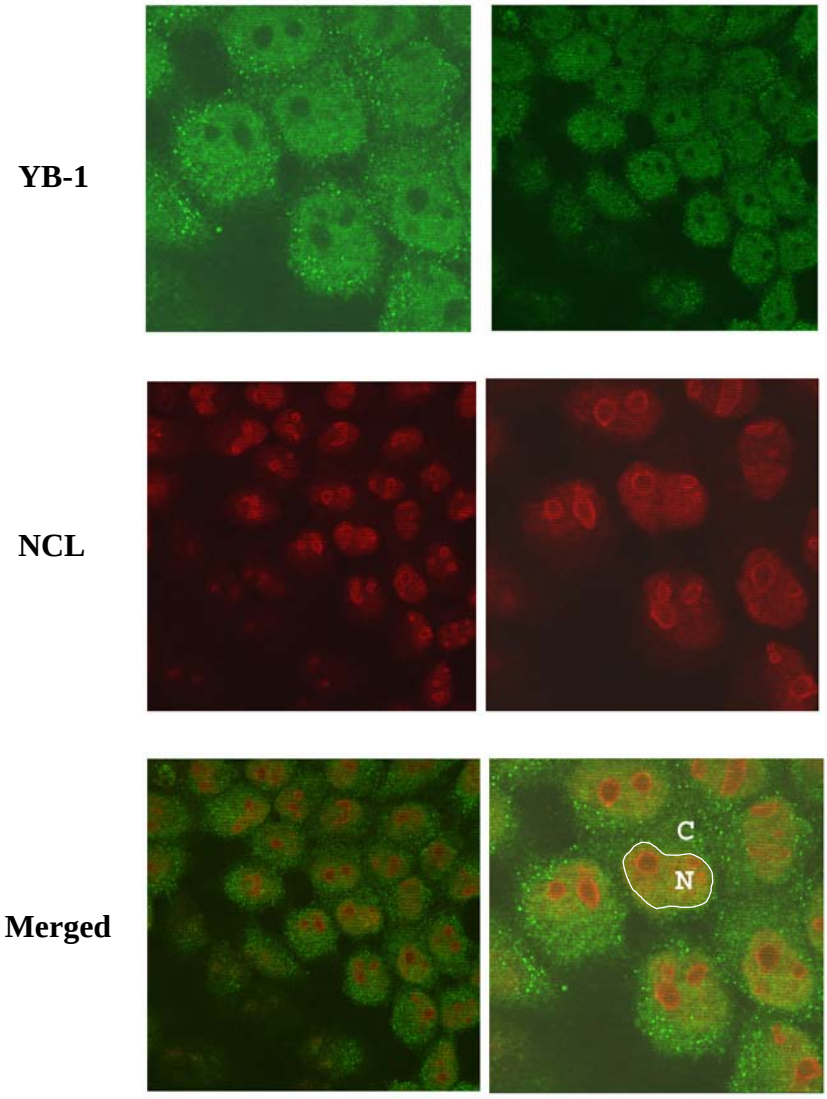


Figure 23 YB-1 and nucleolin colocalize in the nucleus. HeLa cells were immunostained with αYB-1 primary antibody at 1:1000 dilution followed by flourescein-coupled anti rabbit secondary antibody (left panel) or with αNCL primary antibody followed by Texas red coupled anti mouse secondary antibody (middle panel). Top row, probed with αYB-1 antibody; Middle row, Probed with αNCl antibody; Bottom row, Merged images. Left column: 20X magnification; Right column: 60X magnification. N, nucleus; C, cytoplasm.

detection of such an interaction. To test whether NCL could be coimmunoprecipitated with YB-1, HeLa cells were transiently transfected with both HA-NCL and Flag-YB-1 overexpression constructs (Figure 24, lanes 1 and 2). Cell lysate was immunoprecipitated with α-Flag antibody followed by western with α-HA antibody. Control immunoprecipitation was performed with α-His-antibody. No coimmunoprecipitation of nucleolin was detected. As control for efficacy of α-Flag antibody in immunoprecipitation, probing with αFlag antibody in western revealed that α-Flag antibody pulled-down Flag tagged YB-1 protein. Furthermore, cell lysates tested positive for overexpression of the tagged nucleolin protein (Figure 24, lower right panel).

As mentioned earlier, it is conceivable that the overexpressed Flag-YB-1 protein may not have achieved the optimum conformation necessary for interaction with NCL protein. We therefore pursued an alternate approach to detect the interaction of NCl with endogenous YB-1 protein by immunoprecipating NCL with α-YB-1 antibody. HeLa cells were transiently transfected with HA-nucleolin expression construct. Cell lysate was immunoprecipitated with α-YB-1 antibody followed by western with anti-HA antibody. No coimmunoprecipitation of nucleolin was detected (data not shown). Control immunoprecipitation was performed with α-GFP antibody. As control for efficacy of α-YB-1 antibody in immunoprecipitation, probing with α-YB-1 antibody in western revealed that YB-1 antibody pulled-down YB-1 protein. Cell lysates tested positive for overexpression of the tagged nucleolin protein (data not shown).

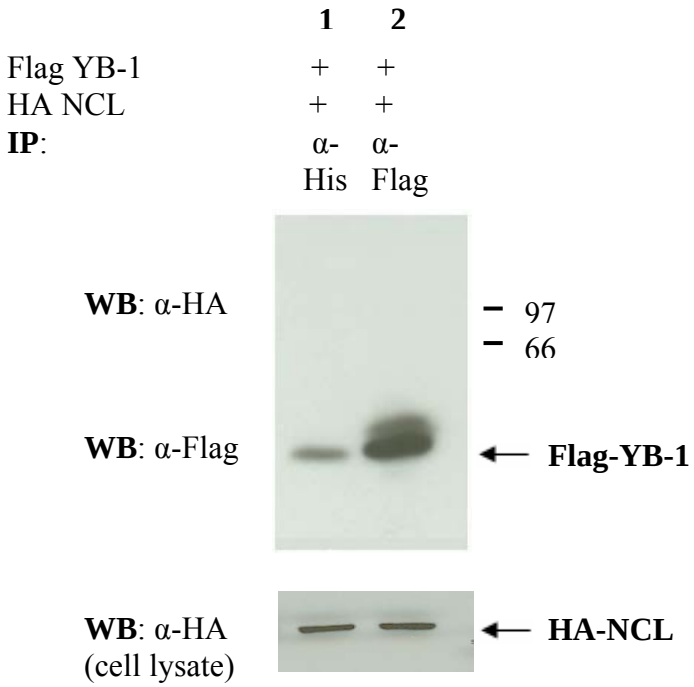


Figure 24 YB-1 and nucleolin do not co-immunoprecipitate. Hela cells were transfected with Flag-YB-1 and HA-NCL constructs as indicated with "+" signs above the panels. In the top left panel, cell lysates were immunoprecipitated with α-His or α-Flag antibodies followed by western with α-Flag or α-HA antibodies. In the lower right panel, western blot analyses on whole cell lysate was done with α-HA antibody.

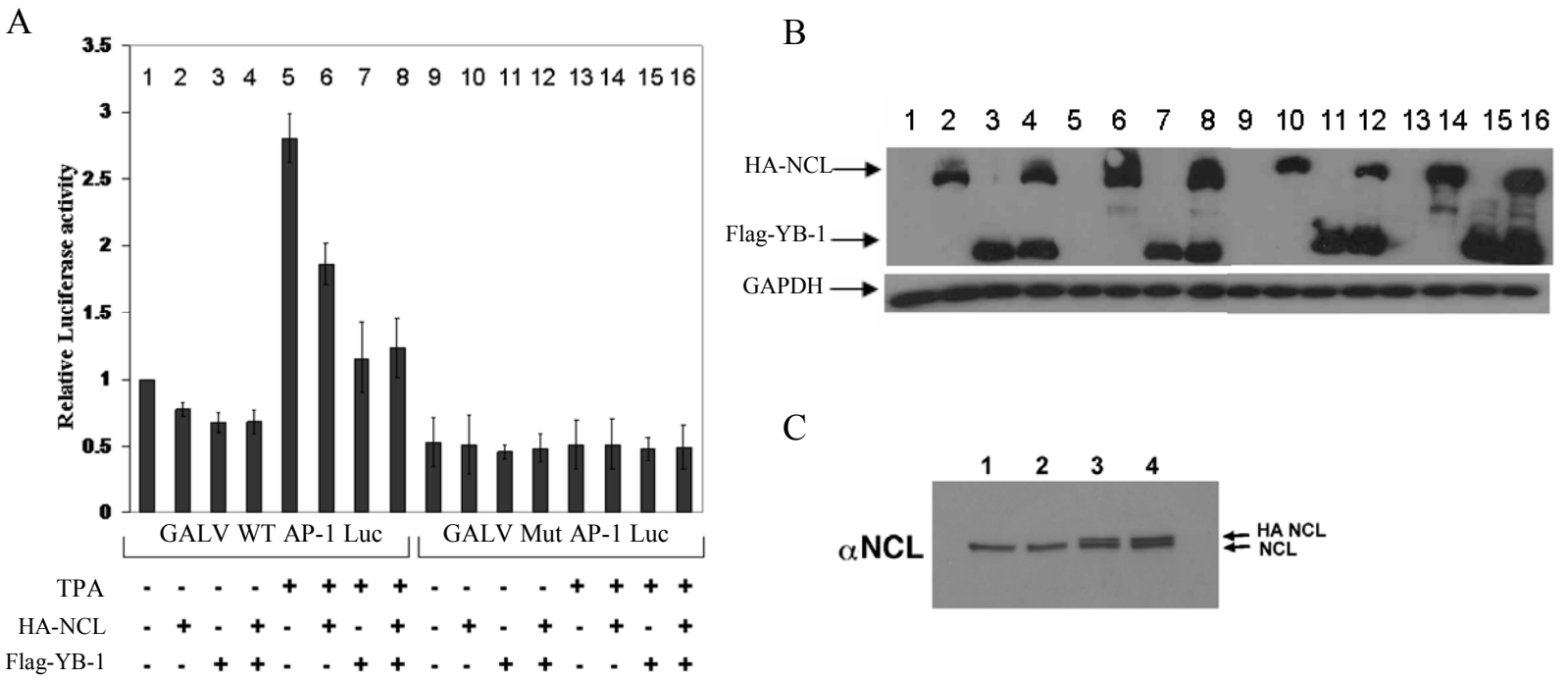


Figure 25 YB-1 and NCL repress TPA induced AP-1 transactivation in HeLa cells. A. 3 x 10^5 HeLa cells were transiently co-transfected with pcDNAFlagYB-1, pNtag-HA-NCL overexpression constructs and wild type or mutant 3XGALV-AP-1-luciferase reporter constructs. Cells were then treated TPA or DMSO solvent control for 24 hours. Presence or absence of TPA or of specific reporter constructs is indicated by "-" and "+" under the bar graphs. Bars bracketed by "GALV WT AP-1 luc", 3X Wild type AP-1-luciferase reporter tranfectants; Bars bracketed by "GALV Mut AP-1 luc", 3X mutant AP-1-luciferase reporter tranfectants. Samples are numerically indicated above the bar graphs. All data were normalized to β-galactosidase expression. Error bars represent standard error of the mean (SEM) of five independent experiments, each performed in duplicate. B. Tagged YB-1 and NCL proteins are overexpressed in the transient transfectants. Overexpression of YB-1 and NCL in transient transfectants were confirmed by immunoblotting of 10μg of lysates from vector control (pcDNA transfectant), YB-1 or NCL transfected cells with αFlag/αHA antibodies. Equal protein loading was verified by immunoblotting with αGAPDH antibody. C. NCL is overexpressed in transient transfectants. Overexpression of NCL in transient transfectants was confirmed by immunoblotting of 10 μg of lysates from (1) vector control (pcDNA transfectant), (2) YB-1, (3) NCL, or (4) YB-1 and NCL transfected cells with αNCL antibodies. Arrows indicate HA-tagged and endogenous NCL proteins.

YB-1 and Nucleolin Repress TPA Induced AP-1 Transactivation

The nuclear localization of YB-1 and NCL and their specific binding to AP-1 DNA raised the possibility that YB-1 and NCL may regulate AP-1-dependent gene expression directly or indirectly. We hypothesized that YB-1 and NCL bind to AP-1 DNA and regulate AP-1 transactivation. If YB-1 and NCL regulate AP-1 transactivation, then activation or repression of an AP-1 containing promoter would be detectable in transactivation assays. Functional studies were therefore performed in HeLa cells to determine the effect of YB-1 and NCL on the transactivation of AP-1 dependent gene expression. The wild type 3XAP-1-GALV-luc reporter gene construct was transiently cotransfected along with the YB-1 (pcDNA-Flag-YB-1) and/or NCL (pNtag-HA-NCL) and β-galactosidase (pSVβgal) expression vectors along with the empty vector control (pcDNA3.1) into HeLa cells.

The data for TPA and c-*fos* transactivation experiments presented in Figures 25A and B, and 26A and B using the GALV AP-1 reporter constructs were generated by Dr. Twizere and are reproduced here with his permission. As shown in Figure 25A, stimulation by TPA resulted in close to 3-fold increase in luciferase activity. YB-1 and NCL, both alone and together, significantly repressed TPA-induced AP-1 transactivation of the reporter construct containing the wildtype AP-1 sequence. NCL caused 52% repression of TPA-induced transactivation (Figure 25A, compare bar graphs 5 and 6), YB-1 caused 92% repression (compare bar graphs 5 and 7), and the combination of overexpressed NCL and YB-1 caused 85% repression (compare bar graphs 5 and 8). In contrast, both YB-1 and NCL did not repress TPA-induced transactivation of the

reporter construct containing mutant AP-1 sequence demonstrating that the repression was AP-1 sequence specific. Also as a control, YB-1 and NCL did not significantly affect transactivation from a constitutive cytomegalovirus luciferase (pCMV-luc) construct, lacking a functional AP-1 site (data not shown). This indicates that the suppressive activity by YB-1 and NCL occur specifically through the AP-1 site. To show that YB-1 and NCL were overexpressed in the transiently transfected cells, whole cells lysates from vector control, Flag-YB-1 and HA-NCL transfected HeLa cells were analyzed by western blot analysis. As shown in Figure 25B, proteins of the predicted size for YB-1 (50 kDa) and NCL (97 KDa) were immunodetected with αFlag and αHA antibodies respectively. Increase in total protein levels (endogenous and transfected) was analyzed by immunoblotting with αYB-1 and αNCL antibodies. While no increase in total intracellular protein level was detectable for YB-1, NCL protein level increased 1.7-fold (Figure 25C). The lack of detection of increase in total YB-1 levels is most likely due to a failure of the αYB-1 antibody to recognize the flag-tagged YB-1 protein. One possible explanation is that the flag tag in the overexpressed YB-1 protein alters the protein conformation such that the αYB-1 antibody epitope on the protein is no longer exposed and/or accessible. To determine whether the YB-1 and NCL mediated transactivational repression was due to decreased cell viability resulting from NCL and YB-1 overexpression constructs, cell viability was assessed following transfections by several independent methods. Trypan blue dye exclusion assays demonstrated that between 97% and 100% of the cells were viable after transfection of NCL and/or YB-1.

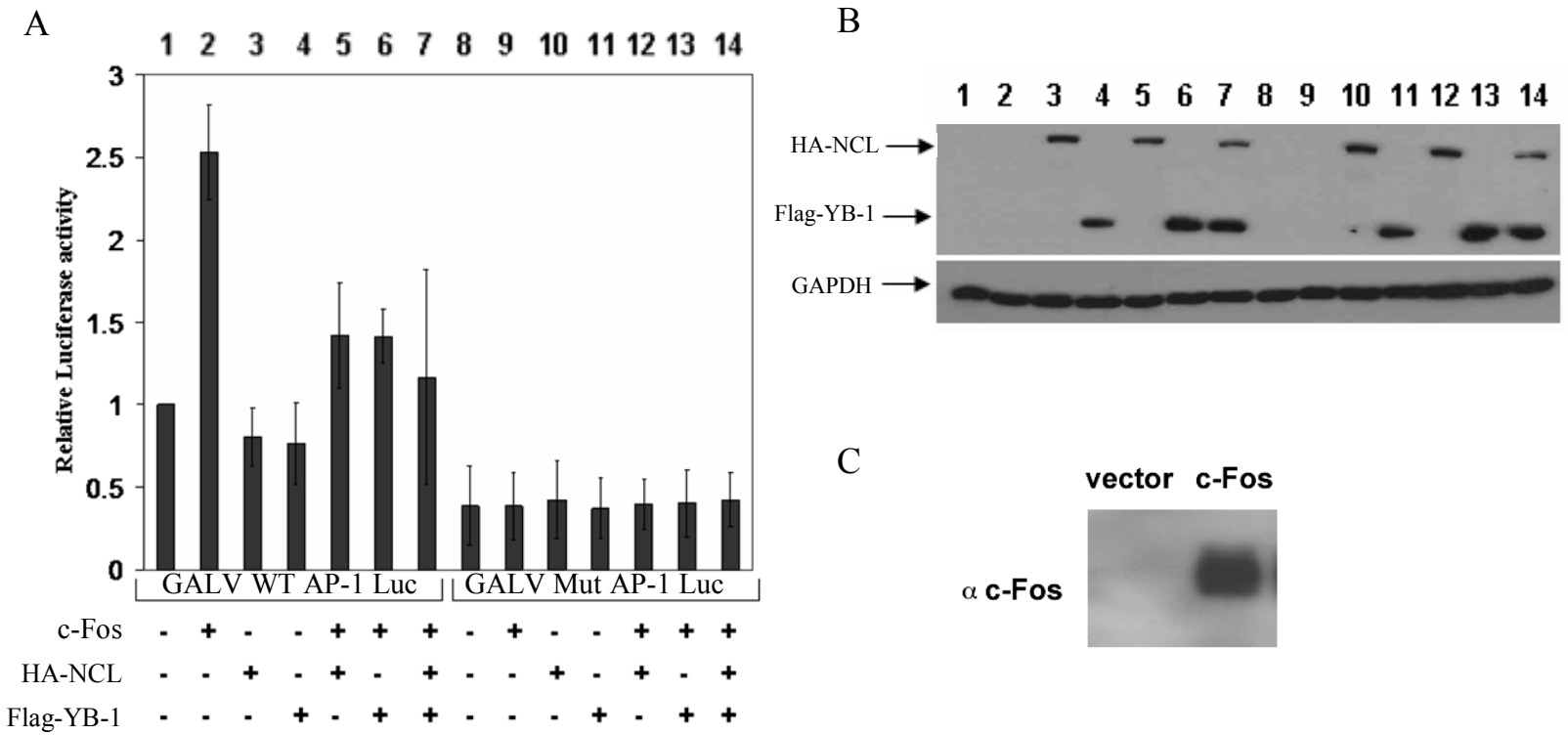


Figure 26 YB-1 and nucleolin repress c-fos induced transactivation in HeLa cells. A. HeLa cells were co-transfected with 10 ng of pcDNAc-fos and 1 µg of pcDNAFlag-YB-1 or pNtag-HA-NCL constructs along with wild type or mutant GALV-AP-1 reporter constructs as described in Materials and Methods. Presence or absence of transfected constructs indicated by "+" and "-" signs. Bars bracketed by "GALV WT AP-1 luc", 3X Wild type AP-1-luciferase reporter tranfectants; Bars bracketed by "GALV Mut AP-1 luc", 3X mutant AP-1-luciferase reporter tranfectants. B. Tagged YB-1 and NCL are overexpressed in the transient transfectants. Overexpression of tagged YB-1 and NCL in transient transfectants was confirmed by immunoblotting of 10 µg of lysates from vector control (pcDNA transfectant) and YB-1 and/or NCL transfected cells with αFlag/αNCL antibodies. Equal protein loading was verified by immunoblotting with αGAPDH antibody. C. Transfection with c-Fos increases total c-Fos protein levels. 10 µg lysates from vector control (pcDNA transfectant) and c-Fos transfected cells were immunoblotted with α c-Fos antibody.

YB-1 and Nucleolin Repress Transactivation by c-Fos in HeLa Cells

The effect of YB-1 and NCL on transactivation by co-transfected c-fos was also examined by Dr. Twizere. Wild type or mutant AP-1 luciferase reporter constructs were transiently cotransfected along with the expression constructs, (1) *c-fos* (pcDNA*c-fos*); (2) YB-1 (pcDNA-Flag-YB-1) and/or NCL (pNtag-HA-NCL) or empty vector (pcDNA3.1) and, (3) β-galactosidase construct (pSVβgal) into HeLa cells. YB-1 and NCL significantly repressed c-Fos induced AP-1 transactivation of luciferase expression at the wild type AP-1 site but not the mutant AP-1 site (Figure 26A). NCL caused 72% repression of *c-fos* mediated transactivation of the wild type AP-1 reporter construct, YB-1 caused 73% repression, and the two proteins together caused 89% repression. Overexpressed c-*fos*, NCL, and YB-1 had no effect on levels of expression of the mutant AP-1 reporter. Overexpression of tagged YB-1 and NCL was confirmed by immunoblotting cell lysates from control and transfected cells with αFlag and αNCL antibodies respectively (Figure 26B). Overexpression of transfected c-Fos was also verified by immunoblotting cell lysates from vector control and c-fos transfected cells with αc-Fos antibody (Figure 26C).

YB-1 but Not Nucleolin Binds Specifically to GCN AP-1 DNA in NAPSTER Assay

We reproducibly observed specific binding of YB-1 and NCL proteins to the GALV AP-1 DNA sequence in NAPSTER assays. To test whether the specific binding of YB-1 and NCL to AP-1 site was limited to the GALV AP-1 DNA sequence, we performed NAPSTER with an AP-1 sequence from yeast GCN promoter (5'-TCGACTATGATGAGTCATGGGGC-3') as competitor oligos (minimal AP-1

sequence is shown underlined). NAPSTER was performed with GALV AP1 DNA sequence on beads, but with wild type or mutant GCN AP-1 oligos as competitors in samples II and III. We also performed NAPSTER assay with wild type or mutant GALV AP-1 oligos as competitors, in parallel. As shown in Figure 27, YB-1 was specifically competed by both the wild type GALV AP-1 and GCN AP-1 oligos but not by the corresponding mutant oligos. NCL was specifically competed by wild type GALV AP-1 but not by GCN AP-1 oligo. The binding of NCL to GCN AP-1 oligo appears to be non-specific in this experiment. These data indicate that the precise binding sites for YB-1 and NCL on the AP-1 sequences may be different. The GALV AP-1 and GCN AP-1 oligos differ in the sequences of their flanking regions. It is possible that the NCL binding site on the GALV AP-1 sequence spans the minimal AP-1 site as well as a portion of the flanking sequence and that specific binding of NCL to the AP-1 site is partially dependent on the sequence of the flaking region. c-Jun binding and transactivation from AP-1 site also depends on the flanking regions of the consensus sequence (Ryseck et al., 1991; Herdegen and Leah, 1998). An interesting observation in this and a few other NAPSTER experiments concerns a slow migrating form of NCL with an apparent molecular weight of about 200 KDa which also binds specifically to the GALV AP-1 DNA in some experiments. The size of this NCL protein form in SDS-PAGE and recognition by αNCL antibody indicate that it is possibly a dimeric form of NCL however, the reasons for the sporadic detection of this form in NAPSTER are not yet clear.

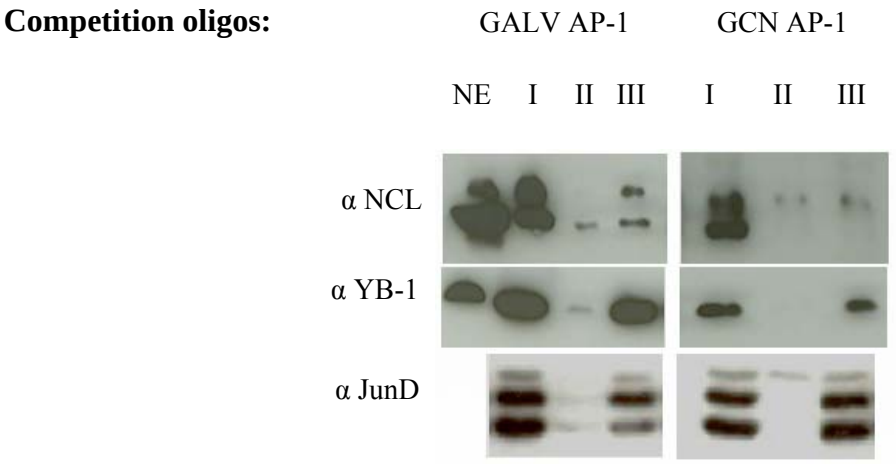
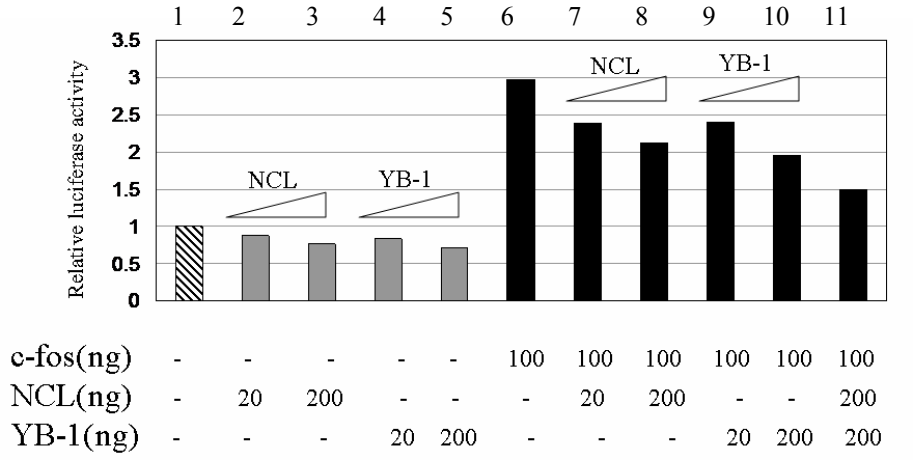


Figure 27 YB-1 but not nucleolin binds specifically to GCN AP-1 DNA in NAPSTER assay. NAPSTER analyses were performed with 300 μg of HT29 NE protein and 20 μg of GALV AP-1 DNA on beads per sample in two sets. In the first set, samples II and III were preincubated with 50 μg of wild type or mutant GALV AP-1 oligos (left panels). In the second set, samples II and III were preincubated with 50 μg of wild type or mutant GCN AP-1 oligos (right panels). Samples I, II and III from both sets were loaded onto SDS-PAGE gels alongside 30 μg of NE protein and subjected to immunoblotting with antibodies against those listed next to each blot.

A

B



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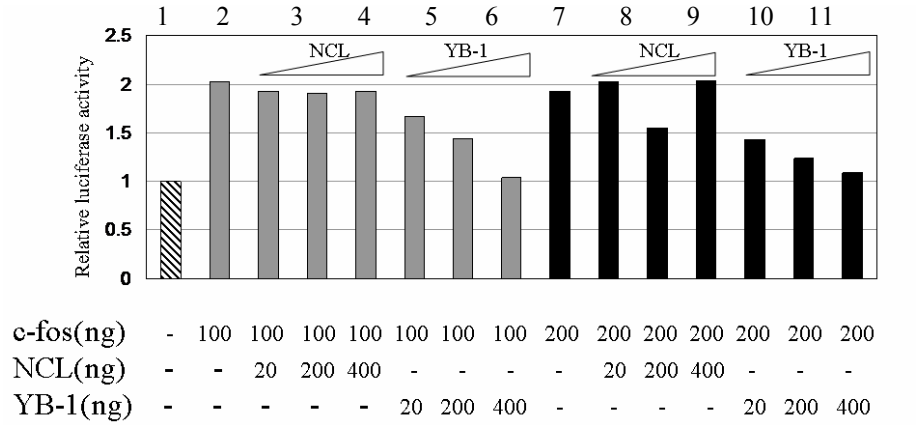


Figure 28 YB-1 but not nucleolin represses c-fos induced transactivation at GCN AP-1 site. A. Schematic of the 4X GCN AP-1 luciferase repoter construct used. B. HeLa cells were transfected with 4XGCN-AP-1 reporter constructs along with none or 100 ng of pcDNAc-fos and various amounts of pcDNAFlag-YB-1 and/or pNtag-HA-NCL constructs as shown below corresponding bar graphs in the Figure. Samples are numerically indicated above the bar graphs. All data were normalized to β-galactosidase expression. C. HeLa cells were transfected with 4XGCN-AP-1 reporter constructs along with 100 or 200 ng of pcDNAc-fos and increasing amounts of pcDNAFlag-YB-1 and/or pNtag-HA-NCL constructs as shown below corresponding bar graphs in the Figure. Samples are numerically indicated above the bar graphs. Data represent two independent experiments.

YB-1 but Not Nucleolin Represses Transactivation from GCN AP-1 Reporter

YB-1 and NCL mediated AP-1 transrepression with a GALV AP-1 DNA reporter construct. To test whether YB-1 and NCL mediated AP-1 transrepression would occur at an AP-1 site from an alternate promoter, transactivation assays were performed with a 4X GCN AP-1 luciferase reporter construct (4X GCN AP-1 luc). Since the binding of YB-1 to GCN AP-1 sequence was specific, but that of NCL to GCN AP-1 sequence was non-specific (Figure 28), we hypothesized that YB-1 but not NCL would transrepress the GCN AP-1 reporter expression. The 4X GCN AP-1 luc consisted of four tandem GCN AP-1 sequences upstream of a luciferase reporter gene. 4X GCN AP-1 luc construct was transiently cotransfected along with the expression constructs for (1) c-Fos (pcDNAc-fos); (2) YB-1 (pcDNA-Flag-YB-1) and/or NCL (pNtag-HA-NCL); (3) β-galactosidase (pSVβ gal) or the empty vector (pcDNA3.1) into HeLa cells. Consistent with our hypothesis, YB-1 significantly repressed *c-fos* induced AP-1 transactivation of luciferase expression at all concentrations tested (Figure 28B and C). Furthermore, YB-1 mediated repression of AP-1 transactivation was YB-1 dose-dependent with an increase in repression observed as a function of increased dosage of YB-1 (Figure 28C, compare bars 6, 7 and 8 with 2; compare bars 13, 14 and 15 with 9). YB-1 caused up to 95% repression of c-fos mediated transactivation of the reporter at the highest dose (400 ng of transfected DNA) tested. The results for NCL were inconclusive. NCL repressed AP-1 transactivation in one out of three experiments (Figure 28B, compare bars 7 and 8 with 6) while NCL did not show significant repression or any dose dependent correlation of AP-1 transrepression with the GCN AP-1 reporter, in two out of three experiments

(Figure 28C, compare bars 3, 4 and 5 with 2; compare bars 10, 11 and 12 with 9). The repression data for NCL in the two experiments correlate with the non-specific binding of NCL to competitor GCN AP-1 sequences observed in NAPSTER assays (Figure 27).

YB-1 and/or Nucleolin Overexpression Does Not Affect AP-1 Protein Level

YB-1 and NCL mediated repression of AP-1 transactivation could potentially occur in several different ways. One possible mechanism could be via YB-1 or NCL mediated decrease in the levels of AP-1 proteins. To examine whether the coexpression of YB-1 or NCL affected the intracellular level of any of the AP-1 subunits, whole cell lysates were generated from pcDNA control and Flag-YB-1 or HA-NCL transfected HeLa cells treated with or without TPA. The expression levels of all seven AP-1 proteins were measured following overexpression of YB-1 and NCL proteins. As shown in Figure 29A and B, the levels of the Jun and Fos family members were not significantly affected by overexpression of YB-1 or NCL. Additionally, the TPA inducibility and gel-migration pattern of all the seven AP-1 subunits was consistent with previously published data (Kovary and Bravo, 1991; Bernstein et al., 1992). Overexpression of YB-1 and NCL were confirmed by immunoblotting with αFlag and αHA antibodies respectively (Figure 29C). These data demonstrate that YB-1 and NCL mediated repression of AP-1 transactivation does not occur via decrease in AP-1 protein levels.

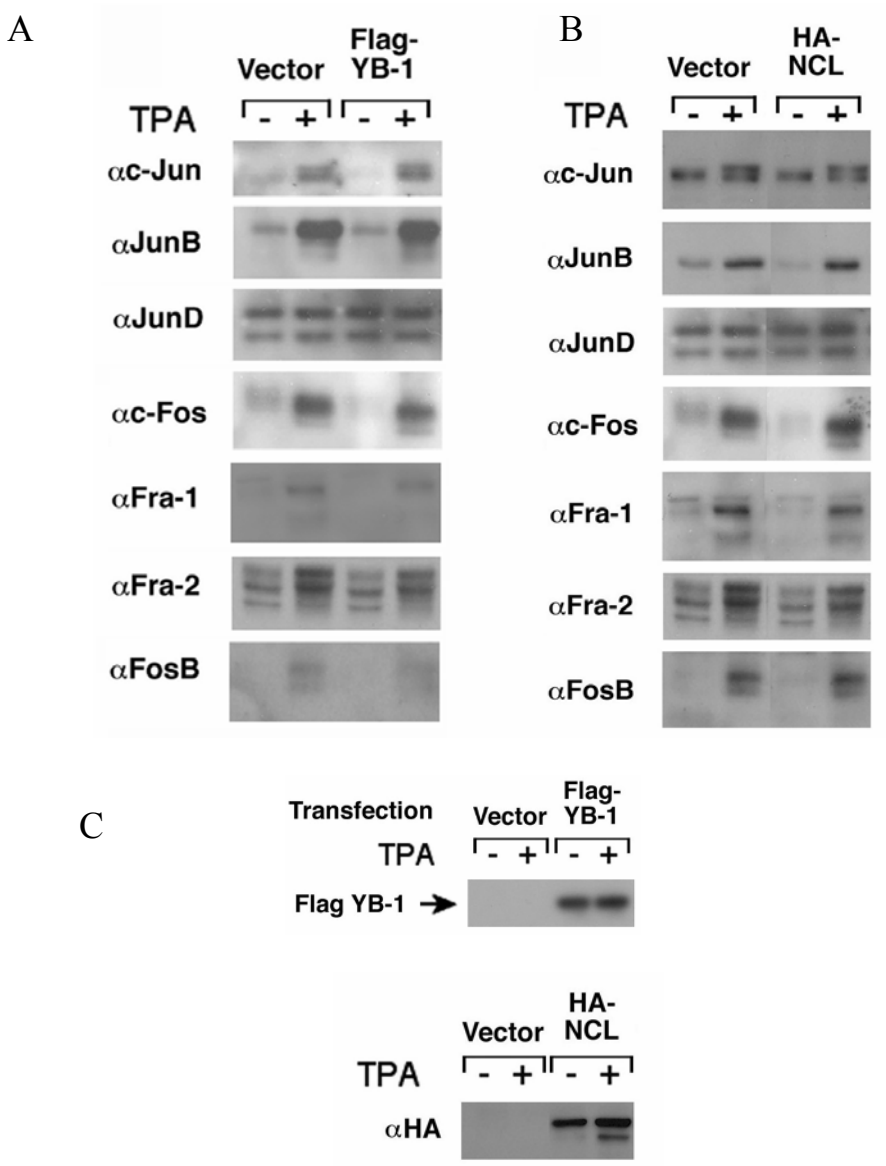


Figure 29 Overexpression of YB-1 and nucleolin do not affect the intracellular levels of AP-1 subunits. A. and B. 4.5 x 10^5 HeLa cells plated in 150 mm tissue culture dishes were transiently transfected with 20 µg of pcDNA3.1 vector control or with pcDNAFlag-YB-1 or pNtag-HA-NCL constructs. Whole cell lysates were prepared after treating the cells with 100ng/ml of TPA for 24 hours. 10 µg of total proteins per sample were separated on SDS-PAGE and immunoblotted with antibodies against all the seven AP-1 subunits. C. Transient transfectants overexpress tagged YB-1 and nucleolin proteins. 10 µg of protein per sample were subjected to SDS PAGE followed by western blot analysis with αFlag or αHA antibody. Brackets indicate the constructs used in the transfections. "-", no TPA; "+", with TPA.

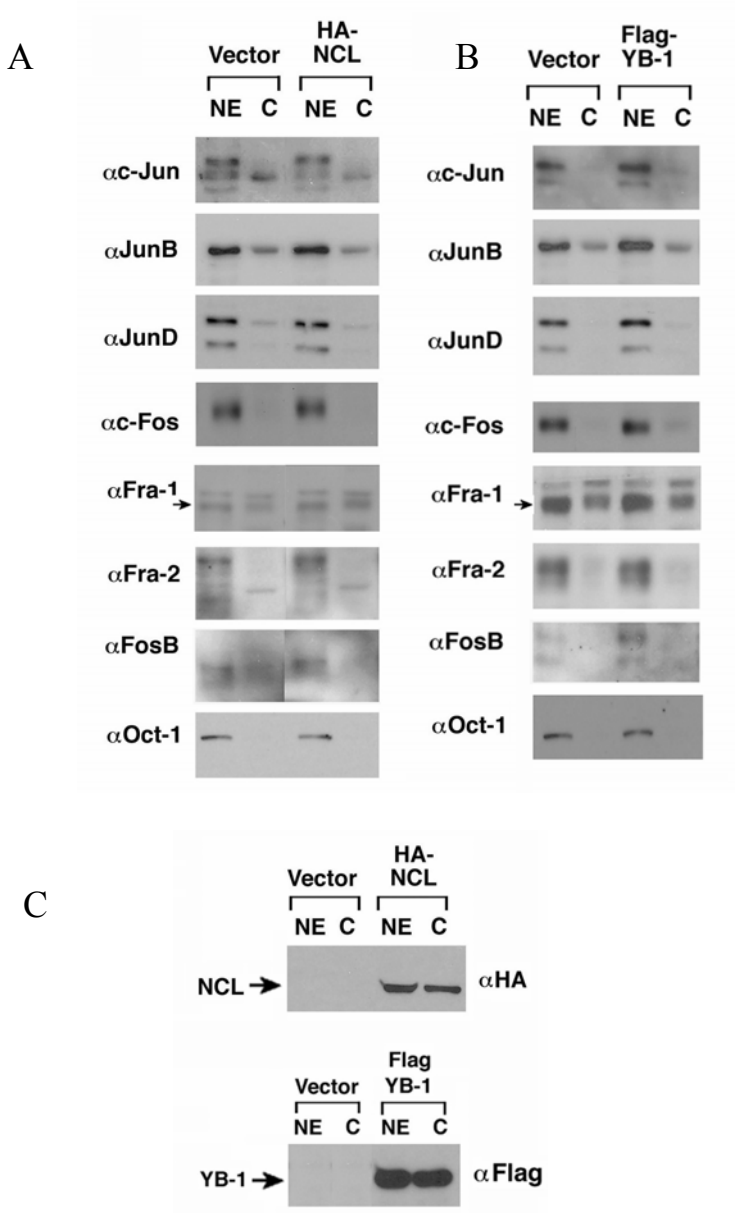


Figure 30 Overexpression of YB-1 and nucleolin do not affect the subcellular localization of AP-1 subunits. A. and B. Hela cells were transfected with vector control or flag YB-1 or HA-NCL and treated with TPA for 24 hours. The cells then underwent extraction to isolate the cytosolic and nuclear fractions. Extracts from each fraction corresponding to 4 x 10^5 cells per sample was subjected to SDS-PAGE followed by immunoblotting with antibodies against all the seven AP-1 subunits and with αOct-1 antibody. NE, nuclear extract; C, cytosol. C. Transfected cells overexpress tagged YB-1 and nucleolin protein in nucleus and cytosol. 10 μg of total protein per sample of nuclear extract and cytosol from Flag YB-1 and HA-NCL transfected cells were subjected to SDS PAGE followed by western blot analysis with αFlag or αHA antibody.

YB-1 and Nucleolin Overexpression Does Not Inhibit Nuclear Localization of AP-1 Proteins

A second possible mechanism of repression could be via YB-1 or NCL mediated decrease in nuclear localization of the AP-1 proteins. In order for AP-1 proteins to bind to the AP-1 site and transactivate gene expression, they need to translocate from the cytoplasm into the nucleus. To determine whether overexpression of YB-1 or NCL decreased the nuclear localization of the AP-1 proteins, YB-1 or NCL transfected HeLa cells were fractionated into nuclear and cytosolic fractions. Levels of all seven AP-1 components were then analyzed in the two cellular fractions by immunoblotting. Nuclear and cytoplasmic levels of all seven AP-1 components were not altered by overexpression of YB-1 or NCL (Figure 30A and B). Most AP-1 subunits localized to the nucleus. Fra-1 and JunB were also detectable in the cytosolic compartment of both vector and YB-1 or NCL transfected cells, although the role for these transcription factors in the cytosol is unclear. Additionally, two bands were detected for Fra-1 protein both in the cytosol and nuclear compartments with the faster migrating form detected at higher levels in the nuclear fraction. Three isoforms of c-Jun with different electrophoretic mobilities were detected and possibly represent differentially phosphorylated forms of the protein. Immunoblotting with ♕Flag and ♕HA antibodies confirmed that tagged YB-1 and nucleolin were overexpressed, with transfected proteins detectable in both cellular compartments (Figure 30C). Immunobloting with an antibody against Oct-1 (a nuclear transcription factor) demonstrated exclusive localization of Oct-1 to the nucleus, confirming that nuclear and cytosolic fractions were free from cross-

contamination. These data demonstrate that YB-1 and NCL mediated repression of AP-1 transactivation does not occur via changes in AP-1 protein localization in the cytosolic and nuclear compartments.

Establishing Equilibrium Binding Conditions for YB-1, Nucleolin and AP-1 Jun Subunits

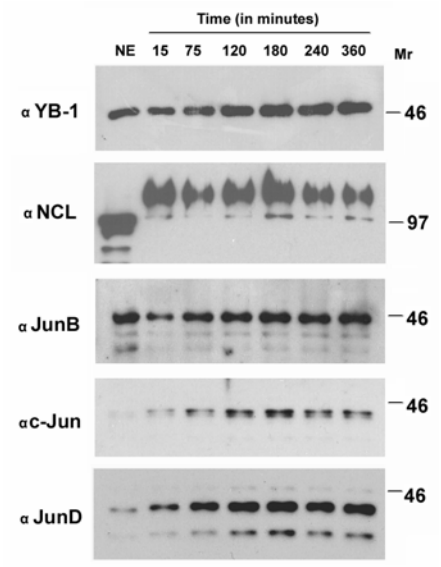
Having ruled out YB-1 and NCL mediated decrease in AP-1 protein levels and localization in the subcellular compartments as possible mechanisms of repression, we hypothesized that YB-1 and NCL mediate repression of AP-1 transactivation via decreasing AP-1 binding to AP-1 site. One method to test this notion would be to analyze the binding of each of the AP-1 subunits to AP-1 DNA, by the NAPSTER assay, in cells overexpressing YB-1 or NCL proteins. The prediction then would be that overexpression of YB-1 and NCL would cause decrease in AP-1 DNA binding. However, this seemingly simple experiment posed several challenges. The first issue was the sheer size of the experiment of precisely analyzing the binding of each of the seven AP-1 subunits in a quantitative manner. However, because Fos subunits can only bind to the AP-1 DNA following heterodimerization with one of the Jun family members, analysis of the binding of just the three Jun members would be equally informative.

The second issue pertained to possible differences in DNA binding kinetics of each of the proteins. The incubation time of the DNA-protein interactions should be sufficient to reach binding equilibrium for each of the proteins. Additionally, the concentration of DNA and protein had to be optimized. In order to observe any change in binding of the jun proteins to AP-1 DNA following YB-1 and NCL overexpression,

concentrations of DNA should be limiting for each of the Jun proteins. This involved titrations of various DNA-protein ratios. These two critical parameters needed to be optimized.

Since both YB-1 and NCL are fairly abundant proteins in cells, we reasoned that in order to detect a decrease in the binding of AP-1 subunits to the AP-1 DNA, the DNA binding conditions would have to be in equilibrium for all the proteins concerned including YB-1, NCL and the AP-1 proteins. To begin, we sought to determine such an equilibrium binding condition. AP-1 DNA affinity chromatography (NAPSTER sample I) was performed using increasing durations of DNA-protein interaction times. An initial titration of concentrations of DNA and NE proteins revealed that DNA was limiting for all proteins including YB-1, NCL, JunB, c-Jun and JunD at a 1:40 ratio (data not shown). We restricted our analyses to the Jun AP-1 subunits because the Fos subunits bind to AP-1 DNA only as heterodimers with Jun subunits. Therefore for the following experiment, DNA and NE proteins were used at 1:40 ratio and the incubations were performed for 15, 75, 120, 180, 240 and 360 minutes. Western blot analysis of the affinity purified samples was carried out with αYB-1, αNCL and αJun antibodies. Qualitative and densitometric analysis of protein bands at various time points revealed that by 180 minutes, all of the proteins tested had reached binding equilibrium (Figure 31A and B).

A



B

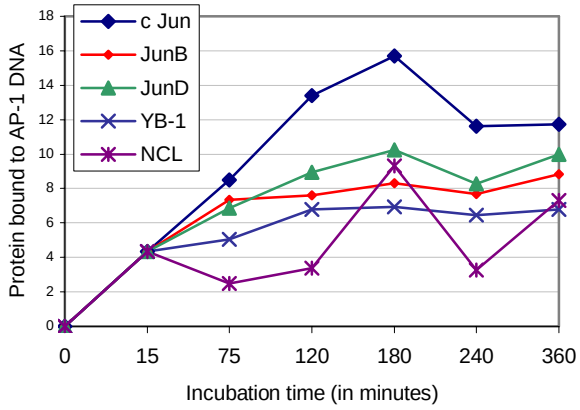


Figure 31 Equilibrium binding curves for YB-1, NCL and AP-1 jun subunits. A. AP-1 DNA affinity chromatography (NAPSTER sample I) was performed using 120 µg of AP-1 DNA on beads and 4800 µg of HeLa NE protein. Protein samples were removed at 15, 75, 120, 180, 240 and 360 minutes of DNA-protein incubation followed by washes, SDS PAGE and then immunoblotted with αYB-1, αNCL, αc-jun, αJunB and αJunD antibodies. B. Graph displaying the DNA-binding kinetics of YB-1, NCL, c-jun, junD and junB as a function of incubation time. Densitometric quantitation of protein bands was performed using the NIH Image 1.62 program as described in Materials and Methods.

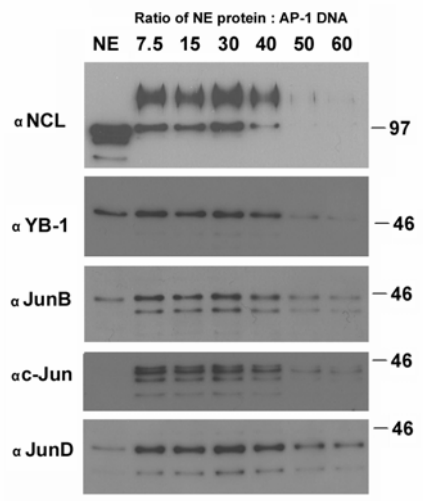
Establishing AP-1 DNA Binding Curve for YB-1, Nucleolin and AP-1 Jun Subunits

Initial titrations of concentrations of DNA:NE proteins had established that DNA was limiting for all proteins including YB-1, NCL, JunB, c-Jun and JunD at a 1:40 ratio (data not shown). The incubation time for this DNA-protein binding reaction used was 75 minutes. To determine if 1:40 ratio was still limiting for all the proteins after 180 minutes equilibrium incubation time, AP-1 DNA affinity chromatography (NAPSTER sample I) was performed using increasing amounts of AP-1 DNA probe and 800 microgram of NE protein. DNA: protein ratios tested included 1:7.5, 1:15, 1:30, 1:40, 1:50 and 1:60 and the incubations were performed for 180 minutes. Western blot analysis of the affinity purified samples was carried out with αYB-1, αNCL and αJun antibodies. Qualitative and densitometric analysis of protein bands at various ratios revealed that at 1:40 ratio, DNA was limiting for all of the proteins tested (Figure 32A and B).

Establishing Competition Curve for YB-1 in NAPSTER Assay

To determine the optimum concentration of competition oligos for NAPSTER assays under the newly standardized conditions of 1:40 ratio (DNA:protein) and 180 minutes (equilibrium incubation time), we performed NAPSTER assays involving preincubation of NE proteins increasing amounts of wild type or mutant AP-1 oligos prior to AP-1 DNA affinity chromatography. Western blot analysis of the affinity purified samples was carried out with αYB-1 antibody. At 5-fold molar excess (compared to AP-1 DNA on beads), both wild type and mutant competition oligos compete almost equally for YB-1 binding (Figure 33).

A



B

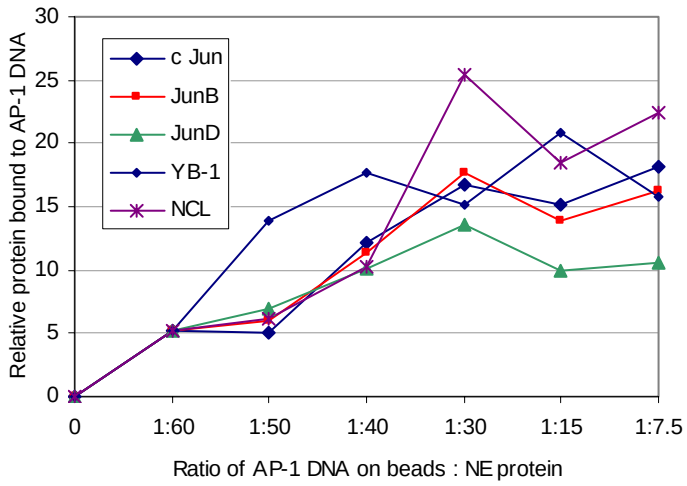


Figure 32 AP-1 DNA binding curve for YB-1, NCL and AP-1 jun subunits. A. NAPSTER analyzes was performed using AP-1 DNA on beads and HeLa NE protein at the ratio of 1:7.5, 1:15, 1:30, 1:40, 1:50 and 1:60. The proteins were incubated with the DNA for 180 minutes followed by washes, SDS PAGE and then immunoblotted with αYB-1, αNCL, αc-jun, αJunB and αJunD antibodies. B. Graph displaying binding of YB-1, NCL, c-jun, junD and junB at various ratios of DNA: protein. Densitometric quantitation of protein bands was performed using the NIH Image 1.62 program as described in Materials and Methods.

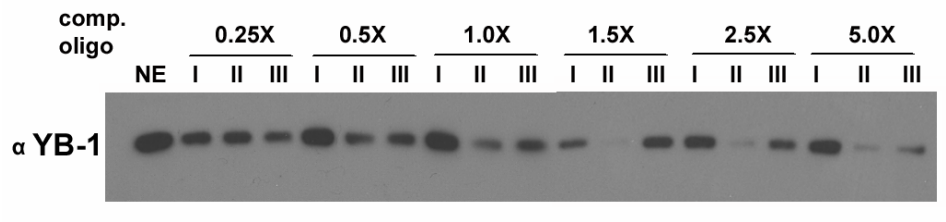


Figure 33 Establishing competition curve for YB-1 in NAPSTER assay. NAPSTER analysis was performed using 20 μg of AP-1 DNA on beads and 800 μg of NE protein following preincubation with increasing fold excess of wild type and mutant AP-1 competition oligos. The proteins were incubated with the DNA on beads for 180 minutes followed by washes, SDS PAGE and then immunoblotted with αYB-1 antibody.

The 2.5-fold molar excess competitions yield optimum specificity of binding, with effective competition clearly detectable with the wild type oligo but not the mutant oligo. Similar results were obtained previously for NCL (data not shown).

Binding of AP-1 Subunits to AP-1 Site in Cells Overexpressing YB-1 and/or Nucleolin

Having determined the equilibrium binding conditions and the limiting DNA concentrations through the experiments described in the previous sections, we tested the hypothesis that YB-1 and NCL mediate repression of AP-1 transactivation via decreases in AP-1 binding to AP-1 site. HeLa cells were transiently transfected with pcDNA3.1 vector or with pcDNAFlag-YB-1 and/or pNtagHA-NCL constructs. Nuclear extracts were prepared following incubation of the cells with 100ng/ml of TPA for 24 hours. NE protein for each of samples I, II and III from vector control and YB-1 and/or NCL transfectants were subjected to NAPSTER analyses. The NE proteins were incubated with the AP-1 DNA on beads at 1:40 ratio for 180 minutes followed by washes, SDS PAGE and then immunoblotted with αYB-1, αNCL, αc-jun, αJunB or αJunD antibodies (Figure 34). The results from this set of experiments were inconclusive due to the non specific binding of NCL to AP-1 DNA (see panel probed with αNCL antibody) and due to high variability in DNA binding within and between independent experiments (compare all panel corresponding to Experiment #1 with #2). Furthermore, we could not detect any increase in the binding of YB-1/Flag-YB-1 or of NCL/HA-NCL protein to the AP-1 DNA in transfectants when compared to control. However blotting with αFlag and αHA antibodies clearly demonstrated that flag-tagged YB-1 and HA-tagged NCL proteins are overexpressed and that Flag YB-1 bound specifically to AP-1

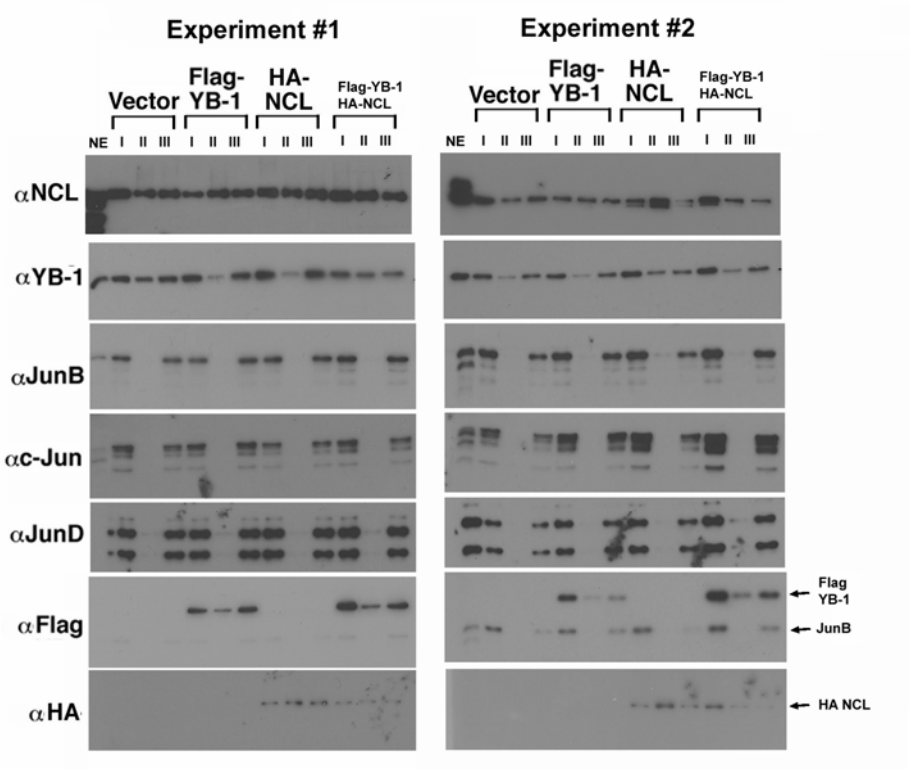


Figure 34 Binding of AP-1 subunits to AP-1 site in cells overexpressing YB-1 and/or nucleolin**.** 4.5 x 10^5 HeLa cells plated in 150 mm tissue culture dishes were transiently transfected with 15 μg of pcDNA3.1 vector control or with pcDNAFlag-YB-1 and or pNtag HA-NCL constructs. Nuclear extracts were prepared after treating the cells with 100ng/ml of TPA for 24 hours. 800 μg of NE protein for each of samples I, II and III from vector control and YB-1/NCL transfectants were subjected to NAPSTER analyses using 20 μg of DNA on beads. The proteins were incubated with the DNA for 180 minutes followed by washes, SDS PAGE and then immunoblotted with αYB-1, αNCL, αc-jun, αJunB, αJunD or αHA antibodies. The JunB membrane from above experiment was reprobed with αFlag antibody and shows residual junB chemiluminiscence in the membrane, indicated with an arrow.

DNA in NAPSTER assay (Figure 34, see panel probed with αFlag antibody). It is possible that the increase in the levels of total nuclear YB-1 and NCL proteins in the transfectants was not sufficient for detection. The results for these experiments are inconclusive and our hypothesis that YB-1 and NCL mediate repression of AP-1 transactivation via decreases in AP-1 binding to AP-1 site remains to be tested possibly by an alternate experimental approach presented in the discussion section.

Discussion

In this study, YB-1 and NCL were identified as novel AP-1 DNA binding transrepressors. We used a microscale analytical AP-1 DNA affinity chromatography method devised previously in our lab (Kumar and Bernstein, 2001) for identification and purification of these proteins. The YB-1 study had a fortuitous beginning. During the course of isolation of the p97 band (later identified as nucleolin) using the NAPSTER assay by Twizere, I noticed two coomassie stainable bands at 47 and 49 KDa in addition to the 97 KDa protein band that Dr. Twizere was attempting to isolate, that bound specifically to the AP-1 DNA. Using MS/MS sequencing, I identified the 49 KDa protein as YB-1 and our data suggest that 47 KDa protein is most likely a degradation product of YB-1 protein. Dr. Twizere identified the 97 KDa protein as nucleolin.

The present study is the first report demonstrating that YB-1 and NCL bind specifically to the AP-1 DNA sequence. This conclusion was supported both by MS/MS sequencing of specific coomassie stainable NAPSTER bands and by immunoblotting detection of specific bands with YB-1 and NCL antibodies. Since YB-1 and NCL are abundant mammalian proteins, their binding specificity for the AP-1 site is intriguing.

The binding of these abundant proteins to the AP-1 site seems to have been missed since AP-1 was first discovered.

YB-1 proteins were originally found by screening cDNA expression libraries for proteins that could bind to DNA sequences containing an inverted CCAAT element (Didier et al., 1988). Studies have shown that YB-1 protein can also bind to other sequences including the W-box, B-box, and apurinic DNA and a triplex forming H-DNA (Hasegawa et al., 1991; Cohen et al., 1991; Lenz et al., 1990; Horwitz et al., 1994) which accounts for its diverse roles in transcriptional activation, repression, DNA repair and translational control. Nucleolin has also been implicated in a variety of cellular processes including, regulation of rRNA transcription and processing, mRNA stability, chromatin decondensation, transcription, immunoglobulin switching, cell cycle regulation and signal transduction (see Ginisty et al., 1999 and Kohno et al., 2003 for review). NCL also binds to a variety of RNA and DNA sequencing including the 3' UTR of APP (Amyloid Precursor Protein), human preprorenin mRNA, the B motif of the alpha-1 acid glycoprotein promoter, matrix attachment region (MAR), telomeric DNA sequence, human papilloma virus-18 (HPV-18) enhancer, mRNA in Xenopus oocytes, 3' non-coding region of poliovirus, and the core binding site of the B cell specific LR1 complex (reviewed in Ginisty, et al., 1999).

The observation that YB-1 and NCL bind specifically to AP-1 DNA, led us hypothesize that sufficient levels of nucleolin and YB-1 would be detectable in the nucleoplasm in HeLa cells. We investigated the localization of these proteins in the cell by confocal microscopy, and both YB-1 and NCL were detectable in the nucleus. Based

on this observation we further hypothesized that YB-1 and NCL may interact with each other through protein-protein interactions. Our attempts to coimmunoprecipitate NCL with YB-1 using both αYB-1 and α-Flag antibodies were unsuccessful. Reciprocal immunoprecipitation of YB-1 with NCL using αNCL has not yet been tested due non-availability of effective immunoprecipitating NCL antibodies. Detection of YB-1 and NCL interaction is further complicated because of possible requirement of DNA for YB-1-NCL association. Chen et al. demonstrated that nucleolin and YB-1 could be coprecipitated with each other from crude cytoplasmic extracts of stimulated Jurkat cells, but the interaction was abolished by RNase treatment (Chen et al., 2000), indicating the critical role of RNA.

Another novel finding made in this study is that YB-1 and NCL repress transactivation of AP-1 dependent gene expression. Using a trimer of the wild type or mutant AP-1 target sequence linked to a luciferase reporter, Dr. Twizere demonstrated that both NCL and YB-1 site specifically represses transactivation of AP-1 dependent gene expression from the wild type but not the mutant AP-1 target sequence. Additionally we demonstrated that YB-1 represses *c-fos* induced AP-1 transactivation at a GCN AP-1 site.

There is increasing evidence that YB-1 and NCL bind DNA and serve as specific transcriptional regulators. YB-1 binds to the promoter of several genes including genes for matrix metalloproteinase 2 (Mertens et al., 1997), MDR 1 (Oda et al., 1998; Bargou et al., 1997), chick collagen α2 (Bayarsaihan et al., 1996), GRP78 (Li et al., 1997). Recent studies have shown that YB-1 functions as a transcriptional repressor. YB-1

binds to a Y-box sequence in the human MHC II (-90 bp to -40 bp) promoter and functions as a repressor (Ting et al., 1994). Furthermore, YB-1 represses the expression of Fas (Lasham et al, 2000) and collagen (Norman et al., 2001). NCL represses transcription by RNA polymerase I by inhibiting the transcriptional machinery and/or via interactions with promoter sequences in rDNA (Roger et al., 2002). NCL binds the B motif of the alpha-1 acid glycoprotein promoter represses mRNA transcription (Yang et al., 1994). For many transcriptional factors, additional proteins have been found to contribute to their DNA binding and/or trans-activation functions (Auwerx and Sassone-Corsi, 1992). These include: MyoD that is regulated by Id (Benezra et al., 1990), the glucocorticoid receptor by hsp90 (Picard et al., 1988) and Cfl-a by Drosophila I-POU (Treacy et al., 1991).

Given that YB-1 and NCL bind to AP-1 sequence, our initial hypothesis is that they transrepress by a mechanism involving DNA binding, but other mechanisms may also be operative. The mechanism of YB-1 and NCL mediated repression of AP-1 transactivation may theoretically involve either/or a combination of the following mechanisms;

(i) Decrease in the levels of AP-1 proteins in the nucleus. It is conceivable that decreases in the AP-1 protein levels in the nucleus could result in decreased AP-1 mediated transactivation at the AP-1 DNA binding site, because of the non-availability of sufficient amounts of AP-1 proteins. The expression level of AP-1 proteins in the nucleus may be governed by one or more of the following processes;

(a) Decreased transcription of genes encoding AP-1 subunits

(b) Changes in AP-1 mRNA processing resulting in decreased translocation of AP-1 transcripts into the cytoplasm for translation

(c) Decreased stability of AP-1 transcripts/increased degradation

(d) Decreased translation of AP-1 transcripts

(e) Decreased stability of AP-1 proteins

(f) Decreased transport of AP-1 proteins from cytoplasm into the nucleus

Our data rule out all of the above mechanisms through experiments that demonstrate that (a) YB-1 and NCL overexpression does not downregulate of the levels of any of the AP-1 proteins and, (b) YB-1 and NCL do not affect the subcellular localization of any of the AP-1 proteins.

(ii) Phosphorylation status of AP-1 proteins. Regulation of protein phosphorylation is a critical mechanism in the control of protein activity. c-Jun transcriptional activity has been reported to be stimulated by phosphorylation of its Ser-63 and Ser-73 residues in its activation domain (Smeal et al., 1991 and Arias et al., 1994).

(iii) Dimerization of AP-1 subunits. The DNA binding, stability and transactivation potential of AP-1 proteins can be substantially altered by changes in composition of AP-1 dimers. While homodimers of c-Jun have strong affinity for AP-1 DNA, heterodimers of c-Fos:c-Jun are more stable and bind AP-1 DNA with higher affinity (Herdegen and Leah, 1998). De Cesare demonstrated that while c-Jun: ATF2 dimers bind to a TRE-like element of the human urokinase enhancer and stimulate

transcription, c-Jun:c-Fos dimers appear to repress transcription of the enhancer at the same site (De Cesare et al., 1995).

Experiments to test the effects of YB-1 and/or NCL on the phosphorylation and dimerization status of all AP-1 subunits would be labor intensive and have not yet been tested. While a role for phosphorylation and/or dimerization of AP-1 proteins in repression has not been ruled out, based on the observation that YB-1 and NCL bind specifically to the AP-1 DNA, we hypothesize that YB-1 and NCL mediate repression of AP-1 by competing against the AP-1 proteins for binding to the AP-1 site.

(iv) Decreased DNA binding of AP-1 proteins. Decreased DNA binding of AP-1 proteins to the AP-1 site would directly decrease AP-1 transactivation. The binding of AP-1 proteins to AP-1 DNA may be altered through several processes including those mentioned in above sections, i.e., phosphorylation and dimerization. Alternatively, YB-1 and NCL may bind to the AP-1 site and competitively or sterically inhibit direct interaction between AP-1 proteins and AP-1 site. Competition for DNA binding has been shown to be an important mechanism of transcriptional regulation. The human factor NF-E binds to the CCAAT box in the promoter of fetal γ-globin gene and inhibits the binding of the transcriptional activator, CP-1, thus preventing the expression of this gene in adults (Superti-Furga et al., 1988). Similarly, cAMP response element binding factor (CREB) downregulates AP-1 transactivation by competing with AP-1 proteins for AP-1 consensus sequence (Masquilier and Sassone-Corsi et al., 1992).

Our strategy to test the notion that YB-1 and NCL decrease AP-1 binding to AP-

1 site was to analyze the binding of each of the AP-1 subunits to AP-1 DNA by the NAPSTER assay, in cells overexpressing YB-1 and/or NCL proteins. Effect of YB-1 and NCL overexpression on AP-1 binding to AP-1 site in NAPSTER assay was compared in two independent experiments. The results from these experiments were inconclusive due to the high variability in DNA binding within and between experiments. Furthermore, we were unable to detect significant increase in total levels of YB-1 and/or NCL protein binding to the AP-1 site. Immunoblotting with αFlag and αHA antibodies clearly demonstrated that flag-YB-1 and HA-NCL were overexpressed and bound to AP-1 site in the transfected cells.

(v) Decreased AP-1 transactivation by other mechanisms. This may include direct quenching or masking of the AP-1 transactivation domains by YB-1 and NCL following binding to the AP-1 site, recruitment a corepressor molecule(s) that could then inhibit AP-1 transactivation, interaction with proteins of the general transcriptional machinery and prevention of AP-1 dependent initiation of transcription, and YB-1 and/or NCL mediated modification of the local chromatin architecture to render transcription site inaccessible to the transcriptional machinery. The contribution of one or more of these mechanisms in YB-1 and NCL mediated AP-1 transactivational repression has not been ruled out and remains to be tested.

Further studies are required to unravel the precise mechanism of YB-1 and NCL mediated AP-1 repression. Firstly, overexpression of YB-1 protein in the flag YB-1 transfectants needs to be verified using an alternate αYB-1 antibody. Secondly, an alternate method of quantitation of DNA-protein interaction needs to be adopted. While

the NAPSTER assay is a rapid and sensitive method for accurate identification of specific DNA binding proteins, its application to quantitative comparisons of DNA binding between samples seems to be limited. Electrophoretic mobility shift assay (also called gel shift assays) qualifies as an equally rapid and more sensitive method of identification of specific DNA binding proteins and represents a more accurate assay for quantitative comparison of DNA binding. Gel shift assays are generally performed under conditions of low protein:DNA concentrations and therefore may favor detection of high affinity DNA:protein interaction and consequently suffer from reduced sensitivity to detection of low affinity DNA:protein interactions. Additionally, the applicability of supershift assays, hinges upon availability of strong supershifting antibodies. Nevertheless, we attempted both gel shift and supershift assays as an alternative to NAPSTER for analyses of YB-1 and NCL binding to the AP-1 site.

Our efforts to detect YB-1 and NCL supershifting activity were unsuccessful under numerous gel shift conditions including variations of temperature, ionic conditions, protein concentrations, electrophoretic conditions and two different YB-1 and NCL antibodies (data not shown). We speculate that this may in part be due to low affinity of the DNA:protein interaction. Super shift assays, in conjunction with UV crosslinking represents a powerful approach to resolving this problem. UV crosslinking may serve to stabilize the DNA-protein interaction. Specificity of binding to the AP-1 site may be tested by using wild type and mutant competition oligos and specific protein interactions could then identified by using specific antibodies. Minimal handling of samples during the assay may reduce variability within and between experiments and

yield more quantifiable data.

It is possible that the negative regulation exerted by YB-1 and NCL proteins require the interactions with additional factors binding to nearby sites on the DNA that differ between the regulatory regions of different genes, or that variations in the flanking sequences surrounding a given AP-1 site favor the binding of particular YB-1 and/or NCL complexes with different transcriptional potential. Location and site-specific factors may influence the activity of YB-1 and NCL protein within the context of a particular promoter region. We attempted to characterize the precise binding site for YB-1 and NCL within the GALV AP-1 sequence using DNAse I footprinting, but these attempts were unsuccessful. We were unable to demonstrate specific protection of any residues when double stranded template was used, presumably due to the low affinity of YB-1 and NCL binding. Similar results have been previously reported for YB-1 at the R2 element (Mertens et al., 1999) and the RE-1 element (Mertens et al., 1997).

The precise mechanism of YB-1 and nucleolin mediated repression is currently under investigation in our laboratory. Further studies will unravel the biological significance of transrepression by YB-1 and NCL at the AP-1 site. YB-1 and NCL may control the expression of specific target genes at the AP-1 site to regulate cell proliferation, anchorage independent growth and other processes integral to carcinogenesis.

Limitations of Present Study

This study used the NAPSTER assay as the means to identifying YB-1 and NCL as novel AP-1 DNA binding proteins. While several attributes of the NAPSTER assay

make it more suitable than conventional gel shift assays for identifying low affinity DNA-protein interactions, a major limitation of the NAPSTER assay however, is that it is not suited to quantitative comparisons between sample sets. Our efforts at using the NAPSTER assay to compare the binding of AP-1 proteins to the AP-1 DNA in control and YB-1 and/or NCL transfectants were inconclusive, primarily because we were unable to make quantitative comparisons between the two sets of NAPSTER samples due to the large variations within and between experiments. A key limitation of this study was inapplicability of gel shift and super shift assays in further analyses of the YB-1 and NCL binding to AP-1 site. This is perhaps because the low affinity YB-1/NCL-AP-1 DNA interaction was unstable under the gel shift conditions tested in the study.

Suggestions for Future Work

In the present study, a combination of DNA affinity chromatography and MS/MS sequencing identified YB-1 and NCL as specific AP-1 DNA binding proteins. Reporter gene assays showed that YB-1 and NCL play a critical role in mediating repression at the AP-1 site. Data generated in this study rule out changes in AP-1 levels and localization as mediators of YB-1 and NCL mediated repression of AP-1 dependent gene expression. However, further studies are needed to understand the precise mechanism by which YB-1 and NCL mediate AP-1 transactivational repression.

Apart from the mechanisms of repression addressed in this study and alluded to in the discussion section, quite a few other mechanisms of repression are possible. One such mechanism is by binding of YB-1 and NCL to the AP-1 site and recruitment of

corepressor molecule/s that reduce AP-1 transactivation (squelching). Also, YB-1 and NCL may bind to AP-1 proteins and prevent binding of AP-1 proteins to the AP-1 DNA (sequestering). Conceptually, physical interactions between YB-1/NCL and AP-1 may also result in occlusion of the AP-1 transactivation domain. Additional experiments to determine whether YB-1/NCL interacts with AP-1 transcription factors may include coimmunoprecipitation of YB-1/NCL and AP-1 proteins. Roles for YB-1 and NCL as competitive inhibitors may be studied by titrating recombinant YB-1/NCL and/or AP-1. If interaction between YB-1/NCL and AP-1 is detected, precise mapping of the interacting regions between YB-1/NCL and AP-1 will be necessary to determine whether the interacting domain occludes AP-1 transactivation domain. Understanding the physiological significance of such interactions and the mechanism by which they modulate AP-1 activity will be challenging.

Further analyses also need to be performed in order to localize the region of YB-1/NCL proteins that are necessary and sufficient for mediating DNA binding and repression of AP-1 transactivation. A systematic deletion analysis of the protein sequence would probably be the best approach to achieving these aims. Wild type YB-1/NCL protein and/or YB-1/NCL derivatives lacking various protein domain/s may be used to determine which derivative is necessary for AP-1 DNA binding. Furthermore, wild type YB-1/NCL protein and/or YB-1/NCL derivatives lacking various protein domain/s may be cotransfected with AP-1 reporter to assess which derivative is necessary and/or sufficient to repress AP-1 activated transcription.

More studies are also needed to identify the in-vivo AP-1 DNA sequences and

the endogenous target genes that are regulated by YB-1 and NCL binding and to elucidate the molecular events that trigger YB-1 and nucleolin mediated transactivational repression. Experiments employing the chromatin immunoprecipitation (ChIP) assays followed by PCR analyses are currently underway in our laboratory to verify gene sequences to which YB-1 binds in vivo in a native chromatin context. As more information on transactivational repression by YB-1 and NCL becomes available, the mechanisms by which genes containing AP-1 site are downregulated by these proteins should become clearer, and may provide valuable insights into mechanisms of regulation of gene expression in general.

Finally and most importantly, possible roles for YB-1 and NCL in cancer need to be investigated. Because AP-1 is implicated in cancer, and since data from this study clearly demonstrate transrepression of AP-1 by YB-1 and NCL, it is logical to postulate a tumor suppressor role for YB-1 and NCL. Preliminary soft agar transformation assays in our laboratory (Twizere, Kim and Bernstein, unpublished data) demonstrated that overexpression of YB-1 inhibits neoplastic transformation of P+ tumor promotion sensitive JB6 cells treated with tumor promoter TPA. The effect of NCL overexpression on neoplastic transformation of TPA treated P+ JB6 cells remains to be tested.

CHAPTER IV

CONCLUSION

The core subject of this research was gene expression. In the search for key molecules that regulate gene expression and the mechanism by which changes in gene expression govern physiological and pathological processes in the cell, we have pursued two complementary approaches. First, we used large scale gene expression profiling to identify genes whose altered expression is related or causal to neoplastic transformation. By combining gene array technology with the JB6 mouse model of susceptibility and resistance to neoplastic transformation, we have identified differential expression of several candidate genes that contribute to tumor promotion sensitivity and resistance. Several of the genes we identified are implicated for the first time in cancer. Overall, our findings from this study have added substantially to our understanding of the pathways that may lead to tumor promoter induced transformation.

As a complementary approach, we studied regulation of gene expression. The expression of specific genes in particular cell types or tissues is regulated by protein binding to specific DNA motifs present within the promoter or enhancer elements of those genes. We have used a DNA affinity chromatography based protocol to identify proteins that bind to AP-1 site. AP-1 is known to bind and transactivate genes that are involved in cell growth, differentiation, signalling and cell death. Aberrant expression of these genes is implicated in several human cancers. The rationale for our study was that once identified, these proteins can be used as targets in preclinical and clinical studies to correct dysregulated expression of AP-1 target genes in cancer. Using the

DNA-affinity chromatography based assay termed the Nucleotide Affinity Preincubation Specificity Test of Recognition (NAPSTER) assay devised previously in our laboratory, we purified two proteins that bound specifically to AP-1 DNA binding site. Analyses of the NAPSTER purified proteins by mass spectrometric sequencing determined the identities of two of these proteins as nucleolin and Y-box binding Protein 1 (YB-1). We further tested the hypothesis that these proteins bind to the AP-1 site and regulate transactivation at the AP-1 site. Overexpression of nucleolin and YB-1, both alone and in combination, was found to repress AP-1 dependent gene expression. To further understand the mechanism of transrepression, we analysed whether overexpression of nucleolin and/or YB-1 affected the levels and/or disrupted the intracellular localization of the AP-1 subunit proteins. Western blot analysis of all the AP-1 species revealed that intracellular levels of AP-1 were unaffected. Furthermore, cell fractionation followed by western blot analysis confirmed that the AP-1 levels were not altered in the cytoplasmic or nuclear compartments. Further studies are underway to elucidate the precise mechanism of repression mediated by these proteins at the AP-1 site.

To summarize, we have identified and characterized nucleolin and YB-1 as novel AP-1 DNA binding proteins. Strong evidence is presented that they repress AP-1 transactivation. These data open a new area of investigation on the mechanisms of transcriptional regulation by these proteins. Our studies provide the basis for several avenues of future investigations both in the fields of regulation of gene expression at the AP-1 site and in TPA induced neoplastic transformation.

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