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AP-1 REGULATION DURING MALIGNANT PROGRESSION
OF MOUSE KERATINOCYTE CELLS

by

Elizabeth Joseloff

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GRADUATE INTERDISCIPLINARY PROGRAM IN CANCER BIOLOGY

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THE UNIVERSITY OF ARIZONA

1997
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Elizabeth Joseloff entitled AP-1 Regulation During Malignant Progression of Mouse Keratinocyte Cells and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

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ABSTRACT

The mouse skin model that has been used to study skin carcinogenesis can be divided into three stages: initiation, promotion, and progression. One genetic change observed during tumor promotion and malignant progression is increased transactivation of the transcription factor AP-1. AP-1 consists of Jun (c-Jun, Jun B, Jun D) and Fos (c-Fos, Fos B, Fra-1, Fra-2) proteins that form Jun:Jun homodimers or Jun:Fos heterodimers. AP-1 binds to a consensus cis-promoter element, the TRE, and transcriptionally regulate a number of genes with various biological functions.

By studying the benign mouse keratinocyte cells, 308, and its malignant variant, 10Gy5, it has been shown that 10Gy5 cells have elevated AP-1 activity compared to 308 cells. Reduced AP-1 transactivation in 10Gy5 cells has been correlated with suppression of its malignant phenotype. This research examined the differential AP-1 transactivation in benign 308 and malignant 10Gy5 cells.

By examining mechanisms of AP-1 regulation in the two cell lines, differences were observed with post-translational modifications of AP-1. There were differences in phosphorylation of one of the AP-1 family members, Jun B. In addition, AP-1 proteins in 10Gy5 cells appear to be in a fully reduced state, unlike AP-1 proteins in 308 cells. A third difference that was observed was in Jun B steady state protein levels, with decreased Jun B protein in malignant 10Gy5 compared to benign 308 cells. Reduced Jun B protein in 10Gy5 cells was the result of decreased Jun B protein synthesis.

Jun B protein may inhibit AP-1 transactivation and cell proliferation. Experiments were performed to determine whether Jun B protein could modulate AP-1 transactivation.
cell growth, and tumor formation in 308 and 10Gy5 cells. Altering Jun B protein levels in these keratinocytes affected AP-1 transactivation. Overexpression of Jun B protein in malignant 10Gy5 cells corresponded to an inhibition of cell growth and tumor development. However, overexpression of Jun B protein in 10Gy5 cells was not sufficient to reverse the malignancy, indicating that additional genetic changes are involved in malignant conversion of these keratinocytes. The results of this research suggest that Jun B protein levels may be important during malignant progression of mouse skin.
I. INTRODUCTION

While skin cancers are often not fatal, they are the most frequently diagnosed cancers in the United States (Parker 1996). It is estimated that there are between 700,000 and 1.2 million patients diagnosed every year with either basal cell carcinoma or squamous cell carcinoma (Boring et al. 1994, Miller and Weinstock 1994), and of these cases, there are approximately 1200 to 2500 deaths (Boring et al. 1994, Weinstock 1993). The number of cases in the United States of malignant melanoma increases each year more rapidly than other cancers (Parker 1997). With 80 out of 100,000 people being diagnosed with melanoma, it is the eighth most common cancer in the United States (Lee 1992, Elwood 1993, Friedman et al. 1991). There is evidence that people who have had basal cell carcinoma or squamous cell carcinoma have a higher risk of developing malignant melanoma (Marghoob et al. 1995). With people living longer and having increased exposure UV irradiation, in part as a result of the depletion of the ozone layer, it is thought that the incidence of non-melanoma skin cancers will continue to rise (Gloster et al. 1996). It is important to understand the molecular mechanisms that regulate the progression of malignant skin cancers so that in the future we may be able to reverse the development of these common and rapidly growing types of cancer.
Organization of the Stratified Epidermis

The epidermis is composed of several different compartments (Figure 1). Epithelial cells differentiate from a proliferating basal cell to a non-proliferating squamous cell (Yuspa 1994, Fuchs 1990). The basal cells are attached to a basement membrane which separates the dermis from the epidermis. The basal cells are in contact with the basement membrane through integrin specific interactions, through the integrin α6β4 (Yuspa 1994, Hertle et al. 1991). The basal compartment is also characterized by the expression of cytokeratins 5 and 14 (Yuspa 1994). Basal cell progression to the spinous layer of the epidermis correlates with a loss of proliferation and a change in expression of keratins from K5/K14 to K1/K10 (Yuspa 1994). Associated with the basal and suprabasal cell boundaries is expression of α3β1 integrin. Retention of this integrin receptor complex but loss of α6β4 integrin is thought to be a critical factor in progression from proliferative to differentiated keratinocytes (Tennenbaum et al. 1996).

Further epithelial maturation involves migration of cells from the spinous compartment to the granular layer. Markers for the granular layer include a change from K1 and K10 expression to up-regulation of differentiation markers such as loricrin, filaggrin, and keratinocyte transglutaminase (Yuspa 1994). In addition to expression of these differentiation markers, there is down-regulation of β1 integrin and a lack of cell adhesion to extracellular matrix components such as fibronectin, laminins 1 and 5, and collagen IV (Tennenbaum et al. 1996).
Figure 1: Organization of the stratified epidermis. Proliferative basal cells attach to the basement membrane. The basal cells mature into non-proliferating layers: the spinous, granular, and cornified compartments. Changes in markers and gene expression that are specific for each compartment are indicated (Yuspa 1994).
The outer-most compartment of the stratified epidermis is the cornified layer where cell death of mature squame cells takes place. The cornified envelope, which surrounds the cells of the cornified layer consists of a filagrin matrix and loricrin crosslinks which is the result of transglutaminase activity (Yuspa 1994). There is evidence that protein kinase C (PKC) activity, specifically activation of PKCa, PKCe, and PKCd, may lead to alterations in expression of genes involved in differentiation, such as suppressing K1 and K10 and enhancing expression of granular markers (Dlugosz and Yuspa 1993), as well as establishment of the cornified envelope (Stanwell et al. 1996).

In addition to PKC activity being important in regulating keratinocyte differentiation, growth factors play a role in this process. Induction of K8/K18 has been associated with suppression of K1 which could be mediated by transforming growth factor α (TGFα) and epidermal growth factor (EGF) through the EGF receptor (Cheng et al. 1993, King et al. 1990, Vassar and Fuchs 1991). TGFα has also been shown to counteract tumor necrosis factor α (TNFα) induced apoptosis in keratinocytes (Reinartz et al. 1996). Proliferation can be inhibited in the basal layer by TGFβ1, and in the suprabasal layers by TGFβ2 and down-regulation of EGF receptors (King et al. 1990, Vassar and Fuchs 1991, Glick et al. 1993). Other signals such as Ca^{2+} and vitamin A influence the differentiation process of epithelial cells. In association with keratinocyte differentiation from basal cells to the cornified layer, increasing Ca^{2+} and decreasing vitamin A gradients have been observed (Menon et al. 1985, Darmon 1991).
The development of squamous cell carcinoma from a normal keratinocyte cell occurs in three distinct stages: initiation, promotion, and progression. During the progression to a squamous cell carcinoma alterations in epidermal differentiation occur. Cell undergoing tumor promotion have reduced K1/K10 expression and enhanced loricrin and filaggrin expression (Yuspa 1994, Lee et al. 1992, Dulgosz and Yuspa 1993). During papilloma progression, K13 is expressed in place of K1 (Yuspa 1994). In addition, α6β4 integrin receptor is expressed in both the basal and suprabasal layers in papillomas as well as carcinomas instead of just in the basal cells as seen in normal epidermis (Yuspa 1994, Tennenbaum et al. 1992, Tennenbaum et al. 1993, De Luca et al. 1990).

**Mouse Skin as a Model for Malignant Progression to Squamous Cell Carcinoma**

The mouse skin model has been used to study the multistep process of skin carcinogenesis. In this system, the stages of initiation, promotion, and progression during squamous cell carcinoma development involve a number of genetic alterations (Hennings et al. 1983) (Figure 2). Proto-oncogene activation as a result of somatic mutations, as well as increased cell proliferation, are involved in initiation and promotion (Hennings et al. 1983, Slaga 1991). In addition initiated keratinocytes in vitro have enhanced proliferative capabilities as they are less responsive to molecular signals for terminal differentiation (Yuspa and Morgan 1981, Kulesz-Martin et al. 1980, Kilkenny et al. 1985). A genetic mutation resulting in an initiated cell is irreversible while additional genetic insults that result from exposure to tumor promoters are reversible during the
Figure 2: The multistep process of mouse skin carcinogenesis. The different stages of the mouse skin carcinogenesis model and the genetic changes associated with progression from an initiated cell to a squamous cell carcinoma are presented.
early stages of papilloma development. During promotion, tumor promoting agents lead to clonal expansion of the initiated cell to give rise to a benign papilloma (Yuspa 1994, Boutwell 1974, Deamant and Iannaccone 1987, Iannaccone et al. 1987, Finch et al. 1996).

While the development of an early papilloma is promoter dependent, premalignant progression of the papilloma is correlated with spontaneous chromosomal alterations which appear to be promoter independent (Yuspa 1994, Hennings et al. 1983, Aldaz et al. 1987). Exposure to a complete carcinogen or clonal expansion can increase the probability that an initiated cell undergoes premalignant progression (Yuspa 1994). In addition, there are certain papillomas that genetically have a higher risk for premalignant progression (Yuspa 1994, Hennings et al. 1990, Hennings et al. 1985). Malignant conversion of a benign papilloma takes place through additional genetic changes such as activation of oncogenes (Yuspa 1994). Malignant conversion, like premalignant progression, is a rare event with less than 5% of benign papillomas developing to squamous cell carcinomas (Hennings et al. 1983).

One genetic alteration during squamous cell carcinoma formation involves the c-Ha-ras gene. Ras family members (Ha-Ras, K-Ras, and N-Ras) are GTP-binding proteins which can be activated by binding GTP in response to guanine nucleotide releasing protein GNP (Marshall 1995). Signaling through Ras can involved a number of kinase signal transduction pathways that can regulate expression and activation of transcription factors important in the carcinogenesis process (Whitmarsh and Davis 1996, Denhart 1996). A mutation in c-Ha-ras can lead to an initiated keratinocyte cell which can then develop into a papilloma and then further to a carcinoma. When v-Ha-ras is transfected into normal
cultured mouse keratinocytes, the cells are then able to form papillomas when grafted on athymic nude mice (Roop et al. 1986). In culture, these v-Ha-ras expressing mouse keratinocytes have an increased rate of proliferation, possibly as a result of an elevated expression of TGFα (Yuspa 1994, Glick et al. 1991, Imamoto et al. 1991). In addition, these transfected cells have constitutively enhanced diacylglycerol levels, reduced K1 and K10 expression, and enhanced loricrin and filaggrin mRNA levels in a similar manner as cells treated with a tumor promoter phorbol ester (Yuspa 1994, Lee et al. 1992, Dulgosz and Yuspa 1993). When v-Ha-ras is introduced into normal human keratinocytes, the cells lose their dependency on growth factors for proliferation (Boukamp et al. 1990). However, the human keratinocytes need to be immortalized prior to transfection with v-Ha-ras for tumor development indicating that in human cells, additional mutations are needed (Boukamp et al. 1990).

Specific PKC isoforms may play different roles in mediating the initiated phenotype in keratinocyte cells. Keratinocytes expressing v-Ha-ras or that had ras mutations as a result of initiation with carcinogens, have higher PKCα activity but reduced PKCδ activity (Yuspa 1994, Denning et al. 1993). There is evidence that the decrease in PKCδ activity is the result of PKCδ being phosphorylated. Utilizing a tyrosine/serine kinase inhibitor such as staurosporine, PKCδ became unphosphorylated and v-Ha-ras expressing epidermal cells were then able to undergo terminal differentiation (Yuspa 1994, Denning et al. 1993, Dlugosz and Yuspa 1991). Furthermore, in response to EGF receptor signaling through TGF, a src tyrosine kinase family member can phosphorylate PKCδ and inactivate it (Denning et al. 1996). TGFα has also been shown to selectively
suppress expression of PKCδ (Geiges et al. 1995). These cell culture experiments have been correlated with what happens in vivo. Treatment with staurosporine in vivo prevented cells grafted on the backs of athymic nude mice from forming papillomas (Strickland et al. 1993).

The next step in the formation of squamous cell carcinoma (SCC) in mouse skin is premalignant progression. Genomic instability and chromosomal aberrations with trisomies at chromosomes 6 and 7 are often observed (Yuspa 1994, Bremner and Balmain 1990). Along with the c-Ha-ras gene being mapped to chromosome 7, a putative tumor suppressor gene CATR1 has also been located on this chromosome. Through transfection and antisense studies regulating expression of this gene in a human squamous carcinoma cell line, CATR1 levels have been shown to potentially be involved in malignant conversion (Li et al. 1996).

For premalignant progression, papillomas are characterized as either high risk or low risk, with high risk papillomas being large tumors which occur early after promotion and do not regress if promotion ceases (Hennings et al. 1985). There are a number of genetic differences between high risk and low risk papillomas. One difference is in the distribution of the α6β4 integrin. This integrin receptor has been observed in both the basal and suprabasal layers of the skin in high risk papillomas while only present in basal cells in low risk papillomas and normal epidermis (Yuspa 1994, Tennenbaum et al. 1992, Tennenbaum et al. 1993, De Luca et al. 1990). Cytokeratin expression varies with these two papilloma types as K13 expression in place of K1 has been seen in high risk papillomas in comparison to low risk papillomas (Yuspa 1994). In addition, in low risk
papillomas and normal skin TGFβ1 has been localized to the basal layer and TGFβ2 has localized to suprabasal cells, while in high risk papillomas neither TGFβ1 nor TGFβ2 could be detected by immunohistochemistry (Yuspa 1994, Glick et al. 1993). As a result of these experiments, it has been proposed that TGFβ1 and TGFβ2 may be tumor suppressors and that the absence of these growth inhibitors may be important to allow for premalignant progression (Yuspa 1994).

Resistance to the antiproliferative effects of TGFβ1 in mouse keratinocyte cells has been associated with expression of the mutated tumor suppressor gene p53 (Reiss et al. 1993, Donehower et al. 1992). The level of p53 protein is correlated with the differentiated state of keratinocytes. Cultured mouse keratinocytes induced to undergo terminal differentiation by treatment with Ca²⁺ have lower levels of p53 protein while papilloma cells that do not undergo differentiation do not show a reduction in p53 levels in response to Ca²⁺ (Weinberg et al. 1995). Surprisingly, while differentiated keratinocytes expressed lower amounts of p53, p53 has increased transactivation capabilities in these cells. This was demonstrated with an induction of p53 regulated cell cycle genes p21waf-1 and mdm-2 in differentiating keratinocytes in comparison to proliferating keratinocytes (Weinberg et al. 1995). In support of this finding, immunohistochemistry of human squamous cell carcinomas indicated that Waf-1 protein was overexpressed in these tumor sections in the differentiated cells compared to the proliferative basal keratinocytes (Tron et al. 1996).
As well as with premalignant progression, mutations in p53 have been correlated with the development of carcinomas. Proteins that bind p53 are different in normal keratinocytes and squamous cell carcinomas, and these p53-binding proteins may play a role in malignant conversion. Unique proteins with molecular weights of 30 kDa, 16 kDa, and 15 kDa have been identified by immunoprecipitation in human SCCs compared to normal cells (Kumar and Spandau 1995). When v-Ha-ras was introduced into keratinocytes from p53 null mice and p53 heterozygous mutant mice, the keratinocytes from the heterozygous mice formed papillomas when grafted on the backs of athymic nude mice while the p53 null mice formed carcinomas (Donehower et al. 1992).

As well as playing a role in the early stages of skin carcinogenesis, an amplification of an A to T transversion at the 61st codon in the c-Ha-ras gene is correlated with increased invasiveness of chemically induced mouse skin carcinomas (Buchmann et al. 1991, Quintanilla et al. 1986). In both in vitro and in vivo experiments, the gene dosage of normal c-Ha-ras to mutant c-Ha-ras appears to be important for malignant progression of mouse skin. In studying the malignant mouse keratinocyte cell line PDV, which was generated by treatment of epidermal cells in culture with 7, 12-dimethylbenz(a)anthracene (DMBA), it was observed that this cell line has a 2 to 1 normal to mutant c-Ha-ras gene ratio (Quintanilla et al. 1991). PDV C57, a more aggressive variant cell line of PDV, has a 1 to 2 normal to mutant c-Ha-ras gene ratio (Quintanilla et al. 1991).

While there is evidence to suggest that TGFβ1 may inhibit benign papilloma formation, it has also been shown that TGFβ1 can promote progression to squamous cell carcinomas and the formation of the more invasive spindle cell carcinomas. This biphasic
effect of TGFβ1 in the multistep process of skin carcinogenesis is also observed in in vivo transgenic mice studies. The expression of the TGFβ1 transgene in keratinocytes allows for a delay in tumor formation of benign papillomas but more rapid malignant conversion to squamous cell carcinomas and an increase in the occurrence of spindle cell carcinomas (Chui et al. 1996).

Alterations in the expression of growth factor receptors are implicated in hyperproliferative epithelial diseases and with malignant progression of epithelial cells. An increase in the number of epidermal growth factor receptors (EGFR) has been associated with hyperproliferative skin diseases such as psoriasis vulgaris and Bowen’s disease (Amagai et al. 1988). Both an amplification of EGFR has been observed in SCC cell lines (Yamamoto et al. 1986, Hirai et al. 1988) and in patients with progressed esophageal squamous cell carcinomas (Ozawa et al. 1989). Reduced expression of retinoic acid receptors (RARα and RARγ) is correlated with malignant conversion of mouse skin. This progressive decrease in RARs from normal keratinocytes to high risk papillomas to squamous cell carcinomas appears to be associated with c-Ha-ras activation and protein kinase C activity (Darwiche et al. 1996). While there is a reduction in RARs, other retinoic receptors, RXRα and RXRβ, have increased expression during skin tumor progression (Darwiche et al. 1995).

The redistribution of the α6β4 integrin with expression in both the basal and suprabasal layers of the epidermis has been observed in carcinomas as well as in high risk papillomas (Yuspa 1994). This redistribution is thought to result from an increase in α6 mRNA levels. Associated with the increase in α6 mRNA in squamous cell carcinomas is
the presence of a splice variant of the α6 mRNA (Hogervorst et al. 1993, Tamura et al. 1991). The presence of the α6 spliced mRNA variant (Yuspa 1994, Tennenbaum et al. 1995) and cell surface matrix receptor CD44 variants (Salmi et al. 1993, Gunthert et al. 1991) has been correlated with the activation of nuclear oncogenes that allow for malignant conversion.

In carcinomas, expression of proteases may be important for the invasive and metastatic potential, allowing for extracellular matrix degradation and cell mobility (Liotta and Kohn 1990). Higher levels of the metalloprotease stromelysin have been shown in high metastatic SCCs compared to low metastatic SCCs, papillomas, and normal keratinocytes (Matrisian et al. 1986a, Ostrowski et al. 1988). In addition, expression of stromelysin was limited to stromal cells in papillomas and SCCs, but was expressed by tumor cells in spindle cell carcinomas (Wright et al. 1994). A cooperation between c-Ha-Ras and EGF can lead to proliferation in human keratinocytes and 92 kDa gelatinase/matrix metalloproteinase (MMP-9) expression (Chen et al. 1993). EGF receptor activation through TGFα can also induce proliferation and migration of keratinocytes and lead to the induction of the protease urokinase-type plasminogen activator (uPA) (Jensen and Rodeck 1993).

Cell-cell contact can effect protease expression, differentiation, and invasiveness in squamous cell carcinomas. Cell-cell interactions between squamous cell carcinoma tumor cells and dermal fibroblasts can lead to induction of proteases such as matrilysin, 72 kDa type IV collagenase, 92 kDa type IV collagenase, and uPA (Borchers et al. 1994). This study also showed that there was a soluble factor from the fibroblasts that could influence
proteases expression. The cell-cell adhesion ligand E-cadherin appears to be necessary for expression of the matrix metalloprotease matrilysin (MMP-7) (Borchers et al. 1997), but decreased E-cadherin levels is needed for reduced differentiation and increased invasive potential (Navarro et al. 1991). β-catenin, a protein involved in cadherin mediated cell adhesion, may have a role in regulating progression of melanoma. High expression of β-catenin with the absence or mutations of the adenomatous polyposis coli tumor suppressor protein (APC) has been observed in human melanoma cell lines, stressing a correlation with cell adhesion and malignant progression of skin cancers (Rubinfeld et al. 1997, Robbins et al. 1996).

The Transcription Factor Complex Activator Protein-1 (AP-1)

The transcription factor complex activator protein-1 (AP-1) has been shown to be involved in the regulation of malignant conversion, perhaps by playing a role in protease transcription (Thevenin et al. 1991, Holladay et al. 1992, Yokoo and Kitamura 1996, Irigoyen et al. 1997). There are seven AP-1 family member proteins that can dimerize to form the AP-1 complex: the Jun proteins (c-Jun, Jun B, Jun D) and the Fos proteins (c-Fos, Fos B, Fra-1, Fra-2) (Figure 3). The AP-1 complex consists of either Jun:Jun protein homodimers or Jun:Fos protein heterodimers which can bind to a promoter element called the TPA responsive element (TRE) that is present in the promoter region of a number of genes (Vogt and Bos 1990). The AP-1 family members dimerize at a leucine-zipper
Figure 3: The AP-1 transcription factor complex. The AP-1 transcription factor consists of dimers of either Jun:Jun family member proteins or Jun:Fos family member proteins. The AP-1 dimer can bind specifically to a cis-element (TRE) in the promoter region of a number of genes that are involved in a range of cellular functions such as cell proliferation, differentiation, and metastasis. AP-1 DNA binding can influence transcriptional activation of these genes.
structural motif, have an upstream C-terminal basic region that allows for DNA binding, and have a N-terminal domain that regulates transactivation (Angel and Karin 1991). Dimerization of AP-1 produces a conformational change in the basic region of the proteins that enhances binding with the general transcription factors TFIIE-34 and TFIIF, allowing for transcription initiation (Martin et al. 1996).

There is evidence that cell growth and tumorigenicity may be mediated through AP-1 transactivation. Elevated AP-1 activity has been correlated with decreased cell differentiation and increased invasion and metastasis. As determined by gel mobility shift assays, undifferentiated human keratinocytes have 80-90% higher AP-1 DNA binding capabilities in comparison to highly differentiated human keratinocytes (Briata et al. 1993). A number of genes that have been shown to be transcriptionally regulated through AP-1 are thought to be involved in invasion and metastasis such as the proteases collagenase (Angel et al. 1987) and stromelysin (Matrisian et al. 1986b).

There are a number of experiments suggesting that AP-1 activity is also involved in tumor promotion as well as malignant conversion. In the JB6 tumor promotion-sensitive (P+) mouse epidermal cell line, blocking AP-1 activity through the use of a dominant negative mutant Jun which lacks the transactivation domain, TAM-67, inhibits transformation by TPA and EGF stimulation (Dong et al. 1994). Elevated AP-1 activity as well as increased expression of the AP-1 family members has been observed in the benign mouse keratinocyte cell line 308 treated with the tumor promoter okadiac acid (Rosenberger and Bowden 1996). Two malignant mouse epidermal cell lines, PDV and 10Gy5, have enhanced AP-1 activity in comparison to the benign 308 cell line (Domann et
Transfection of the dominant negative mutant Jun TAM-67 into PDV and 10Gy5 cells reduces AP-1 activity and suppresses squamous cell carcinoma development, correlating increased AP-1 transactivation with malignant conversion (Domann et al. 1994b).

Signal Transduction Pathways Regulating AP-1

Signal transduction regulating AP-1 activity involves several complex pathways which often cross-talk with each other. As a result of signals to the cell such as stress responses, growth factors and cytokines, UV irradiation, and oxidation-reduction, four major kinase signaling pathways can be activated through Ras (Figure 4). Three of these pathways involve dual threonine tyrosine mitogen-activated kinase (MAPK) families which include ERK, JNK, and p38 signaling which can lead to AP-1 gene transcription and AP-1 transactivation (Whitmarsh and Davis 1996). Signaling through Ras/Rac has also been shown to lead to both MAPK dependent and MAPK independent signaling to AP-1 (Collins et al. 1996, Denhart 1996, Irani et al. 1997). The kinase cascades from Ras have three levels: the MAPKKK (Raf, MEKK, MLK-3), MAPKK (MEK, MKK4/SEK1, MKK3,6,MEK6,SAPKK2,3), and MAPK (ERK1,2, JNK/SAPK, p38). The MAPKs are phosphorylated by the MAPK kinases at the motifs Thr-Glu-Tyr, Thr-Phe-Tyr, or Thr-Gly-Tyr (Denhart 1996).

Signal transduction through Ras can lead to ERK activation by phosphorylation (Moodie 1993), and activated ERK can in turn lead to increased AP-1 transcription or
Figure 4: Signal transduction pathways involved in regulating AP-1.
Three major kinase pathways have been shown to be involved in AP-1 signal transduction: MAPK, JNK, and p38. Ras/Rac signaling may also be involved in regulating AP-1. Growth factor, ceramide, UV, and reactive oxygen species are some signals effecting AP-1 transcription and AP-1 transactivation.
post-translational modification (Pulverer et al. 1991, Gille et al. 1992). Upstream signaling molecules, such as growth factors, can transduce a signal through activated Ras by Ras protein interacting with downstream kinases such as Raf proteins. Raf is recruited to the plasma membrane to physically interact with Ras and become phosphorylated (Marais et al. 1995). The C-terminus of Ras is farnesylated to bind to the membrane and this post-translational modification of Ras is necessary for Raf serine threonine kinase activation (Kikuchi and Williams 1994). Phosphorylation of Raf is a critical point of regulation. Hyperphosphorylation of membrane associated Raf can act in a negative feedback loop for Raf activation (Wartmann et al. 1997). Downstream MEK1,2 specifically associate with Raf protein at a proline-rich sequence. A phosphorylation site within this region of MEK1 is necessary for its activation and its ability to phosphorylate ERKs (Catling et al. 1995). ERK1,2 activation can then lead to downstream signaling to transcriptionally activate AP-1 such as c-Fos. SRE transactivation of the c-Fos promoter has been shown to be enhanced by wild-type ERK expression enhanced while inhibited by kinase-defective ERK expression (Westwick et al. 1994).

While growth factors predominantly signal through the ERK pathway, stress activates the JNK kinase cascade. The initial kinase activation step in the stress-activated protein kinase (SAPK) or c-Jun amino-terminal kinases (JNK) is MEKK1,2,3,4 activation. The kinase domain of MEKK binds to GTP bound Ras and leads to MEKK phosphorylation as demonstrated through MEKK1 studies (Russell et al. 1995). MEKK1,2,3 can all activate MEK1,2 (Blank et al. 1996, Ellinger-Ziegelbauer et al. 1997). with MEKK2 having a preference for activating JNK and MEKK3 for activating ERK1,2
MEKK4 (Gerwins et al. 1997), MAPK-upstream kinase MUK (Hirai 1996), and TAK-1 (Shirakabe et al. 1997) are all members of the MAPK kinase kinase family which only leads to JNK activation. MKK4 is downstream of MEKKs and can directly phosphorylate JNK, resulting in JNK activation and AP-1 transcriptional activation (Yang et al. 1997). JNK has been shown to complex with c-Jun at its transactivation domain and phosphorylate residues Ser 63 and Ser 73, enhancing AP-1 transactivation capabilities (Derijard et al. 1994, Miller et al. 1996).

A third MAPK signaling pathway involves activation of p38 kinase which has also been shown to be activated by stress signals such as environmental stress and cytokines (Raingeaud et al. 1996). MLK-3 (mixed lineage kinase-3) is a serine threonine kinase which can activate both p38 and JNK pathways but cannot lead to ERK activation (Tibbles et al. 1996). MLK-3 can co-precipitate specifically with downstream signaling components MKK4/SEK1 and MKK6 supporting the role of this kinase in both JNK and p38 signaling (Tibbles et al. 1996). There are several identified MAP kinase kinases that lead to p38 activation. MKK4 can activate both JNK and p38 while MKK3 and MKK6 specifically phosphorylate and activate only p38 (Han et al. 1996, Raingeaud et al. 1996). Two other stress-activated protein kinase kinases, SAPKK2 and SAPKK3, are 78% and 97% identical respectively to MKK3 and also only activate p38 kinase (Cuenda et al. 1996). Recently, another p38 specific MAPKK that is homologous to MKK3 has been cloned, MEK6 (Stein et al. 1996). In addition, a downstream substrate for p38 kinase called MAPK-activated protein kinase-2, MAPKAPK2, has been identified (Bogoyevitch et al. 1996, Sanghera et al. 1996). Activation through the p38 signal transduction
pathway has been shown to elevate AP-1 transactivation (Wesselborg et al. 1997, Whitmarsh and Davis 1996).

Ras related signaling can also take place through interactions through the small G protein Rac. Signaling through Rac has been shown to cross-talk with the JNK and p38 pathways (Wesselborg et al. 1997, Collins et al. 1996). Rac activation has also been implicated in downstream signal transduction independent of ERK and JNK pathways in response to reactive oxygen species (Irani et al. 1997).

While signaling through growth factors such as EGF can lead to AP-1 transactivation through these MAPK pathways, EGF and cytokines can also lead to increased AP-1 activity in a Ras independent manner through induction of c-fos transcription. One cis-element in the c-fos promoter involved in c-fos transcription is the sis-inducible response element (SIE). Independent of Ras, an activated tyrosine kinase growth factor receptor can phosphorylate the transcription factor p91 and phosphorylated p91 protein can then translocate to the nucleus. Phosphorylated p91 can bind to the SIE as part of a sis-inducible factor protein complex (SIF) and lead to c-fos gene transcription (Sadowski et al. 1993, Fu and Zhang 1993, Silvennoinen et al. 1993, Stahl and Yancopoulos 1993).

Stress signals such as UV and oxidative stress predominantly activate JNK and p38 stress kinase pathways as discussed above (Dhar et al. 1996, Mendelson et al. 1996). In addition to these pathways, there is also evidence for ERK involvement in both UV (Radler-Pohl et al. 1993, Liu et al. 1995) and redox regulation (Kamata et al. 1996, Irani et al. 1997) Also, UVB and UVC radiation have also been shown to lead to AP-1 activity.
through atypical PKC activation independent of the EGF receptor (Huang et al. 1997, Huang et al. 1996). The atypical PKCs may feed into the MEK/ERK pathway as dominant negative PKCζ inhibits ERK activation downstream of Ras and Raf and does not effect JNK (Berra et al. 1995, Ueda et al. 1996). The sphingomyelin pathway is also implicated in the activation of Raf. In response to signals like TNFα or interleukin-1β, sphingomyelin is hydrolyzed to ceramide and activates ceramide-activated protein kinase (CAP kinase) which in turn can complex with and phosphorylate Raf (Yao et al. 1995).

Along with kinase activation, phosphatases play a role in regulating these AP-1 signal transduction pathways. MAP kinase phosphatase MKP-1 expression has been correlated with decreased ERK activity (Brondello et al. 1997), decreased JNK activity (Liu et al. 1995), and also dephosphorylation of p38 (Mendelson et al. 1996). While all three mitogen-activated protein kinases can be substrates for MKP-1, there is evidence that this phosphatase has high selectivity for p38 (Mendelson et al. 1996). Other identified MAPK phosphatases are MKP-2 which recognizes ERK and JNK, MKP-3 which is selective for ERK, M3/6 which selectively inactivates JNK, and PAC1 which recognizes ERK and p38 (Chu et al. 1996, Muda et al. 1996). Another protein phosphatase, protein phosphatase 2A (PP2A), can dephosphorylate and deactivate MEK, regulating ERK activation (Heriche et al. 1997). PP2A has also been shown to directly dephosphorylate c-Jun at C-terminal negative regulatory phosphorylation sites leading to increased AP-1 activity (Alberts et al. 1993).
Transcriptional Regulation of AP-1

One mechanism of AP-1 regulation is at the transcriptional level. From in vivo footprinting experiments of the c-jun promoter from HeLa cells and human fibroblasts, several cis-elements have been mapped (Rozek and Pfeifer 1993, Rozek and Pfeifer 1995). Under serum-deprived conditions, proteins bind to DNA at two AP-1-like elements (-71 to -64 and -190 to -183), a CCAAT box (-91 to -87), SP-1 sequences (-115 to -110 and -123 to -118), a nuclear factor jun site (-140 to -132), a RSRF element (related to the serum response element), and a sequence that is bound by a currently unidentified factor (Rozek and Pfeifer 1993, Rozek and Pfeifer 1995). However, for c-jun induction by serum (Rozek Pfeifer 1995) or by UV irradiation (Rozek and Pfeifer 1993), post-translational modifications of already bound trans-acting factors at the promoter of c-jun are observed regulating c-Jun transcription. There is also evidence that the retinoblastoma susceptibility gene product, RB, can transcriptionally regulate c-jun by influencing SP-1 DNA binding. Through gel mobility shift assays with recombinant RB protein and a c-jun SP-1 site DNA oligonucleotide, RB can elevate DNA binding and this enhanced binding can be inhibited by a heat-labile and protease sensitive SP-1 negative regulator (Sp1-I) which can associate with RB (Chen et al. 1994).

Promoter elements that regulate Jun B have also been characterized. Serum response elements SRE (-1452, and +2091), cAMP response elements CRE (+2071, +2116), and a TRE (-949) have been mapped to the murine jun B gene as cis-regulatory domains (Phinney et al. 1994). There is some controversy in the literature about the exact
location of elements for serum stimulation. De Groot et al. mapped the \textit{jun B} promoter region between -848 and -245 to be responsive to PKC and PKA activation (De Groot et al. 1991), Kitabayashi et al. mapped SRE elements between -1451 to -1425 (Kitabayashi et al. 1993), and Perez-Albuermel et al. mapped -91 to +2197 to be responsive to serum and growth factors (Perez-Albuermel et al. 1993). In addition, c-Ets-1 and c-Ets-2 have been shown to bind to \textit{jun B} promoter elements between -848 and -574 and between -196 and -91. It has been shown that activated c-Ha-ras can mediate Ets induced Jun B transcription, at least in part through ERK signaling (Coffer et al. 1994). A negative regulatory element in \textit{jun B} for the attenuation of serum induced transcription has been located between -89 and +32 in v-src transformed rat fibroblast cells (Yu et al. 1995).

Of the three Jun family members, Jun D is the least inducible and is often constitutively expressed. Mapping of the human \textit{jun D} promoter region indicates a TRE, CCAAT box, CRE elements, an Oct site, and SP-1 elements (Berger and Shaul 1994). The enhancer region includes the SP-1 and Oct sites from -83 to -194 (Berger and Shaul 1994). Also, human Jun D appears to be positively autoregulated which could account for its predominantly constitutive expression (Berger and Shaul 1994).

Of the Fos family member proteins, with the exception of c-Fos, little information is known about their transcriptional regulation. The major cis-regulatory elements of the \textit{c-fos} promoter consists of the serum response element SRE and a sis-inducible element SIE. A protein complex, p62 ternary complex factor (p62 TCF) in combination with two serum response factors (SRF) can form a complex that can bind to the SRE element (Ryan et al. 1989, Granham and Gilman 1991, Prywes et al. 1988, Norman et al. 1988, Shaw et
Phosphorylated p91 binds to the SIE element as part of a sis-inducible factor protein complex (SIF) for transcriptional induction of c-fos (Sadowski et al. 1993, Fu and Zhang 1993, Silvennoinen et al. 1993).

In addition to the SRE and SIE, some minor transactivating elements of c-fos have been identified. For cAMP induction of c-fos, it has been shown that a CRE site (-62 to -57) is critical (Fisch et al. 1989, Moens et al. 1993). However, cAMP induction is not as strong as transactivation from the SRE and SIE elements. DNA methylation may regulate transactivation from the CRE motif as methylation will decrease the binding of nuclear proteins to this site (Moens et al. 1993). Under certain conditions, an AP-1 element can regulate c-fos transcription. In HeLa cells, it has been shown that if the SRE element is mutated so that transcription from this element is inhibited, transcription can be regulated by AP-1 (Shaw et al. 1989a). In the murine c-fos promoter, an EIA adenovirus response element has been localized in the promoter region including the CRE site (Gedrich and Engel 1995). Recently, a 1-alpha, 25-dihydroxyvitamin D3 transcription factor response element (VDRE) has been identified (Schrader et al. 1997). A retinoblastoma control element RCE has been mapped between -102 and -71 of the human c-fos promoter and RB can downregulate c-fos through this element (Robbins et al. 1990). It has also been suggested that the transcription factor PEA-3 may be a regulatory factor for serum induction of c-fos (Shaw et al. 1989b).

These elements have been previously thought to transactivate the c-fos gene independently of each other, but transgenic mice studies prove otherwise. Transgenic mice were generated with clusters of point mutations within the different cis-elements
(SRE, SIE, CRE, and AP-1) (Robertson et al. 1995). In these mice, regulation of c-fos gene expression was only possible with all the elements functional, indicating that these promoter regions act in concert with each other for c-fos transcription (Robertson et al. 1995).

**AP-1 mRNA Stability**

AP-1 can be regulated at the level of mRNA stability. This is particularly the case for the c-fos message. In the 3' untranslated region of c-fos mRNA, there is a 75 nucleotide AU-rich RNA destabilizing element, ARE, that is involved in signaling both mRNA deadenylation and degradation. Three AUUUA pentanucleotide sequences in the 5' region of the ARE mediate RNA destabilization, while a 20-nucleotide U-rich sequence at the 3' end of the ARE signals removal of the poly(A)tail (Fort et al. 1987, Rahmsdorf et al. 1987, Kabnick and Housman 1988, Shyu et al. 1989, Shyu et al. 1991, Chen and Shyu 1994). By deleting the U-rich region, it has been shown that there is not only a decrease in the rate of deadenylation, but also greater stability of the mRNA, suggesting that this region may enhance the ability of the ARE to destabilize the c-fos message (Chen and Shyu 1994). As well as with mRNA stabilization, the AU-rich region also appears to correlate with the oncogenic potential of c-Fos. In fibroblasts, removal of the ARE sequence of the c-fos mRNA allows c-Fos to become 20 times more oncogenic (Lee et al. 1988, Meijlink et al. 1985, Raymond et al. 1989).
Along with untranslated regions, there are also mRNA destabilizing elements located within the coding region of \textit{c-fos} mRNA (Schiavi \textit{et al.} 1994, Shyu \textit{et al.} 1989, Shyu \textit{et al.} 1991, Wellington \textit{et al.} 1993). One of these destabilizing elements is a 320 nucleotide sequence coding for the leucine zipper domain of the c-Fos protein. This element can lead to destabilization of the \textit{globin} mRNA in a \textit{globin-fos} chimeric transcript (Shyu \textit{et al.} 1991, Shyu \textit{et al.} 1989). The ability of this region to influence stabilization of \textit{c-fos} mRNA appears to be dependent on RNA structure instead of translation. This is seen as mRNA stabilization is not effected by inserting a single nucleotide in the \textit{globin-fos} chimeric transcript which causes a frameshift mutation (Wellington \textit{et al.} 1993).

It has also been demonstrated that specific RNA binding proteins can influence \textit{c-fos} message stability. A 64 kDa protein associated with polyosomes and a 53 kDa protein which binds at the 5' end of the 320 nucleotide sequence of the \textit{c-fos} coding region have been identified (Chen \textit{et al.} 1992). This 53 kDa protein, which specifically binds to a 56 nucleotide region within the 320 nucleotide sequence, is thought to be necessary but not sufficient for \textit{c-fos} message stability (Chen \textit{et al.} 1992). Deletion of either this 56 nucleotide binding region or the remaining section of the 320 nucleotide sequence both result in a destabilized message (Chen \textit{et al.} 1992).

Recently, an ARE-like message destabilizing element in \textit{c-jun} has been identified (Peng \textit{et al.} 1996). This is unique in that this ARE does not contain AUUUA motifs. This element has three separate domains (I, II, and III) which function interdependently to allow for rapid degradation of the poly(A) tail (Peng \textit{et al.} 1996). While this non-AUUUA
ARE of c-jun has no sequence homology to the ARE element of c-fos, they are functionally similar as determined by domain swapping experiments (Peng et al. 1996).

**AP-1 Protein Stability**

Protein degradation of c-Jun is regulated by a ubiquitin-dependent degradation process. Ubiquitin-dependent proteolysis occurs when ubiquitin polypeptides are bound to the protein at lysine residues via ubiquitin-conjugating enzymes (Chau et al. 1989). There is a 27 amino acid region in the N-terminus of c-Jun protein called the δ domain that signals multiubiquitination and protein degradation as removal of this domain stabilizes c-Jun (Treier et al. 1994). Also, v-Jun protein which is missing the δ domain region, has a longer half-life than wildtype c-Jun and does not undergo ubiquitination (Treier et al. 1994). In addition to ubiquitin-dependent degradation, c-Jun protein degradation appears to be also regulated by phosphorylation. c-Jun phosphorylation by JNK kinase decreases its ubiquitination, leading to a more stable protein (Musti et al. 1997). Interestingly, Jun D protein, which differs from c-Jun in the N-terminal region including the δ domain, is also not efficiently ubiquitinated and is more stable than c-Jun (Musti et al. 1996).

c-Fos protein also has a domain which regulates its stability. A carboxy-terminal PEST motif in c-Fos is necessary for rapid protein degradation (Tsurumi et al. 1995). c-Fos protein, like c-Jun, appears to be degraded in a ubiquitin-dependent manner by the 26S proteasome (Tsurumi et al. 1995). In addition to the C-terminal PEST domain, c-Fos needs to dimerize with phosphorylated c-Jun protein for degradation (Papvassiliou et al.
1992, Tsurumi et al. 1995). The addition of ERK, casein kinase II, and CDC2 kinase in vitro expedites c-Fos degradation (Tsurumi et al. 1995). Similar to what is seen with v-Jun protein, v-Fos which lacks the C-terminal PEST region as a result of a deletion and frameshift mutation (Angel and Karin 1991), is not sensitive to the ubiquitin-dependent degradation (Papavassiliou et al. 1992).

There is evidence for some protease-dependent mechanisms for AP-1 degradation. Calcium-dependent neutral proteases, calpains, have been shown to be able to degrade all the Jun family members as well as c-Fos, Fos B, and to a lesser extent Fra-2 in vitro (Hirai et al. 1991, Carillo et al. 1994). In cultured human fibroblast cells, a inverse correlation has been seen with expression of a cathepsin B-like protease and AP-1 protein stability in vivo (Offord et al. 1993).

Post-translational Modifications of AP-1 Proteins

Phosphorylation is one type of post-translational modification that can effect AP-1 activity. Phosphorylation can positively and negatively regulate AP-1 transactivation. C-terminal phosphorylation of all three Jun family member proteins can inhibit AP-1 DNA binding and transactivation (Boyle et al. 1991; De Groot et al. 1993; Nikolakaki et al. 1993). Serine 243, threonine 231, and serine 249 are the critical residues for C-terminal phosphorylation of c-Jun protein (Boyle et al. 1991). A mutation of serine 243 to phenylalanine prevents phosphorylation of the other two residues and leads to elevated c-Jun DNA binding, indicating that serine 243 may be important in negatively regulating AP-
activity through phosphorylation (Franklin et al. 1993). N-terminal phosphorylation of c-Jun at serines 63 and 73, on the other hand, positively regulates AP-1 transcriptional activity (Smeal et al. 1992). Phosphorylation of these two serine residues is responsible for strong AP-1 transcription capabilities of Jun proteins. For example, Jun B protein does not have serines 63 and 73 phosphorylated and as a result leads to weaker AP-1 transactivation compared to c-Jun (Kallunki et al. 1996). C-terminal phosphorylation of c-Fos protein can negatively regulate its own gene transcription (Sassone-Corsi et al. 1988), and C-terminal phosphorylation of both Fra-1 and Fra-2 leads to enhanced AP-1 DNA binding (Gruda et al. 1994).

Another type of AP-1 post-translational modification is oxidation-reduction regulation which can influence AP-1 DNA binding. AP-1 proteins have increased DNA binding potential when in a reduced state (Abate et al. 1990, Okuno et al. 1993). A cysteine residue that is part of the conserved sequence Lys-Cys-Arg in Jun and Fos proteins is important for redox responsiveness. When cysteine 272 and cysteine 154 of Jun and Fos proteins respectively are mutated to serine residues, AP-1 DNA binding is increased (Abate et al. 1990, Okuno et al. 1993). An apurinic/apyrimidinic endonuclease, Ref-1, can in vitro enhance AP-1 DNA binding in a similar manner as reducing agents as determined with purified Ref-1 protein as well as with recombinant Ref-1 protein (Xanthoudakis and Curran 1992, Xanthoudakis et al. 1992).
AP-1 Protein-Protein Interactions

Another mechanism of AP-1 regulation involves protein-protein interactions with Jun, Fos, or the AP-1 dimer complexing with inhibitory proteins. One AP-1 inhibitory protein is IP-1 which can bind specifically with the AP-1 dimer and prevent it from binding to DNA (Auwerx and Sassone-Corsi 1991). IP-1 is present in both the cytoplasm and nucleus of cells and needs to be in a dephosphorylated state to bind with and inhibit the AP-1 complex (Auwerx and Sassone-Corsi 1991). In vitro, PKA, PKC, Ca\(^{2+}\)/calmodulin-dependent kinases, and serum stimulation have all been shown to phosphorylate IP-1 and lead to elevated AP-1 DNA binding (Auwerx and Sassone-Corsi 1991, Auwerx and Sassone-Corsi 1992).

Another AP-1 inhibitory protein is Jif-1. Jif-1 protein was identified from Jun-binding clones that were isolated from a cDNA library of chicken embryo fibroblast cells (Monteclaro and Vogt 1993). Jif-1 negatively regulates AP-1 DNA binding by associating specifically with c-Jun protein at its leucine zipper and preventing it from complexing with other AP-1 proteins and binding to the TRE (Monteclaro and Vogt 1993). There is evidence that the leucine zipper is important for Jif-1:Jun protein interactions. C-terminal deletions of c-Jun which disrupt the leucine zipper lead to c-Jun being unresponsive to Jif-1 (Monteclaro and Vogt 1993). In addition, increasing concentrations of c-Fos protein can disrupt Jif-1:Jun interactions and restore AP-1 DNA binding capabilities (Monteclaro and Vogt 1993).
There is evidence for another c-Jun specific inhibitory protein. Studies utilizing HeLa TK- cells demonstrated that an inhibitor can complex specifically with the δ region of c-Jun and inhibit transcriptional activation (Biachwal and Tjian 1990). The presence of the δ domain of c-Jun protein appears to be critical for its inhibitory effect (Biachwal and Tjian 1990).

There is also an AP-1 inhibitory protein identified that specifically associates with c-Fos protein. As a result of serum stimulation, c-Fos is translocated from the cytoplasm to the nucleus. There is evidence that there is an inhibitory protein that can specifically interact with c-Fos in the cytoplasm of the cell and prevent this translocation, thus prevent AP-1 DNA binding and activity (Roux et al. 1990). This inhibitor is sensitive to cAMP levels in the cells as seen by experiments with cells treated with a cell-soluble cAMP analog, 8-Br-cAMP (Roux et al. 1990).

Inhibition of Cell Growth, Tumor Formation, and AP-1 Activity by Jun B

Jun B protein expression has been correlated with terminal differentiation. Jun B protein is induced in myeloid cells in response to various differentiation factors such as IL-6, 1,25-dihydroxyvitamin D₃, TPA, and bryostatin 1 (Lord et al. 1990, Datta et al. 1991). A similar pattern is seen in epidermal cells with an increase in Jun B expression at the onset of terminal differentiation of human keratinocyte cells (Gandarillas and Watt 1995). Jun B and c-Jun may play opposite roles in regulating cellular proliferation. Experiments using antisense oligonucleotides in a number of different cell types (3T3 fibroblasts, human
mammary carcinoma cells, and PC12 neuronal cells) have indicated that inhibiting Jun B expression leads to an increase in the rate of cell proliferation while inhibiting c-Jun expression leads to enhanced cell differentiation (Schlingensiepen et al. 1993).

Jun B has been shown to negatively influence transformation and also tumorigenesis in athymic mice. In transformation experiments, transfection of primary rat embryo cells with c-jun and c-Ha-ras led to a significant number of foci (Schutte et al. 1989). In contrast, when these cells were transfected with jun B along with c-jun and c-Ha-ras, the number of foci observed was greatly reduced (Schutte et al. 1989). Also, unlike c-Jun, Jun B overexpression alone did not cause Rat-1A fibroblasts to be transformed or become tumorigenic when injected into athymic nude mice (Schutte et al. 1989).

There is evidence that Jun B can inhibit AP-1 transactivation. It has been shown that by co-transfection of F9 teratocarcinoma cells with both c-jun and jun B, AP-1 activity is reduced in comparison to transfection with only c-jun (Schutte et al. 1989). Jun B is not able to induce transactivation from a single TRE element from the collagenase promoter in F9 and in HeLa cells (Chiu et al. 1989). Also, co-transfection of c-jun and jun B in F9 cells inhibits the transcriptional capabilities of c-Jun from the collagenase TRE element (Chiu et al. 1989). This inhibitory effect of AP-1 transactivation by Jun B can be seen by looking at downstream genes that are transcriptionally regulated by AP-1. For example, in dermal fibroblasts, TGFβ inhibits collagenase gene expression with induction of Jun B while in keratinocytes TGFβ induces collagenase as a result of c-Jun overexpression (Mauviel et al. 1996).
Statement of the Problem

The research discussed in this dissertation involves examining AP-1 regulation and its role in malignant conversion of benign mouse keratinocytes to malignant squamous cell carcinoma. Studies using the benign 308 cell line and its malignant variant 10Gy5 have shown a correlation with AP-1 activity and malignant conversion of skin (Domann et al. 1994a, Domann et al. 1994b). The squamous cell carcinoma-forming 10Gy5 cell line has elevated AP-1 DNA binding and transactivation in comparison to papilloma-forming 308 cells. Reducing AP-1 activity by transfection with a dominant negative c-Jun TAM-67 leads to suppression of squamous cell carcinoma formation in athymic nude mice (Domann et al. 1994a, Domann et al. 1994b). The overall goal of the research outlined in this dissertation was to examine molecular mechanisms of AP-1 regulation that could account for the enhanced AP-1 activity observed in malignant 10Gy5 cells.

Chapter 3 examines mechanisms of AP-1 regulation that may lead to the differential AP-1 activity in these two cell lines. Potential differences in transcriptional regulation and post-translational modifications of the AP-1 proteins, as well as differences in protein-protein interactions and c-Ha-ras gene amplification are addressed.

Chapter 4 addresses the differential Jun B regulation in benign 308 and malignant 10Gy5 cells. Jun B mRNA levels, the presence of mutations in the jun B transcript, Jun B protein translocation, protein degradation, and protein synthesis are examined in these cells.
Chapter 5 discusses the functional role of Jun B protein as a negative regulator of AP-1 activity and tumorigenesis in this mouse keratinocyte cell system. Experiments overexpressing Jun B in malignant 10Gy5 cells and reducing Jun B protein levels in benign 308 cells are described. The effect of these manipulations on AP-1 activity and tumorigenicity are addressed.
II. MATERIALS AND METHODS

Cell Lines

The 308 cell line was derived from Balb/C mouse skin that was treated in vivo with 7,12-dimethylbenz(a)anthracene (DMBA) and selected by resistance to Ca^{2+} induced terminal differentiation (Strickland et al. 1988). These cells form papillomas when grafted to athymic nude mice (Strickland et al. 1988). The 10Gy5 cell line is a malignant variant of 308 cells that sustained exposure to 10 Gy of ^{60}Co gamma irradiation and forms squamous cell carcinomas when subcutaneously injected into athymic nude mice (Domann et al. 1994a). Jun B C6, Jun B C7, Jun B C16, and Jun B C18 were derived from 10Gy5 cells stably transfected with a Jun B expression construct pLLJunB. Mock C12 and mock C13 clonal cell lines were generated by stable transfection with the parental vector of the Jun B expression plasmid pLL78NL. 1A5 cells are a variant of 308 cells that stably express a luciferase reporter construct for the TRE element from the human collagenase gene promoter. PDV cells were derived from mouse skin cells transformed in vitro with DMBA and these cells form squamous cell carcinomas in athymic nude mice (Fusenig et al. 1978). CarB cells were derived from a murine primary spindle cell carcinoma as described (Buchmann et al. 1991). All cell lines were maintained in Minimal Essential Medium (MEM) with 7.5% fetal bovine serum and 2.5% calf serum.
Protein Extraction

Nuclear and cytoplasmic proteins were isolated from 70% confluent cells that were serum starved for 18 hours. In a similar manner as previously described (Dignam et al. 1983), cells were rinsed twice in PBS, scraped and incubated for 30 minutes at 4°C in Buffer A (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT), and lysed using a Dounce homogenizer (tight pestle). Nuclei were pelleted by spinning in a microcentrifuge for 30 seconds and the cytoplasmic protein in the supernatant collected. The nuclei were incubated in Buffer C (20 mM HEPES pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT) for 15 minutes at 4°C. The nuclear debris was pelleted by centrifugation at 4°C for 15 minutes and the supernatant containing nuclear protein was diluted at a 1:6 ratio in Buffer D (20 mM HEPES pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT).

For the studies examining the redox state of AP-1 proteins in 308 and 10Gy5 cells, nuclear proteins were extracted in a similar manner as described above with the exception of Buffer A having varying concentrations of the reducing agent, dithiothreitol (DTT). The concentrations of DTT in Buffer A were 25 mM, 50 mM, 75 mM, or 100 mM.
**Western Analysis**

For western analysis, 20 μg of nuclear or cytoplasmic proteins were separated on 10% SDS-PAGE. Proteins were transferred from the acrylamide gel to Immobilon-P transfer membrane by electroblotting at 50 V at 4°C overnight. The membrane was blocked with 3% milk in TBST (0.05% Tween 20, 20 mM Tris HCl pH 7.4, 0.9% NaCl) for 2 hours and incubated with the primary antibody for 1.5 hours at a 1:3000 dilution in 3% milk-TBST for all the rabbit polyclonal antibodies with the exception of Fos B and c-Fos which were used at a 1:1000 dilution. The blots were washed with TBST, incubated with a secondary horseradish peroxidase conjugated goat anti-rabbit antibody at a 1:35000 dilution in 3% milk-TBST for 1 hour, washed with TBST, and incubated with ECL western blotting detection reagent and then exposed to X-ray film. The antibodies were all purchased from Santa Cruz Biotechnology Inc.

**Gel Mobility Shift Assay**

For antibody clearance gel mobility shift experiments, 3 μg of 10Gy5 nuclear protein and 6 μg of 308 nuclear protein were incubated for 2 hours at room temperature with varying concentrations (5 ng, 150 ng, or 300 ng) of AP-1 rabbit polyclonal antibodies or rabbit serum. The AP-1 protein-antibody complexes were incubated at room temperature for 30 minutes with 3 x 10^5 cpm of ^32P Klenow labeled human collagenase TRE double stranded DNA oligonucleotide and 1 μg of poly(dIdC)-poly(dIdC) in gel shift
buffer (10 mM Tris pH 7.5, 50 mM NaCl, 0.5 mM EDTA, 1 mM MgCl₂, 4% glycerol, 0.5 mM DTT) (Domann et al. 1994a, Domann et al. 1994b, Stein et al. 1992). The human collagenase TRE oligonucleotide sequence 5'agcttgTGAGTCAgccgctag 3' was utilized for this assay. The gel shift DNA binding reactions were resolved on a 5% polyacrylamide-TBE (Tris, Boric acid, EDTA) gel at 30 m amps and the gel dried at 80°C and exposed to X-ray film.

To determine the specificity of AP-1 DNA binding in the gel mobility shift assays, unlabeled TRE oligonucleotide used as a competitor was incubated with nuclear protein prior to the DNA binding assay. Three micrograms of 10Gy5 nuclear extract or 3 ug of nuclear extract from 308 cells stimulated 2 hours with TPA to elevate basal DNA binding were incubated for 2 hours at room temperature with 25X, 50X, or 100X concentrations of unlabeled DNA double stranded human collagenase TRE oligonucleotide prior to the gel mobility shift assay. A mutated human collagenase TRE oligonucleotide with a sequence of 5' TGAATCT 3' was used as a negative control for this experiment.

To examine for the presence of AP-1 inhibitory proteins in the cytoplasm of benign 308 cells, gel mobility shift assays were performed by mixing experiments of nuclear and cytoplasmic proteins. Varying concentrations of cytoplasmic protein from 308 cells (50 ng, 100 ng, 200 ng, 400 ng, 600 ng, 1 μg, 2μg, 4 μg, 8 μg, 16 μg, 32 μg) were incubated for 2 hours at room temperature with 3 μg of 10Gy5 nuclear protein prior to the gel mobility shift assay.
Immunoprecipitation

Cells at 70% confluency were serum starved for 18 hours and washed twice in methionine-free MEM media prior to $^{35}$S labeling. Cells were incubated with 50 $\mu$Ci/ml of $^{35}$S methionine for various time points for continuous labeling experiments. Cells incubated for 30 minutes with $^{35}$S methionine and then incubate in serum-free MEM for pulse-chase experiments. For studies examining overall phosphorylation of AP-1 proteins, cells were starved in phosphate-free MEM for 30 minutes before labeling period with 100 $\mu$Ci/ml of $^{32}$P orthophosphate for 1 hour.

After cells were labeled in tissue culture, cells were washed twice with PBS and lysed by scraping in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% Na Deoxycholate), vortexing, and incubating at 4°C for 20 minutes. The lysates were precleared with 40 $\mu$l of 50% protein-A-sepharose beads in TENN buffer (50 mM Tris pH 7.4, 5 mM EDTA, 0.5% NP-40, 150 mM NaCl) by rotating at 4°C for 10 minutes. $1 \times 10^8$ cpm of pre-cleared $^{32}$P supernatant or 500 $\mu$g of $^{35}$S supernatant were rotated at 4°C overnight with 35 $\mu$l of 50% protein-A-sepharose solution and 1.5 $\mu$g of AP-1 antibody. The protein-antibody-bead complexes were washed 3 times in SNNTE buffer (50 mM Tris pH 7.4, 5 mM EDTA, 5% sucrose, 1% NP-40, 0.5 M NaCl) and twice in RIPA buffer. The protein-antibody complexes were denatured from the protein-A-sepharose beads by boiling for 10 minutes in Laemmli buffer and then resolved on 12.5% SDS-PAGE at 5 m amps overnight. The gel was fixed in 10% methanol/10%
acetic acid for 1 hour and enhanced for 1 hour with 1 M Na salicylate. The gel was dried at 80°C for 1.5 hour, and exposed to x-ray film.

For double immunoprecipitation experiments, ³⁵S labeling and immunoprecipitation were performed as described above with the following modifications. Cells were labeled for 1 hour with 100 μCi/ml of ³⁵S methionine and lysed under non-denaturing conditions with RIPA buffer without SDS and immunoprecipitated initially with a Jun B rabbit polyclonal antibody. The beads were washed and boiled for 10 minutes in denaturing buffer (50 mM Tris-HCl pH 7.5, 0.5% SDS, 70 mM β-mercaptoethanol) in a similar manner as described by Gruda et al. (Gruda et al. 1994). The supernatant was diluted 1:4 with RIPA buffer and immunoprecipitated with the second AP-1 antibody (Jun B, c-Jun, Jun D, c-Fos or Fra-1). The protein-antibody complexes were denatured from the protein-A-speharose beads by boiling in Laemmli buffer and resolving on 12.5% SDS-PAGE. The gel was fixed and enhanced as described above and exposed to X-ray film.

**DNA Isolation**

Genomic DNA was isolated from 308, 10Gy5, PDV, and CarB cells. Cells were lysed by scraping in a phenol lysis buffer (phenol equilibrated with 0.5 M Na Acetate pH 5.2, 0.3 M Na Acetate pH 7.5, 0.5% SDS, 2 mM EDTA) and nucleic acids were extracted with a 24:1 chloroform/isoamyl alcohol mixture and precipitated with 100% ethanol. RNA was precipitated from DNA by treatment with 4 M LiCl and DNA precipitated from
the remaining supernatant with 100% ethanol and then resuspended in a Tris-EDTA buffer.

Plasmid DNA for transfection studies was isolated by a CsCl DNA extraction protocol. Initially, plasmid DNA was transformed into bacterial competent cells. Competent cells and 1 μg of plasmid DNA was incubated on ice for 30 minutes, heat-shocked at 42°C for 45 seconds, and incubated at 4°C for 2 minutes. The culture was grown at 37°C for 1.5 hours after 0.9 ml of SOC media were added. The transformation reaction was plated LB agar plates with ampicillin at a concentration 100 μg/ml and incubated at 37°C overnight. A colony that grew on the LB-ampicillin plate after overnight incubation was selected and grown in 1 liter of LB medium with ampicillin overnight for CsCl plasmid DNA preparation.

For CsCl plasmid DNA isolation, the bacterial cultures were pelleted at 6000 rpm for 10 minutes at 4°C and the pellets resuspended in an alkaline lysis solution. Lysozyme in 10 mM Tris pH 8.0 at a concentration of 12 mg/ml was added and the mixture incubated on ice for 10 minutes. Twelve milliliters of 0.2 M NaOH - 1% SDS solution were added and incubated for 10 minutes at 4°C. A potassium-acetate solution (3 M K, 5 M Acetate) was added and the mixture incubated on ice for 20 minutes prior to centrifugation at 10,000 rpm for 15 minutes at 4°C. The supernatant was filtered from the debris pellet and nucleic acids precipitated with 1 volume isopropanol for 10 minutes at room temperature. After pelleting at 5,000 rpm for 15 minutes at room temperature, the nucleic acids pellet was resuspended in TE buffer. DNA was extracted from RNA by the addition of 5 M LiCl and pelleting RNA by spinning at 10,000 rpm for 10 minutes at 4°C.
DNA was precipitated from the supernatant with isopropanol and resuspended in TE buffer. Four grams of CsCl and 1 mg of ethidium bromide was added to the TE solution and the DNA subjected to a CsCl gradient by spinning at 45,000 rpm overnight at 25°C. The plasmid DNA band was detected with UV light, isolated from the gradient, and ethidium bromide was extracted from the DNA with H₂O-saturated butanol. The plasmid DNA was precipitated with 100% ethanol at -20°C overnight, centrifuged at 10,000 rpm for 10 minutes at 4°C, and the DNA pellet resuspended in TE for quantitation.

**Southern Analysis**

For Xba-I RFLP Southern analysis of the ras gene, DNA was digested with Xba-I restriction enzyme at a concentration of 10 units/µg and 15 µg of the digested DNA were electrophoresed on a 0.8% agarose gel at 30 V. The DNA was transferred to GeneScreen Hybridization Transfer membrane. The blot was hybridized at 42°C overnight with 2 x 10⁶ cpm/ml of ³²P random prime labeled murine c-Ha-ras genomic DNA of exon 1 and 2 of that was cloned into the parental vector pSP65 (Brown et al. 1988). The blot was washed with 2X SSC (NaCl/Na citrate) at room temperature; 2X SSC+1% SDS at 65°C; and 0.1X SSC+1% SDS at 65°C. The blot was then exposed to X-ray film. Xba-I digests the normal c-Ha-ras gene at the 61st codon to yield a DNA fragment of 12 kb, while digestion of an A to T transversion mutation at the 61st codon of c-Ha-ras gives DNA fragments of 8 kb and 4 kb.
RNA Isolation

RNA was isolated from 70% confluent serum deprived cells in the following manner. Cells were serum starved for 18 hours and lysed in guanidinium isothiocyanate buffer (4M guanidinium isothiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1 M β-mercapto-ethanol). RNA was extracted as described by Chomczynski et al. utilizing phenol and chloroform/isoamyl alcohol and precipitated with isopropanol (Chomczynski and Sacchi 1987).

Northern Analysis

Fifteen micrograms of RNA were electrophoresed on a 1% agarose - 40 mM MOPS (3-[N-morpholino]propanesulfonic acid) - 0.66 M formaldehyde gel for 2 hours at 150 V and transferred to GeneScreen Hybridization Transfer membrane for northern analysis. The membrane was prehybridized for 18 hours at 42°C in hybridization buffer (50% formamide, 1% polyvinylpyrrolidone, 1% bovine serum albumin, 50 mM Tris HCl pH 7.5, 0.1% sodium pyrophosphate, 1% SDS, 1 M NaCl, 10% dextran sulfate, 200 μg/ml salmon sperm DNA), hybridized with 3 x 10⁶ cpm/ml of ³²P random-prime labeled cDNA in hybridization buffer for 18 hours, washed with 2X SSC (0.15 M NaCl, 0.015 M sodium citrate) at room temperature, 2X SSC/1% SDS at 65°C, and 0.1X SSC at room temperature, and then exposed to X-ray film.
A 1.5 Kb EcoRI cDNA fragment of the mouse jun B gene (Ryder et al. 1988) was used as a probe to detect jun B message by northern analysis. A 0.75 Kb XbaI-PstI fragment of pHcGAP cDNA for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Tso et al. 1985) was used as a loading control for the RNA samples.

**Transient Transfection**

For transient transfection experiments, cells were trypsinized and seeded in 6 well tissue culture plates at a concentration of 1 x 10^5 cells per well. After 24 hours when cells were approximately 50% confluent, transient transfection took place. A total of 6 μg of plasmid DNA were diluted to 30 μl in HEPES/NaCl buffer (20 mM HEPES, 150 mM NaCl pH 7.4) and 9 μl of transfection reagent DOTAP (Boehringer Mannheim) were diluted to 30 μl in HEPES/NaCl buffer per well. Diluted DNA and diluted DOTAP were mixed together and incubated at room temperature for 10 minutes and then added to 2 ml of serum-free medium. Cells were rinsed once in serum-free medium and the DNA-DOTAP-medium mixture in serum-free medium was added to the well. After approximately 18 hour of transfection, cells were recovered in 2% serum containing MEM for approximately 8 hours, and then serum-starved for 30 hours for the luciferase assay and 48 hours for CAT assay prior to the cells being lysed.

A human collagenase TRE-luciferase reporter construct and its parental vector pGL2-Basic (Promega) were used for luciferase experiments. The collagenase TRE-luciferase plasmid was generated by cloning -73 to +63 base pairs of the human
collagenase I promoter in the multiple cloning site of pGL2-Basic vector. For CAT assays, pTicTacCAT and pΔMCS CAT plasmids were utilized. The pTicTacCAT plasmid was generated by cloning 27 mer of the mouse *stromelysin* promoter which includes the TRE and CACCC motifs into pBLCAT2 vector. The pΔMCS CAT plasmid has the multiple cloning site of pBLCAT2 vector deleted. A Jun B antisense phosphorothioate oligonucleotide and a scrambled phosphorothioate oligonucleotide with similar GC content as a negative control (Schlingensiepen et al. 1993) were transiently introduced into 308 cells at a concentration of 0.8 μM. The cells were initially transiently transfected with the AP-1 luciferase reporter construct and then transiently transfected with the antisense oligonucleotides.

**Luciferase Assay**

Cells from transient transfection experiments were lysed in 100 μl of 1% TritonX100, 25 mM glycyglycine, 15 mM MgSO4, 4 mM EGTA, and 1 mM DTT. Forty micrograms of protein from cell lysate were added to 180 μl of a luciferase assay solution (25 mM glycyglycine, 15 mM K3PO4, 15 mM MgSO4, 4 mM EGTA, 2 mM ATP and 1 mM DTT) and 100 μl of luciferin solution (25 mM glycyglycine, 15 mM MgSO4, 4 mM EGTA, 0.2 mM luciferin, 2 mM DTT) were injected directly to the samples with an ALL Monolight 2010C luminometer. Relative light units were measured by the luminometer with a single photon detection program. Cell lysate from untransfected cells was utilized as a background control for the assay.
Stable Transfection

The 10Gy5 cells were trypsinized and washed twice with HBS buffer (25 mM HEPES, 70 mM NaCl, 0.75 mM Na2PO4). One million cells were resuspended in 1 ml of HBS buffer and incubated with 10 μg of plasmid DNA at 4°C for 5 minutes. The plasmid DNA used for transfection was pLLJun B which is a dicistronic Jun B expression vector of jun B and neomycin resistance transcripts in the same orientation driven by the 5' retroviral LTR of MoMuLV (Moloney Murine Leukemia Virus) (Figure 5). The parental vector pLL78NL has the luciferase transcript in place of the Jun B transcript (Levine et al. 1991). The cells and DNA were electroporated at 250 V and 250 μF. After 2 days of recovery in culture, 800 ng/ml of active G418 were added to MEM. Transfected 10Gy5 transfected cells were cultured under G418 selection until colonies grew to confluency in a 10 cm tissue culture dish. Cells were then trypsinized and seeded at a density of a single cell per well in a 48 well tissue culture dish. The clones were grown to confluency in culture under G418 selection, expanded, and screened for elevated Jun B steady state protein levels by western analysis. Twenty mock (parental vector) transfected 10Gy5 clones and 25 10Gy5-Jun B clones were examined for Jun B protein expression.
Figure 5: Schematic map of the Jun B expression construct pLLJunB. pLLJunB was generated by cloning the Jun B transcript in place of the luciferase gene in the parental vector pLL78NL. pLLJunB plasmid is a dicistronic construct driving the jun B transcript and the neomycin resistance gene in the same orientation by the 5' retroviral LTR of the Moloney Murine Leukemia Virus.
Stable transfection of 308 cells with the human collagenase TRE luciferase pGL2 Basic reporter construct and a neomycin expressing plasmid (transfection done by Joanne Finch) generated the 1A5 cell line. 1 x 10^6 308 cells were electroporated with 8 μg of the collagenase TRE luciferase plasmid and 2 μg of the neomycin plasmid at 400 V and 500 μF. A HEPES Buffered Sucrose buffer was used for the transfection (Chu et al. 1987). Seventy-two hours after electroporation cells were selected with 600 μg/ml of G418 and clones single-cell isolated. The 1A5 cells were shown to stably express the collagenase TRE luciferase reporter construct by 12 hour treatment with 100 ng/ml of TPA and AP-1 activity analyzed by luciferase assay.

**CAT Assay**

CAT lysates were prepared by scraping cells in 200 μl of 250 mM Tris pH 8.0 and repetitive freeze-thawing of the samples with 3 minutes in ethanol/dry ice and 3 minutes at 37°C. The cell debris was pelleted and the supernatant was incubated for 10 minutes at 65°C. The samples were centrifuged and the supernatant or cell lysate was used in CAT assays. Five micrograms of cell lysate, 5 μl of 10 mg/ml of butyl-Co-A, and 20 μCi of 14C chloramphenicol were incubated at 37°C for 7 hours. The incorporated 14C was extracted from the CAT reactions with the addition of 300 μl of mixed xylenes, isomers plus ethylbenzene (Aldrich). The upper xylene phase was extracted and the samples back-extracted with 100 μl of 250 mM Tris pH 8.0. The upper phase was removed and back-
extracted again with 100 µl of 250 mM Tris and 200 µl of the upper xylene phase was then counted in 10 ml of omnifluor(Dupont)/toulene for 5 minutes on a $^{14}$C channel of a scintillation counter. In determining the percent conversion for the CAT assay reactions, 20 µCi of $^{14}$C chloramphenicol was quantitated to be equivalent to 256526 cpm.

**Cell Growth Analysis**

Cells were trypsinized and $1 \times 10^5$ cells seeded in 60 mm tissue culture dishes and grown in the presence of serum. Cells were collected and counted with in duplicate for various time point to determine cell growth. Cell numbers were calculated after cells were grown in culture for 24 hours, 36 hours, 48 hours, and 60 hours.

**Subcutaneous Injection in Athymic Mice**

Cells were trypsinized and washed in sterile PBS. Athymic nude mice were subcutaneously injected with $5 \times 10^6$ cells in 200 µl of sterile PBS per site of injection. Each mouse was injected at 4 sites and 4 mice were injected per cell line. Tumors developed between 12 and 16 weeks following subcutaneous injection. Mice were sacrificed when a tumor burden of at least one tumor having a volume of 2 cm$^3$ was reached. The experiment was completed after 16 weeks after subcutaneous injection.
PDV cells were used as a positive control and the mice injected with PDV cells all developed tumors by 3 weeks.
III. DIFFERENTIAL REGULATION OF AP-1 IN BENIGN AND MALIGNANT MOUSE KERATINOCYTE CELLS

Introduction

There are a number of potential mechanisms that could account for the enhanced AP-1 DNA binding and transactivation observed in malignant 10Gy5 cells compared to benign 308 cells. These mechanisms may involve transcriptional activation of genes that encode for AP-1 or post-translational modifications of AP-1 proteins. Protein-protein interactions of the AP-1 complex with inhibitory proteins is another possible mechanism. In addition, c-Ha-ras gene dosage could play a role in regulating the enhanced AP-1 transcriptional activity as well as the malignant phenotype of 10Gy5 cells.

Post-translational modification can involve either phosphorylation or oxidation-reduction regulation. Phosphorylation can influence AP-1 transactivation in both a positive and a negative manner. N-terminal phosphorylation of c-Jun protein can enhance AP-1 activity (Smeal et al. 1992, Kallunki et al. 1996). Phosphorylation can negatively regulate AP-1 DNA binding and transactivation through C-terminal phosphorylation of Jun family member proteins (Boyle et al. 1991, De Groot et al. 1993, Nikolakaki et al. 1993) and through C-terminal phosphorylation of c-Fos which can negatively regulate its own transcription (Sassone-Corsi et al. 1988). C-terminal phosphorylation of Fra-1 and Fra-2 can lead to increased AP-1 DNA binding (Gruda et al. 1994).
The redox state of AP-1 proteins can also influence its activity. AP-1 proteins have increased DNA binding capabilities when in a reduced state (Abate et al. 1990, Okuno et al. 1993). A cysteine residue that is part of a conserved amino acid sequence, Lys-Cys-Arg, is critical for regulating redox responsiveness of AP-1. By mutating cysteine residue 272 in Jun proteins and cysteine 154 in Fos proteins, it has been observed that there is enhanced AP-1 DNA binding (Abate et al. 1990, Okuno et al. 1993). This indicates that these residues are critical sites for redox regulation. A 37 KDa nuclear protein, Ref-1, has been shown in vitro to act in a similar manner as reducing agents to increase AP-1 DNA binding (Xanthoudakis and Curran 1992).

Protein-protein interactions of AP-1 proteins with inhibitory proteins may negatively regulate AP-1 activity. IP-1 is an inhibitory protein that in a dephosphorylated state can complex with AP-1 and prevent DNA binding (Auwerx and Sassone-Corsi 1991). Another AP-1 inhibitory protein, Jif-1, is capable of specifically binding to c-Jun and preventing it from complexing with other AP-1 proteins and binding to the TRE (Monteclaro and Vogt 1993). A similar type of inhibitory protein has been identified for c-Fos in which the inhibitory protein binds to c-Fos in the cytoplasm preventing it from translocating to the nucleus and being part of the AP-1 complex (Roux et al. 1990).

Amplification of an A to T transversion mutation at the 61st codon of c-Ha-ras has been correlated with induction of carcinoma formation and increased invasiveness (Buchmann et al. 1991, Quintanilla et al. 1986). An increase in the gene dosage of mutant to normal c-Ha-ras has been shown to be associated with malignant progression and a more aggressive phenotype (Quintanilla et al. 1986). Increased signaling through Ras, as
previously discussed, could lead to increased AP-1 transcription and phosphorylation resulting in enhanced AP-1 activity.

These different mechanisms of AP-1 regulation were examined in 308 and 10Gy5 cells. Steady state protein levels of AP-1 proteins were analyzed by western experiments. The presence of AP-1 inhibitory proteins in the benign 308 cells was investigated by gel mobility shift assays with 10Gy5 nuclear protein mixed with 308 cytoplasmic protein. Overall phosphorylation of the AP-1 proteins as well as oxidation-reduction of AP-1 proteins in benign 308 and malignant 10Gy5 cells was also examined. Gene dosage of mutated to normal c-Ha-ras in these cells was determined by RFLP Southern analysis. Finally, dimerization of Jun B protein in benign 308 cells with specific AP-1 family members was analyzed.

Results

Malignant 10Gy5 cells have decreased nuclear steady state Jun B protein levels in comparison to benign 308 cells.

In order to determine whether there are differences in AP-1 protein levels, which could be a result of differential transcription of AP-1, nuclear protein from benign 308 and malignant 10Gy5 cells was analyzed by western (Figure 6). The Jun family member proteins have molecular weights of approximately 39 KDa. Fra-1, Fra-2, and Fos B have molecular weights of 46-48 KDa, and c-Fos has a molecular weight of 55 KDa. The
Figure 6: Nuclear AP-1 protein levels in benign 308 and malignant 10Gy5 cells. 20 μg of nuclear protein from 308 and 10Gy5 cells were used for western analysis for AP-1. Rabbit polyclonal antibodies (Santa Cruz Biotechnology Inc.) for the Jun and Fos family members were utilized in this experiment. The steady state nuclear protein levels are similar in the two cell lines for all the AP-1 family member proteins, with the exception of Jun B. There is a decrease in the level of nuclear Jun B protein in malignant 10Gy5 cells compared to benign 308 cells. Jun family members have molecular weights of 39 kDa. Fos B, Fra-1, and Fra-2 have molecular weights of approximately 46-48 kDa, and c-Fos has a molecular weight of 55 kDa. The AP-1 proteins detected by western analysis are indicated by the arrows.
results of western analysis indicate that there are similar steady state levels of AP-1 proteins present in 308 and 10Gy5 cells with the exception of Jun B. The 10Gy5 cells have reduced steady state nuclear Jun B protein levels compared to 308 cells.

c-Jun, Jun B, Jun D, c-Fos, and Fra-2 can be part of the AP-1 complex that can bind to the TRE in both 10Gy5 and 308 cells.

To examine whether different AP-1 family member proteins are able to bind to a TRE element in benign 308 and malignant 10Gy5 cells, antibody clearance gel mobility shift assays were performed. To determine the specificity of the AP-1 DNA binding, unlabeled collagenase TRE DNA oligonucleotide was incubated with nuclear protein as a competitor prior to the gel mobility shift assay. The TRE gel shift band in 10Gy5 cells and phorbol ester-stimulated 308 cells (TPA 100 ng/ml) decreased significantly with pre-incubation with 25X, 50X, and 100X cold TRE oligonucleotide in the gel mobility shift assay (Figure 7). A negative control mutant TRE oligonucleotide was unable to clear the gel shift band in either cell line. The 308 cells were treated with TPA for 2 hours to increase the DNA binding signal observed by the TRE gel shift assay.

To characterize the specific AP-1 family members that can bind to the TRE in the gel mobility shift assays, 6 µg of 308 nuclear protein and 3 µg of 10Gy5 nuclear protein were incubated with increasing concentrations of AP-1 specific rabbit polyclonal antibodies or rabbit serum and then the protein-antibody complexes were subjected to the DNA binding assay. A strong TRE shift was observed when 6 µg of 308 nuclear protein
Figure 7: AP-1 DNA binding to a double stranded human collagenase TRE oligonucleotide.

Cold competition gel mobility shift experiments verified AP-1 protein specific binding to a DNA collagenase TRE oligonucleotide. 3 μg of nuclear protein from malignant 10Gy5 cells and 3 μg of TPA stimulate benign 308 nuclear protein were incubated for 2 hours at room temperature with increasing concentrations (25X, 50X, and 100X) of non-radioactively labeled TRE DNA prior to gel mobility shift assays with ^32P labeled collagenase TRE oligonucleotide. The unlabeled TRE oligonucleotide gives a clearance in a TRE gel shift with increasing concentrations while the mutated TRE oligonucleotide does not. The human collagenase TRE sequence TGAGTCA and the mutated TRE sequence TGAAATCT were used in this experiments.
were used in these experiments. The c-Jun antibody gave a clearance and Jun B and Jun D antibodies yielded supershifts with 308 nuclear protein (Figure 8A). With the Fos family members in 308 cells, the c-Fos antibody produced a supershift and the Fra-2 antibody gave a clearance (Figure 8B). For 10Gy5 nuclear extracts, c-Jun antibody gave a clearance of the TRE DNA binding while both Jun B and Jun D antibodies produced supershifts (Figure 8C). Increasing concentrations of c-Fos antibody yielded a supershift and increasing concentrations of Fra-2 antibody gave a clearance of the TRE shift in 10Gy5 (Figure 8D). Rabbit serum was used as a negative control in this assay and did not give a clearance of the TRE gel shift in either cell line.

The results of these antibody clearance gel mobility shift assays indicate that the same AP-1 family member proteins in both 308 and 10Gy5 cells are able to bind to the TRE. While there is little Jun B protein detected by western analysis in 10Gy5 cells, Jun B protein can be part of the AP-1 complexes in these cells as shown with antibody clearance experiments. These results suggest that the gel mobility shift assay may be more sensitive than western analysis.

**AP-1 inhibitory proteins are not present in the cytoplasm of benign 308 cells.**

A potential mechanism to account for the increased AP-1 activity in 10Gy5 cells compared to 308 cells is that there are inhibitory proteins in the cytoplasm of 308 cells that prevent the AP-1 complex from binding DNA. To examine this possibility, mixing experiments of cytoplasmic and nuclear proteins from 308 and 10Gy5 cells were analyzed.
Figure 8: AP-1 family members that can bind to a TRE DNA oligonucleotide in 308 and 10Gy5 cells.
Figure 8A: Jun family member proteins that can bind to a TRE DNA oligonucleotide in benign 308 cells.
6 µg of 308 nuclear protein were incubated with 50 ng, 150 ng, and 300 ng of c-Jun, Jun B, and Jun D rabbit polyclonal antibodies and rabbit serum as a negative control. The protein-antibody complexes were incubated with a $^{32}$P labeled human collagenase TRE oligonucleotide for a gel mobility shift assay. c-Jun antibody gave a slight clearance while Jun B and Jun D antibodies gave a supershift of the 308 TRE gel shift. Rabbit serum alone did not produce an antibody clearance or a supershift.
Figure 8B: Fos family member proteins that can bind to a TRE DNA oligonucleotide in 308 cells. 6 μg of 308 nuclear protein were incubated with 50 ng, 150 ng, and 300 ng of c-Fos, Fos B, Fra-1 and Fra-2 rabbit polyclonal antibodies and rabbit serum prior to a TRE gel mobility shift assay. The c-Fos antibody gave a supershift and the Fra-2 antibody yielded a clearance of the 308 TRE gel shift. Rabbit serum alone did not give an antibody clearance or a supershift.
Figure 8C: Jun family member proteins that can bind to a TRE DNA oligonucleotide in malignant 10Gy5 cells.

Three micrograms of 10Gy5 nuclear protein were incubated with 50 ng, 150 ng, and 300 ng of c-Jun, Jun B, and Jun D rabbit polyclonal antibodies and rabbit serum for 2 hours at room temperature before a TRE gel mobility shift assay with a $^{32}$P labeled human collagenase TRE oligonucleotide. Similar to what was seen with 308 nuclear protein, the c-Jun antibody gave a clearance and the Jun B and Jun D antibodies gave supershifts. Increasing concentrations of rabbit serum did not give an antibody clearance or a supershift.
Figure 8D: Fos family member proteins that can bind to the TRE in malignant 10Gy5 cells.

Increasing concentrations of Fos family rabbit polyclonal antibodies were used for antibody clearance gel mobility shift experiments. Three micrograms of 10Gy5 nuclear protein were incubated with 50 ng, 150 ng, and 300 ng of c-Fos, Fos B, Fra-1, and Fra-2 antibodies for 2 hours at room temperature prior to the TRE gel mobility shift assay. c-Fos antibody gave a slight supershift and an antibody clearance and the Fra-2 antibody produced a clearance.
by gel mobility shift assays. These experiments were done in a similar manner as utilized by Auwerx et al. to identify the AP-1 inhibitory protein, IP-1 (Auwerx and Sassone-Corsi 1991). To determine whether cytoplasmic protein from 308 cells would inhibit 10Gy5 gel shift, increasing concentrations of 308 cytoplasmic proteins from 50 ng to 32 μg were incubated with 3 μg of 10Gy5 nuclear protein prior to the TRE gel mobility shift assay (Figure 9A). With increasing concentrations of 308 cytoplasmic proteins added to 10Gy5 nuclear protein, there was an enhanced TRE gel shift. In a similar manner as with 308 cytoplasmic proteins, increasing concentrations of 10Gy5 cytoplasmic proteins to 10Gy5 nuclear protein led to elevated AP-1 DNA binding (Figure 9B).

These experiments indicate that AP-1 inhibitory proteins are not present in the cytoplasm of benign 308 cells. In addition, these results suggest that AP-1 proteins present in the cytoplasm of both 308 and 10Gy5 cells are able to bind to DNA but have not yet been translocated to the nucleus.

**Similar steady state protein levels of Ref-1 are observed in 308 and 10Gy5 cells.**

Oxidation-reduction regulation is one type of post-translational modification that can influence AP-1 activity. AP-1 proteins have elevated DNA binding capabilities when in a reduced state (Abate et al. 1990, Okuno et al. 1993). The 37 KDa nuclear protein Ref-1 has been shown in vitro to act in a similar manner as reducing agents to increase AP-1 DNA binding (Xanthoudakis and Curran 1992).
Figure 9: AP-1 inhibitory proteins in the cytoplasm of benign 308 cells.
A. Cytoplasmic proteins from 308 cells (50 ng to 32 μg) were incubated with 3 μg of
10Gy5 nuclear protein prior to a TRE gel mobility shift assay. Increasing concentrations
of 308 cytoplasmic protein enhanced the TRE gel shift in malignant 10Gy5 nuclear
protien.
B. Cytoplasmic proteins from 10Gy5 were added to 3 μg of 10Gy5 nuclear protein prior
to a TRE gel mobility shift assay as a control. Similar to what was seen with 308
cytoplasmic protein, 10Gy5 cytoplasmic protein led to an increase in the 10Gy5 nuclear
TRE gel shift.
Western analyses were performed to determine the steady state levels of Ref-1 protein in 308 and 10Gy5 cells (Figure 10). These experiments show that there are similar amounts of Ref-1 protein present in the nucleus of both benign 308 and malignant 10Gy5 cells.

There are differences in the level of reduction of AP-1 proteins in 308 and 10Gy5 cells.

While western analysis did not show differences in the steady state protein levels of Ref-1 in 308 and 10Gy5 cells, the reducing capability of Ref-1 in these cells was not examined. It is therefore possible that there are alterations in the reduced state of AP-1 in 308 and 10Gy5 cells. To analyze this, gel mobility assays were performed with nuclear protein from 308 and 10Gy5 nuclear extracts treated with varying concentrations of the reducing agent dithiothreitol (DTT) (25 mM, 50 mM, 75 mM, and 100 mM) (Figure 11).

With increasing concentrations of DTT, the TRE gel mobility shift observed in benign 308 cells was enhanced while a similar effect was not seen with DTT treated extracts from 10Gy5 cells. However, even at the highest concentration of DTT, the 308 TRE gel shift was not nearly as strong as the TRE shift from 10Gy5 extracts in the presence or absence of DTT. These results indicate that oxidation-reduction regulation of AP-1 is different in these two mouse keratinocyte cell lines. AP-1 proteins in 308 cells are not fully reduced unlike AP-1 proteins in the 10Gy5 cell line.
Figure 10: Ref-1 steady state protein levels in benign 308 and malignant 10Gy5 cells. 20 μg of nuclear and cytoplasmic protein from 308 and 10Gy5 cells were analyzed by western for Ref-1 protein levels. A Ref-1 rabbit polyclonal antibody (Santa Cruz Biotechnology Inc.) was used for western blotting at a 1:3000 dilution. Similar amounts of steady state Ref-1 protein was observed in both cell lines. Ref-1 protein has a molecular weight of 37 kDa and is indicated by the arrow.
Figure 11: The reduced state of AP-1 proteins in benign 308 and malignant 10Gy5 cells. Nuclear protein extracted from the both cell lines in the absence of the reducing agent DTT or in the presence of 25m, 50, 75, or 100 mM DTT was used in a TRE gel shift assay. 3 μg of nuclear protein were used in the gel shift assay to examine AP-1 DNA binding. The addition of DTT to 308 nuclear protein gave an increase in the AP-1 gel shift compared to 308 nuclear protein without reducing agent. However, the 308 TRE gel shift even in the presence of reducing agent is not as great as the gel shift observed from 10Gy5 nuclear protein. The TRE gel shift observed in 10Gy5 cells is the same in the absence or presence of DTT.
The overall phosphorylation of Jun B protein is different in 308 and 10Gy5 cells.

Another mechanism of post-translational modification that can regulate AP-1 activity is phosphorylation. Phosphorylation can both positively and negatively regulate AP-1 DNA binding and AP-1 transactivation. Overall phosphorylation of AP-1 proteins was examined by $^{32}$P orthophosphate labeling of benign 308 and malignant 10Gy5 cells and immunoprecipitation for the specific AP-1 family members from total cell lysate. Similar amounts of phosphorylated Jun B and Jun D protein were seen in both cell lines while no phosphorylated c-Jun was detected in either cell line (Figure 12A). In addition, there were similar amounts of phosphorylated Fra-1 protein and no phosphorylated c-Fos or Fra-2 protein in these cells (Figure 12B).

These results suggest that, with the exception of Jun B, there are no differences in overall phosphorylation of AP-1 in 308 and 10Gy5 cells. Jun B is either hyperphosphorylated in 10Gy5 or hypophosphorylated in 308 since there are similar amounts of phosphorylated Jun B protein in the two cell lines but there is a decrease in steady state Jun B protein levels in 10Gy5 cells.

There is no amplification of the mutated c-Ha-ras gene and/or loss of the normal allele in malignant 10Gy5 cells in comparison to benign 308 cells.

There is a correlation between the gene dosage of mutated to normal c-Ha-ras in malignant conversion of benign mouse skin as well as increased invasive potential
Figure 12: Phosphorylation of AP-1 proteins in 308 and 10Gy5 cells.
Overall phosphorylation of AP-1 proteins was analyzed by \(^{32}\text{P}\) orthophosphate labeling of cells for 1 hour and immunoprecipitation for the specific AP-1 family members with protein-A-sepharose beads and AP-1 rabbit polyclonal antibodies.

A. 308 and 10Gy5 \(^{32}\text{P}\) lysates (1 x 10\(^8\) cpm) were immunoprecipitated for c-Jun, Jun B, and Jun D with 1.5 \(\mu\)g AP-1 specific antibodies. Similar amounts of phosphorylated Jun B and Jun D proteins are seen in both cell lines. Neither cell line has significant amounts of phosphorylated c-Jun protein.

B. 308 and 10Gy5 \(^{32}\text{P}\) lysates (1 x 10\(^8\) cpm) were immunoprecipitated for c-Fos, Fra-1 and Fra-2. Similar amounts of phosphorylated Fra-1 protein are observed in 308 and 10Gy5 cells while no phosphorylated c-Fos or Fra-2 proteins are detected.
(Buchmann et al. 1991, Quintanilla et al. 1986, Quintanilla et al. 1991). Signaling through c-Ha-ras can lead to increased AP-1 activity through enhanced transcription and phosphorylation (Pulverer et al. 1991, Gille et al. 1992, Whitmarsh and Davis 1996, Irani et al. 1997). RFLP Southern analysis (Quintanilla et al. 1991) was utilized to examine the gene dosage of mutated to normal c-Ha-ras in 308 and 10Gy5 cells (Figure 13). DNA was digested with the restriction enzyme Xba-I for RFLP analysis of the A to T transversion mutation at the 61st codon of c-Ha-ras.

For Southern analysis 15 µg of DNA digested with Xba-I from 308 and 10Gy5 cells were examined by Southern blotting using a probe of exon 1 and exon 2 of murine c-Ha-ras genomic DNA. DNA extracted from the PDV malignant squamous cell carcinoma mouse cell line and from the CarB spindle cell carcinoma mouse cell line were used as controls in this experiment. It has been previously shown that PDV cells are heterozygous for normal and mutated c-Ha-ras gene and CarB cells are homozygous for the mutated c-Ha-ras allele (Buchmann et al. 1991, Fusenig et al. 1978).

The results of this experiment show that CarB cells have a loss of the normal (12 Kb DNA fragment) c-Ha-ras allele and PDV, 308, and 10Gy5 all have both normal (12 Kb) and mutated (8 Kb and 4 Kb) ras alleles. Previous studies indicated that PDV cells had a 2:1 normal to mutated c-Ha-ras alleles (Fusenig et al. 1978). However from Figure 13, it is not possible to determine differences in the intensities of the DNA bands for the normal and mutated c-Ha-ras alleles. Therefore differences in gene dosage are not distinguishable by this Southern analysis. While conclusions can not be made about c-Ha-ras gene dosage in 308 and 10Gy5 cells, it can be concluded that there is not a gross
Figure 13: Mutated and normal c-Ha-ras gene expression in benign 308 and malignant 10Gy5 cells. 
15 μg of DNA digested with Xba-I restriction enzyme was used for Southern analysis. 
Hybridization conditions were at 42°C with 2 x 10^6 cpm/ml of 32P labeled exon 1 and 
exon 2 of murine c-Ha-ras genomic DNA. PDV cells and CarB cells were used as 
positive controls in this experiment. PDV cells are heterozygous for mutated c-Ha-ras at 
the 61st codon and CarB cells are homozygous for mutated c-Ha-ras at the 61st codon. 
PDV, 308, and 10Gy5 cells show both a 12 Kb DNA fragment for the normal allele and 
the 8 Kb and 4 Kb DNA fragments for the mutated allele. CarB cells have a loss of the 
normal c-Ha-ras allele showing only the 8 Kb and 4Kb DNA fragments.
amplification of the mutated allele and/or a loss of the normal allele of \textit{c-Ha-ras} in malignant 10Gy5 cells.

\textbf{Jun B protein specifically associates with c-Fos protein in benign 308 cells.}

In order to determine with which AP-1 family members Jun B protein dimerizes in benign 308 cells, double immunoprecipitation experiments were performed (Figure 14). 308 cells were labeled in culture with $^{35}$S methionine and total cell lysates were prepared by lysing under non-denaturing conditions in RIPA buffer without SDS. Jun B protein was immunoprecipitated under non-denaturing conditions to retain JunB:AP-1 dimers from 308 and 10Gy5 lysates. Immunoprecipitations were then performed under denaturing conditions for c-Fos, Fra-1, or c-Jun. The results of this double immunoprecipitation experiment show that Jun B protein in 308 cells specifically associates with c-Fos protein.

\textbf{Discussion}

By examining AP-1 regulation, some potential mechanisms do not appear to play a role in leading to the enhanced AP-1 activity in malignant 10Gy5 cells. Similar AP-1 family members can form AP-1 dimer complexes in 308 and 10Gy5 cells (Figures 8). AP-1 inhibitory proteins, such as IP-1 or Jif-1, that could complex with AP-1 and prevent DNA binding are not present in the cytoplasm of benign 308 cells (Figure 9). A gross amplification of the A to T transversion mutation at the 61st codon of \textit{c-Ha-ras} or a loss
Figure 14: Jun B protein dimerization with c-Fos protein in 308 cells. Double immunoprecipitation experiments were used to determine specific protein:protein associations with Jun B in benign 308 cells. 308 cells labeled for 1 hour with 100 μCi/ml of $^{35}$S methionine were lysed and immunoprecipitated under non-denaturing conditions with a rabbit Jun B polyclonal antibody. The second immunoprecipitation was done under denaturing conditions with rabbit polyclonal antibodies for either Jun B, c-Jun, Jun D, c-Fos, or Fra-1. In 308 cells, Jun B protein specifically associates with c-Fos protein.
of the normal c-Ha-ras allele is not seen in 10Gy5 cells (Figure 13). Also, both cell lines have similar steady state Ref-1 protein levels (Figure 10).

While certain AP-1 regulatory mechanisms do not appear to be important in influencing AP-1 activity in these cells, three differences were observed. AP-1 proteins in 308 cells are not fully reduced unlike in 10Gy5 cells (Figure 11), but this does not appear to be a major form of differential AP-1 regulation in these two cell lines. A difference was also observed with Jun B phosphorylation. Jun B protein is either hyperphosphorylated in 10Gy5 cells or under-phosphorylated in 308 cells (Figure 12). Unlike c-Jun, Jun B protein is not phosphorylated at serines 63 and 73, resulting in lower AP-1 transactivating capabilities (Kallunki et al. 1996). One possibility to explain the differential Jun B phosphorylation observed in 308 and 10Gy5 cells is that the N-terminus of Jun B protein in 10Gy5 is mutated in such a manner that serines 63 and 73 are able to be phosphorylated, leading to enhanced AP-1 transactivation. Finally, there is a decrease in nuclear steady state Jun B protein levels in malignant 10Gy5 cells in comparison to benign 308 cells which will be discussed below (Figure 6).

The final part of these experiments was to determine what AP-1 proteinsJun B specifically associates with in 308 cells. c-Fos protein specifically dimerizes with Jun B in 308 cells. This is interesting since there is evidence that c-Fos may be important for malignant conversion. Expression of an activated fos oncogene (v-fos or 5' c-fos/3' v-fos construct) in benign 308 and SP-1 mouse keratinocytes led to malignant progression (Greenhalgh and Yuspa 1988), transgenic mice that overexpress exogenous c-fos develop osteosarcomas (Ruther et al. 1989), and papillomas that form in transgenic mice deficient
for c-fos can not be converted to malignant squamous cell carcinomas unlike the wild-type and heterozygous mice (Saez et al. 1995). It is possible that in benign 308 cells Jun B protein is sequestering c-Fos from being part of the AP-1 complex. As c-Fos may be important for the malignant phenotype, it would be of interest to determine whether Jun B protein is influencing AP-1 activity and malignancy in 308 cells by preventing c-Fos from binding to the TRE in the promoter region of genes important for malignant progression.
IV. JUN B REGULATION IN BENIGN 308 AND MALIGNANT 10GY5 MOUSE KERATINOCYTE CELLS

Introduction

Jun B protein expression has been correlated with terminal differentiation and reduced cellular proliferation, inhibition of transformation and tumorigenesis, and decreased AP-1 activity (Gandarillas and Watt 1995, Schlingensiepen et al. 1993, Schutte et al. 1989, Chiu et al. 1989). When mechanisms of AP-1 regulation were examined in benign 308 and malignant 10Gy5 cells, differences in Jun B steady state protein levels were observed. The 10Gy5 cells have reduced Jun B protein compared to 308 cells. The variation in Jun B protein levels may account for the differences in AP-1 activity and tumorigenicity in these two cell lines.

Jun B may be regulated at the levels of transcription, mRNA stability, protein synthesis, or protein stability. For jun B transcriptional regulation, cis-regulatory elements such as SRE elements, CRE elements, c-Ets binding sites, and a TRE element have been shown to be important (De Groot et al. 1991, Kitabayashi et al. 1993, Perez-Albuerne et al. 1993, Phinney et al. 1994). In addition, a negative regulatory element for jun B transcription has been identified in the promoter region of the jun B gene in v-src transformed rat fibroblast cells (Yu et al. 1995). For mRNA stability, an ARE-like mRNA destabilizing element has been identified in the c-jun message (Peng et al. 1996), and a similar message destabilizing effect may be seen in jun B mRNA. Ubiquitin-dependent
proteolysis has been shown to regulate protein degradation of the Jun family members (Chau et al. 1989, Treier et al. 1994, Musti et al. 1996). Deposphorylation of amino acids in the N-terminus of Jun proteins that appear to signal this ubiquitin-dependent protein degradation (Musti et al. 1996). In addition, Jun B protein degradation has also been shown to be regulated by a calcium-dependent proteases, calpain (Hirai et al. 1991, Carillo et al. 1994).

To investigate why Jun B steady state protein levels are reduced in malignant 10Gy5 cells, Jun B was examined at both the message and protein levels. Jun B protein translocation from the cytoplasm to the nucleus was analyzed. To determine whether there are differences in Jun B transcription or Jun B mRNA stability, Jun B message levels were examined. The rates of Jun B protein degradation and protein synthesis were also determined for 308 and 10Gy5 cells.

Results

The decrease in nuclear Jun B steady state protein in malignant 10Gy5 cells is not a result of aberrant Jun B protein translocation from the cytoplasm to the nucleus.

A possible explanation for why there is a decrease in nuclear Jun B protein in 10Gy5 cells compared to 308 cells is that Jun B protein is not being efficiently translocated from the cytoplasm to the nucleus in 10Gy5 cells. To address this, Jun B western analysis was performed on cytoplasmic and nuclear extracts from 308 and 10Gy5
cells. Jun B western experiments with 15, 20, and 30 μg of cytoplasmic protein showed the presence of Jun B protein in cytoplasm of 308 cells but not in the cytoplasm of 10Gy5 cells (Figure 15). Based on this data, there does not appear to be a defect in translocation of Jun B protein from the cytoplasm to the nucleus in malignant 10Gy5 cells that could account for the decrease in steady state nuclear Jun B protein.

There are no differences in jun B transcription or mRNA stability in 308 and 10Gy5 cells.

To examine jun B message levels in the two cell lines, RNA was isolated and analyzed by northern experiments for jun B and GAPDH mRNA levels (Figure 16). A 1.5 kb EcoRI cDNA fragment of the mouse jun B gene (Ryder et al. 1988) was used to detect jun B message by northern analysis. A 0.75 kb XbaI-PstI fragment of pHcGAP cDNA for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Tso et al. 1985) was used as a loading and transfer control for the RNA samples. After normalizing for GAPDH levels, the jun B message levels in both 308 and 10Gy5 cells were similar. The differential Jun B regulation observed in 308 and 10Gy5, therefore, was not a result of alterations in jun B transcription or mRNA stability. This suggests that the reduced Jun B protein level in malignant 10Gy5 cells is a result of variations in Jun B protein stability or translation.
Figure 15: Steady state cytoplasmic Jun B protein levels in benign 308 and malignant 10Gy5 keratinocytes. 15 µg, 20 µg, and 30 µg of cytoplasmic protein from 308 and 10Gy5 cells were used for western analysis. For comparison, 20 µg of nuclear protein from both cell lines were also resolved on SDS-PAGE, transblotted, and incubated with a primary Jun B rabbit polyclonal antibody at a dilution of 1:3000. A secondary horseradish peroxidase-conjugated goat anti-rabbit antibody was used at a dilution of 1:35000. Jun B protein was detected by using ECL western blotting detection reagent. Detection of Jun B protein with a molecular weight of 39 kDa is indicated by the arrow.
Figure 16: Steady state jun B mRNA levels in 308 and 10Gy5 cells. For northern analysis, 15 µg of RNA from 308 and 10Gy5 cells were used for detecting jun B message. A 1.5 kb EcoRI fragment of mouse jun B cDNA was used as a probe for jun B mRNA. A 0.75 kb Xba-I/PstI fragment of human GAPDH cDNA was used as a probe for GAPDH message. GAPDH is a normalizing factor for loading and transfer during the northern experiment. After normalizing, similar amounts of jun B mRNA was detected in 308 and 10Gy5 cells.
The rates of Jun B protein degradation in the two cell lines are similar.

One possible mechanism of differential Jun B protein regulation in 308 and 10Gy5 cells is that there is a difference in the rates of Jun B protein degradation. In order to examine Jun B protein degradation, cells were labeled with $^{35}$S methionine and then incubated with non-radioactive serum-containing medium for pulse-chase experiments. Total cellular protein was isolated and Jun B immunoprecipitated and resolved on SDS-PAGE. Figure 17 shows the amount of labeled Jun B protein in 308 and 10Gy5 over time after the pulse-chase as determined from three independent experiments. The error bars indicate the standard deviation of the mean. The rate of Jun B protein degradation appeared to be similar in the two cell lines with the half-life of Jun B protein calculated to be approximately 2.5 hours in both 308 and 10Gy5.

There is a decrease in the rate of Jun B protein synthesis in malignant 10Gy5 cells compared with benign 308 cells.

The results of the Jun B northern data and Jun B pulse-chase experiments suggest that there are not differences in Jun B transcription, Jun B mRNA stability, or the rate of Jun B protein degradation in malignant 10Gy5 cells compared to benign 308 cells. Alterations in the rate of Jun B protein synthesis in the two cell lines could account for the decrease in Jun B steady state protein levels in 10Gy5.
Figure 17: The rate of Jun B protein degradation in benign 308 and malignant 10Gy5 cells.

Cells were labeled with 50 μCi/ml of $^{35}$S methionine for 30 minutes then chased with serum-free MEM for various time points to examine Jun B protein degradation. Protein was isolated every hour after the chase for 5 hours and 500 μg of Jun B protein immunoprecipitated with a Jun B rabbit polyclonal antibody and protein-A-sepharose beads. The immunoprecipitated Jun B protein was resolved on 12.5% SDS-PAGE, the gel dried and the amount of labeled Jun B protein determined by phosphoimager. The results of the pulse-chase experiment indicate that the half-life of Jun B protein is similar in 308 and 10Gy5 cells with most of the Jun B protein degraded by 5 hours.
In order to examine Jun B protein translation, 308 and 10Gy5 cells were continuously labeled with $^{35}$S methionine and Jun B protein immunoprecipitated after 1, 2, 3, 4, and 5 hours of labeling. The labeled Jun B protein was then resolved by SDS-PAGE. A reduction in the accumulation of $^{35}$S labeled Jun B protein in 10Gy5 cells was observed in comparison to 308 cells (Figure 18). Since the rates of Jun B protein degradation were similar in the two cell lines, these results suggest that Jun B protein synthesis is reduced in 10Gy5 compared to 308 cells. It is possible, however, that there may be differences in the $^{35}$S labeling index in the two cell lines which could be addressed by examining labeling of another AP-1 family member protein besides Jun B. A decreased rate of Jun B protein synthesis in malignant 10Gy5 cells could account for the lower steady state Jun B protein levels observed by western analysis. The decreased rate of Jun B protein synthesis in 10Gy5 cells may either be a result of mutations in the jun B transcript that effect translation or alterations in the translational machinery.
Figure 18: Continuous $^{35}$S methionine labeling of cells to determine the rate of Jun B protein synthesis in 308 and 10Gy5 cells.

308 and 10Gy5 cells are metabolically labeled with 50 μCi/ml of $^{35}$S methionine. After labeling for various time, protein is isolated. 400 μg of protein lysate are immunoprecipitated with a Jun B rabbit polyclonal antibody and protein-A-sepharose beads. The immunoprecipitated Jun B protein is resolved on 12.5% SDS-PAGE, the gel dried, and exposed to x-ray film. There was a reduction in the amount of labeled Jun B protein that accumulated over time in malignant 10Gy5 cells compared to benign 308 cells.
Discussion

The difference in nuclear Jun B protein levels in benign 308 and malignant 10Gy5 could result from a number of potential mechanisms of Jun B regulation. Aberrant Jun B protein translocation in which Jun B protein is sequestered in the cytoplasm could account for the decrease in Jun B nuclear protein in 10Gy5 cells. As determined by western analysis, this did not appear to be the case as Jun B protein was not present in the cytoplasm of 10Gy5 (Figure 15). However, Jun B protein was detected in both the cytoplasm and nucleus of 308 cells.

Another possible mechanism of Jun B regulation is at the mRNA level, indicating differences in jun B transcription or Jun B mRNA stability. Steady state jun B mRNA levels can be examined in the two cell lines by northern analysis (Figure 16). After normalizing for GAPDH message, there appeared to be no difference in the amount of jun B mRNA in 308 and 10Gy5 cells. These results suggest that the differential regulation of Jun B is at the level of translation or protein stability.

The rate of Jun B protein degradation in the cells was examined by pulse-chase experiments. The rates of Jun B protein degradation in 308 and 10Gy5 cells were similar (Figure 17). However, there was a decrease in the accumulation of 35S labeled Jun B protein over time after continuous 35S methionine labeling in 10Gy5, suggesting that there is a decrease in the rate of Jun B protein synthesis in 10Gy5 cells compared to 308 (Figure 18).
There are several possibilities for why there is a decrease in Jun B translation in 10Gy5 cells. Some of these mechanisms may involve modifications of proteins that are part of the translational machinery. Phosphorylation of initiation factors can influence protein translation. For example, dephosphorylation of eIF-2 can inhibit protein synthesis (Rhoads 1993, Pain 1986, Jansen et al. 1995). Translational regulation of a particular mRNA or a class of mRNAs is most often at the level of initiation, specifically with the accessibility of the cap-binding protein, eIF-4E (Hershey 1991, Jansen et al. 1995). Trans-acting factors can also affect protein synthesis. These factors are either messenger ribonucleoprotein particles (mRNP) which can modulate availability of the mRNA to polysomes or RNA binding proteins (RNA BP) which can mimic secondary structure of mRNA in the 5' leader region, such as with iron regulation of ferritin expression (Jansen et al. 1995, Melefors and Hentze 1993).

While regulation of the translation machinery of the cell is often a general effect, sequence changes in the 5' and 3' untranslated regions as well as the coding region can have a more specific influence on a translation of a particular mRNA. A 5' leader region with a high percentage of GC residues can form secondary structures that can impede the translational machinery (Kozak 1989, Jansen et al. 1995). "Leaky scanning" of the ribosome can occur when there are upstream AUGs from the initiator AUG in the 5' leader region which can inhibit protein synthesis, such as with the β2-adrenergic receptor (Parola and Kobika 1994). As a result of leaky scanning, some genes such as c-myc have multiple initiation codons causing translation of a N-terminal truncated protein or an elongated protein (Hann et al. 1992, Jansen et al. 1995).
Sequences within the coding region of the gene can lead to frameshifts. An example of this is ornithine decarboxylase antizyme, a repressor of ornithine decarboxylase. The in-frame translation of ornithine decarboxylase antizyme mRNA leads to a premature stop, but in the presence of high concentrations of polyamines, a +1 frameshift takes place and antizyme is translated (Matsufuji et al. 1995, Ichiba et al. 1995).

Modifications of the trailer or 3' untranslated region can also affect translation. Long poly(A) tails can lead to enhanced protein synthesis of mRNA (Jansen et al. 1995). Sequences within the trailer 3' untranslated regions can either stimulate or repress translation. AU-rich elements in the 3' untranslated regions have been shown to lead to reduced translation by shortening of the poly(A) tail of c-fos (Wilson and Treisman 1988) and of human interferon-β (Grafi et al. 1993).

In future studies, the jun B sequence from benign 308 and malignant 10Gy5 cells could be analyzed. These experiments could give an indication of mechanisms involved in jun B translational regulation in these mouse keratinocytes.
V. THE ROLE OF JUN B AS A NEGATIVE REGULATOR OF AP-1 ACTIVITY AND TUMORIGENICITY IN BENIGN 308 AND MALIGNANT 10GY5 MOUSE KERATINOCYTES

Introduction

There is evidence to suggest that Jun B protein expression may play a role in reducing cellular proliferation, inhibiting transformation and tumorigenesis, and decreasing AP-1 activity. Jun B protein is induced in myeloid cells in response to various differentiation factors such as IL-6, 1,25-dihydroxyvitamin D₃, TPA, and bryostatin 1 (Lord 1990, Datta 1991) and in terminally differentiating human keratinocytes (Gandarillas and Watt 1995). Experiments using antisense oligonucleotides in a number of different types of cells (3T3 fibroblasts, human mammary carcinoma cells, and PC12 neuronal cells) have indicated that inhibiting Jun B expression leads to an increase in the rate of cell proliferation, while inhibiting c-Jun expression leads to enhanced cell differentiation (Schlingensiepen et al. 1993).

Jun B has been shown to negatively influence both transformation and tumorigenesis in athymic mice. As assayed by a transformation experiment, transfection of primary rat embryo cells with c-jun and c-Ha-ras lead to significant foci formation (Schutte et al. 1989). In contrast, when these cells are transfected with jun B along with c-jun and c-Ha-ras, the number of foci observed in a transformation assay is greatly reduced (Schutte et al. 1989). Jun B overexpression does not cause Rat-1A fibroblasts to
be transformed or tumorigenic, unlike what was observed with c-Jun overexpression
(Schutte et al. 1989).

Jun B can also inhibit AP-1 transactivation. It has been shown that by co-
transfection of F9 teratocarcinoma cells with both c-jun and jun B, AP-1 activity can be
reduced to a lower level of transactivation in comparison to transfection with only c-jun
(Schutte et al. 1989). Jun B does not have as strong transactivating ability from a single
TRE element from the collagenase promoter in F9 and in Hela cells in comparison to c-
Jun (Chiu et al. 1989). Also, co-transfection of c-jun and jun B in F9 cells inhibits c-Jun
transactivation capabilities from the collagenase TRE element (Chiu et al. 1989).

In order to determine the functional role of Jun B protein in the 308 and 10Gy5
mouse keratinocyte system, Jun B protein was down-regulated in benign 308 cells using
antisense phosphorothioate oligonucleotides. 308 cells were transiently transfected with a
Jun B antisense oligonucleotide or a scrambled blocked oligonucleotide and analyzed by
an AP-1 transactivation luciferase experiment.

In addition, the influence of Jun B on AP-1 transcription by stably overexpressing
Jun B protein in malignant 10Gy5 cells was determined by AP-1 CAT assays. Cell growth
curves and subcutaneous injection into athymic nude mice of the stable 10Gy5 Jun B
overexpressing clones demonstrated the effect of Jun B expression on cell growth and
tumorigenesis.
Results

**Jun B steady state protein levels are selectively reduced in benign 308 cells by introducing a Jun B antisense phosphorothioate oligonucleotide.**

A Jun B specific antisense oligonucleotide or a scrambled oligonucleotide as a negative control as described by Schlingensiepen and co-workers (Schlingensiepen et al. 1993) were introduced into 308 cells at a concentration of 0.8 μM. The 308 cells that stably express a luciferase reporter construct for the TRE human collagenase gene promoter region, designated 1A5 cells, were transiently transfected with the blocked antisense oligonucleotides using DOTAP transfection reagent. A second method used to express the blocked antisense oligonucleotides in 308 cells was to transiently transfect both the luciferase reporter plasmid and the oligonucleotides. The reporter construct was first transfected into the cells and then subsequently the phosphorothioate oligonucleotides were transiently transfected into 308 cells. Western analysis for Jun B showed transient repression of Jun B protein by the antisense phosphorothioate oligonucleotides (Figures 19A, 19B). Selective repression of steady state Jun B nuclear protein was observed with the Jun B antisense oligo compared to the scrambled control oligo with both of these methods. Twenty micrograms of nuclear protein were used in the western experiments.
A. Western analysis of Jun B protein levels in IA5 cells. IA5 cells were transiently transfected with either a Jun B specific antisense oligonucleotide or a scrambled antisense oligonucleotide as a negative control. 20 μg of nuclear protein are resolved on 12.5% SDS-PAGE and transblotted to nitrocellulose membrane for western analysis. Jun B protein was detected with a Jun B rabbit polyclonal antibody. The Jun B antisense oligonucleotide specifically reduced Jun B steady state protein levels in IA5 cells.

B. Western analysis of Jun B protein levels in 308 cells transiently transfected with both a collagenase TRE-luciferase reporter construct and antisense oligonucleotides. 308 cells were first transiently transfected with the TRE-luciferase reporter construct and then transiently transfected with antisense oligonucleotides. Jun B western analysis showed a selective decrease of Jun B steady state protein levels with the Jun B blocked antisense oligonucleotide.

Figure 19: Steady state Jun B protein levels after transient transfection with an antisense Jun B phosphorothioate oligonucleotide.
A. Western analysis of Jun B protein levels in IA5 cells. IA5 cells were transiently transfected with either a Jun B specific antisense oligonucleotide or a scrambled antisense oligonucleotide as a negative control. 20 μg of nuclear protein are resolved on 12.5% SDS-PAGE and transblotted to nitrocellulose membrane for western analysis. Jun B protein was detected with a Jun B rabbit polyclonal antibody. The Jun B antisense oligonucleotide specifically reduced Jun B steady state protein levels in IA5 cells.
B. Western analysis of Jun B protein levels in 308 cells transiently transfected with both a collagenase TRE-luciferase reporter construct and antisense oligonucleotides. 308 cells were first transiently transfected with the TRE-luciferase reporter construct and then transiently transfected with antisense oligonucleotides. Jun B western analysis showed a selective decrease of Jun B steady state protein levels with the Jun B blocked antisense oligonucleotide.
Transfection of keratinocyte cells with a Jun B antisense phosphorothioate oligonucleotide leads to elevated AP-1 transactivation.

By selectively reducing Jun B steady state protein levels with blocked antisense oligonucleotides (Figures 19A, 19B), AP-1 transcriptional activity increased as determined by luciferase assays. IA5 cells transiently transfected with the Jun B blocked antisense oligonucleotide had approximately a 6 fold increase in AP-1 transactivation in comparison to untransfected IA5 cells or cells transfected with the scrambled oligonucleotide (Figure 20A). Similar results were also observed when the TRE luciferase reporter construct was transiently transfected into 308 cells and then subsequently transiently transfected with the antisense oligonucleotides. In this experiment, elevated AP-1 transcriptional activity was seen in the 308 cells transfected with the Jun B antisense oligo compared to untransfected 308 cells or cells transfected with the scrambled oligo (Figure 20B). These results demonstrate that reducing Jun B protein in benign 308 cells can lead to enhanced AP-1 transactivation.

Clonal selection of malignant 10Gy5 cells that stably overexpress Jun B protein.

10Gy5 cells were stably transfected with a Jun B expression vector by electroporation. Electroporation conditions of 250 V and 250 μF gave a 50% cell kill in 10Gy5 cells (Figure 21). The amount of cell death after electroporation can be used to determine transfection efficiency of electroporation conditions, with a 50% cell survival
Figure 20: AP-1 transactivation in benign keratinocytes transiently transfected with Jun B antisense phosphorothioate oligonucleotides.

Figure 20A: Luciferase assay for AP-1 transactivation in 1A5 cells transiently transfected with Jun B antisense phosphorothioate oligonucleotides.

After transient transfection with antisense oligonucleotides, 1A5 cells were lysed and cell lysate used in a luciferase reaction. AP-1 luciferase activity was measured by relative light units using a ALL Monolight 2010C luminometer. Relative light units measured from 308 cell lysates not expressing the luciferase reporter construct showed the background of the assay. By reducing Jun B protein in 1A5 cells with the Jun B antisense oligonucleotide, AP-1 activity increased 6 fold in comparison to untransfected 1A5 cells or cells transfected with the scrambled oligonucleotide.
Figure 20B: AP-1 transactivation in 308 cells transiently transfected with a TRE-luciferase reporter construct and Jun B antisense phosphorothioate oligonucleotides. 308 cells transiently transfected with both the AP-1 luciferase reporter construct and the antisense oligonucleotides were analyzed by a luciferase assay for AP-1 transactivation. 10Gy5 cells transiently transfected with the AP-1 luciferase reporter construct showed approximately a 3 fold induction over 308 cells and 308 cells transfected with the scrambled control oligo. 308 cells transfected with the Jun B antisense oligonucleotide gave about a 2 fold increase in AP-1 transactivation.
Figure 21: Cell survival curve of 10Gy5 cells after electroporation.
One million 10Gy5 cells were electroporated at either 250 μF, 500 μF, or 960 μF at various voltage up to 450 volts. Cell were grown for 48 hours and then trypsinized and counted using a Coulter counter to determine the amount of cell kill under the different electroporation conditions. The electroporation conditions of 250 μFarads and 250 volts yielded approximately 50% cell death in 10Gy5 cells. These conditions were used as an indicator of maximum plasmid DNA uptake by cells for establishing 10Gy5 Jun B stable transfectants.
as an indication of maximum transfection efficiency. The Jun B expression vector is a dicistronic plasmid driving the jun B transcript and neomycin resistance gene in the same orientation by the MoMuLV (Moloney murine leukemia virus) promoter. For mock transfected control in this experiment, 10Gy5 cells were transfected with the parental vector that expresses luciferase in place of jun B. These transfection studies generated 25 G418 resistant clones of Jun B-transfected 10Gy5 cells and 20 clones of vector only transfected 10Gy5 cells.

Jun B western analysis of 20 µg of nuclear protein from the 10Gy5 Jun B stable clones indicated that clones Jun B C6 and Jun B C7 expressed intermediate Jun B protein levels while clones Jun B C16 and Jun B C18 expressed high steady state Jun B protein levels (Figure 22). None of the mock transfected 10Gy5 clones had high levels of Jun B expression, however, one of these clones had a slight increase of steady state Jun B protein compared to untransfected 10Gy5 cells. The level of Jun B protein in this mock control clone, though, was significantly lower than in the Jun B overexpressing 10Gy5 clonal cell lines.

**Jun B overexpression in 10Gy5 cells affects AP-1 transactivation.**

AP-1 transcriptional activity in the 10Gy5 Jun B overexpressing stable cell lines was examined from a TRE element of the mouse stromelysin promoter by CAT assay experiments. The intermediate Jun B expressing 10Gy5 clones (Jun B C6, Jun B C7) showed reduced AP-1 transactivation in comparison to the parent 10Gy5 cells or 10Gy5
Figure 22: Jun B western analysis of 10Gy5 Jun B stable transfected clones. Nuclear protein isolated from 10Gy5 cells, 10Gy5 cells transfected with a Jun B expression plasmid, and 10Gy5 cells transfected with the parental (mock) vector were analyzed by western for Jun B steady state protein levels. Western analysis of 25 G418 resistant 10Gy5 clones transfected with the Jun B expression construct and 20 mock 10Gy5 clones identified 10Gy5 Jun B overexpressing clones. Jun B C6 and Jun B C7 express intermediate steady state Jun B protein levels and clones Jun B C16 and Jun B C18 express high amounts of Jun B protein. The arrow indicates Jun B protein.
clones stably expressing the vector alone, mocks C12 and C13 (Figure 23). The level of reduction in TRE activation was to the level of 308 basal AP-1 transactivation. The higher Jun B expressing 10Gy5 clones Jun B C16 and Jun B C18, however, had sustained AP-1 transactivation.

**Jun B overexpression in 10Gy5 cells leads to a decrease in cell growth.**

In order to determine whether Jun B protein expression can affect cell growth, growth curve analysis was done on 10Gy5 Jun B overexpressing clones (Figure 24). At the beginning of the experiment, $1 \times 10^5$ cells were plated and the cells were counted after growing in culture for 24 hours, 36 hours, 48 hours, and 60 hours. Jun B C6 was used as a representative intermediate Jun B expressing clone, Jun B C16 was used as a representative high expressing 10Gy5 Jun B clone, and mock C12 was a representative vector only transfected 10Gy5 clone for this experiment. The mock C12 and untransfected 10Gy5 cells grew at similar rates while with increasing Jun B protein expression cells grew slower, with the high expressing Jun B C16 cell line growing the slowest.

The benign 308 cells grew a slower rate than the parental malignant 10Gy5 cells. However, while 308 cells express relatively high amounts of Jun B protein, the cell growth rate of 308 cells was faster than the 10Gy5 clones which overexpress Jun B protein. This suggests that there are additional changes besides Jun B protein levels between the 308 and 10Gy5 cells that account for the differences in cell growth.
Figure 23: AP-1 transactivation in 10Gy5-Jun B overexpressing stable cell lines. CAT assays were used to examine AP-1 transactivation in 10Gy5 Jun B overexpressing stable clones. The cell lines were transiently transfected with either the pTicTacCAT plasmid containing a region of the mouse stromelysin promoter including the TRE and CACCC motifs or with the control pAMCS CAT plasmid which has the multiple cloning site deleted from the vector. Five micrograms of protein were used in CAT reactions. The incorporated $^{14}$C in the CAT reactions was extracted with xylene and the liquid phase CAT reactions quantitated using a scintillation counter (cpm). The intermediate Jun B expressing cell lines Jun B C6 and Jun BC7 had reduced AP-1 transactivation, while the higher Jun B expressing cells Jun B C16 and Jun B C18 did not show a decrease in AP-1 transcriptional activity. Altering Jun B steady state protein levels may lead to a deregulation of AP-1 transactivation. This data is representative of two independent experiments.
Figure 24: Cell growth curves of 10Gy5-Jun B overexpressing cells. One hundred thousand cells were cultured for 24 hours, 36 hours, 48 hours, and 60 hours. At the various time points cell numbers were determined. The increase in cell number over time was similar for untransfected 10Gy5 and mock C12 cells. The intermediate Jun B expressing 10Gy5 clone, Jun B C6, had a decreased growth rate compared to the parental 10Gy5 and mock C12 cells. The high Jun B expressing 10Gy5 clone, Jun B C16, had the slowest growth rate. These results indicate that with increasing Jun B protein expression, cells grow slower.
Jun B overexpression in malignant 10Gy5 cells leads to increased latency of tumor formation in a mouse tumorigenicity study.

Subcutaneous injection of athymic nude mice with the Jun B overexpressing 10Gy5 stable clones addresses whether increased Jun B expression can affect tumorigenesis of mouse skin. In this tumorigenicity assay, the benign 308 cells did not form tumors throughout the duration of the experiment. Increasing levels of Jun B protein in 10Gy5 cells caused a delay in tumor formation in athymic nude mice after subcutaneous injection (Figure 25). At both 9 and 12 weeks post injection, the parental 10Gy5 cells, the mock transfected 10Gy5 clones, and the intermediate Jun B expressing clones all had approximately 50% of the injection sites forming tumors. The high Jun B expressing cells had less injection sites with tumors, with only 19% for Jun B C16 and no tumors developing for Jun B C18. By 12 weeks after subcutaneous injection, the tumor incidence increased to 25% of injection sites with tumors for Jun B C16 and 33% for Jun B C18.

Jun B overexpression in 10Gy5 cells causes a reduction in tumor size in tumors developed by subcutaneous injection in athymic nude mice.

By 12 weeks, with increasing Jun B protein expression a larger proportion of injection sites of athymic nude mice developed only small tumors or nodules (tumor volumes less than 15 mm³), while 10Gy5, mock C12, and mock C13 all had large tumors
Figure 25: Tumor incidence in athymic nude mice subcutaneously injected with Jun B overexpressing 10Gy5 cell lines.
Tumor development was examined at 9 and 12 weeks after subcutaneous injection of athymic nude mice with Jun B expressing 10Gy5 clones. The 10Gy5 cells, mock transfected 10Gy5 clones, and the intermediate Jun B expressing clones all had approximately 40% of injection sites with tumors at week 9 and 50% of injection sites with tumors at week 12. One of the high Jun B expressing clones, Jun B C16, had a tumor incidence of 19% while the other high Jun B expressing cell line, Jun B C18, did not have tumors at any injection site at week 9. The high Jun B expressing 10Gy5 clones had a tumor incidence of about 30% at 12 weeks after injection. There was enhanced latency in tumor formation with increasing Jun B expression. Tumors did not develop in mice injected with benign 308 cells.
forming by 9 weeks post subcutaneous injection. At week 9, the intermediate Jun B expressing clones had about 50% of injection sites with nodules. One of the Jun B high expressing clones, Jun B C16, had only small tumors or nodules developing (19% of the total injection sites) and the other Jun B high expressing 10Gy5 clone, Jun B C18, had neither nodules or large tumors forming by 9 weeks. Mice were sacrificed in this experiment when at least one tumor had a volume larger than 2 cm³. By 12 weeks, the 10Gy5 and vector only mock control injected mice all had a large tumor burden and were sacrificed. Between 12 and 14 weeks the mice injected with the intermediate Jun B expressing 10Gy5 cells were sacrificed. All the mice injected with the Jun B high expressing clones were still alive with only developing nodules by 16 weeks when the experiment was terminated. At week 16, 75% of all the sites of injection for Jun B C16 had nodules and 83% of all the injection sites for Jun B C18 had nodules.

Figure 26 displays these data as the percent of tumors classified as either small tumors (nodules) or large tumors. At 12 weeks after subcutaneous injection into athymic nude mice, 100% of the tumors that formed with the parental 10Gy5 and mock transfected 10Gy5 cells were classified as large tumors. The intermediate Jun B expressing 10Gy5 clones, Jun B C6 and Jun B C7, had 67% of the tumors that formed classified as large tumors and 33% as nodules. For one of the high Jun B overexpressing 10Gy5 cell lines, Jun B C16, 50% of the tumors were nodules and for the other high Jun B-10Gy5 clone, Jun B C18, 100% of the tumors were nodules.
Figure 26: Size distribution of tumors in athymic nude mice at 12 weeks after subcutaneous injection with 10Gy5 Jun B overexpressing cells.
The parental and mock transfected 10Gy5 cells all had 100% of the injection sites developing tumors by 12 weeks post subcutaneous injection. The percent of lesions that were classified as nodules, a tumor volume less than approximately 15 mm$^3$, was greater with increasing amount of Jun B protein expressed in 10Gy5 cells. Increasing Jun B expression led to reduced tumor burden in athymic nude mice.
Discussion

Altering Jun B protein levels can affect AP-1 transactivation, cell growth, and tumorigenicity in a mouse skin carcinogenesis model system. By transiently transfecting benign 308 cells with a Jun B antisense phosphorothioate oligonucleotide, Jun B steady state protein levels were reduced (Figure 19) and AP-1 transcriptional activity was elevated (Figure 20). Increasing Jun B protein expression stably in malignant 10Gy5 cells also led to a deregulation of AP-1 transactivation. Intermediate Jun B overexpressing 10Gy5 clones (Jun B C6 and Jun B C7) showed decreased AP-1 transcriptional activity from a mouse stromelysin promoter region which includes a TRE element in comparison to the parental 10Gy5 and mock transfected 10Gy5 cells (Figure 23). High expressors of Jun B protein in 10Gy5, clones Jun B C16 and Jun B C18, however, did not show reduced AP-1 transactivation (Figure 23). With elevated Jun B protein, there appeared to be a decrease in cell growth (Figure 24). In the mouse tumorigenicity studies, there was an increase in the latency of tumor formation and a decrease in tumor burden with increasing expression of Jun B protein in 10Gy5 cells (Figure 25, Figure 26).

The majority of the data suggest a correlation with reduced Jun B protein levels and increased AP-1 transcriptional activity, cell growth, and tumor formation. However, AP-1 transactivation studies with the stable Jun B overexpressing 10Gy5 cells showed a decrease in AP-1 activity with intermediate Jun B protein levels, but sustained AP-1 transactivation with higher Jun B protein expression (Figure 23). Chiu and co-workers have shown that Jun B does not have strong transcriptional capabilities from a single TRE.
element (Chiu 1989). One potential explanation for the maintained AP-1 transactivation in the high Jun B overexpressing 10Gy5 clones is that the CAT assay experiments were detecting a large accumulation of Jun B low affinity TRE binding protein complexes.

Another possibility is that Jun B may be sequestering certain AP-1 family members and by altering the levels of Jun B protein in the cells, there are changes in the ability of the different AP-1 members to dimerizing with each other. There is evidence the composition of the AP-1 complex can specifically influence transactivation. In an osteosarcoma cell line, overexpressing Fra-2 and Jun D showed increased AP-1 transcriptional activity, overexpressing c-Jun and any of the Fos family members led to a decrease in transactivation, and overexpressing Fra-1 and Jun B had no effect (McCabe et al. 1996). Inducing c-Fos and Fra-1 expression in human papilloma virus immortalized keratinocytes altered the composition of the AP-1 complex from predominantly c-Jun protein homodimers to c-Jun:Fra-1 and c-Jun:Jun B complexes (Rosl et al. 1997).

A third plausible explanation for the maintained AP-1 transactivation in the high Jun B overexpressing 10Gy5 clones is that an isolated TRE element from the stromelysin promoter was utilized in the CAT assays and analyzing TRE activation from various gene promoters or promoter regions may give different responses. Examination of osteocalcin gene transcription, a marker of osteoblast differentiation, showed differential AP-1 transactivation depending on the promoter region analyzed. AP-1 elements are present within the osteocalcin promoter and can modulate gene transcription differently: the TRE site within the vitamin D-responsive element (VDRE) showed reduced AP-1 transactivation and the AP-1 element within the TGF-β responsive element (TGRE)
conferred enhanced AP-1 transcriptional activity (McCabe et al. 1996). Chicken embryo fibroblasts expressing either c-Jun or v-Jun had different transcriptional capabilities from various point mutated AP-1 and CREB sites, suggesting the importance of specific DNA sequences and the composition of the AP-1 complex (Hadman et al. 1993).

The observed decrease in cell growth with increasing Jun B protein expression in 10Gy5 cells may be a result of Jun B modifying the AP-1 complex in such a manner that there is reduced transcription of genes for cell proliferation. Another possibility is that AP-1 complexes containing Jun B may lead to an induction of genes involved in regulating differentiation. There is evidence, as previously discussed, for a role of Jun B in leading to increased differentiation such as in myeloid cells (Lord et al. 1990, Datta et al. 1991) and human differentiating keratinocytes (Gandarillas and Watt 1995). Finally, it is possible that Jun B protein may be specifically dimerizing with an AP-1 family member, such as c-Fos, that is important for tumorigenesis and sequestering it from being part of the AP-1 complex. Of interest, c-Fos protein specifically dimerizes with Jun B in benign 308 cells (Figure 14). There is evidence to suggest that c-Fos may be a critical factor for the malignant phenotype as discussed in Chapter 3 (Greenhalgh and Yuspa 1988, Ruther et al. 1989, Saez et al. 1995).

The results from the athymic nude mice tumor experiment suggest that Jun B expression is important for tumorigenesis in keratinocytes as indicated by the reduced tumor incidence and tumor burden with elevated Jun B protein levels in malignant 10Gy5 (Figure 25, Figure 26). However, increasing Jun B protein was not sufficient to reverse the malignant phenotype of 10Gy5 cells. Unlike experiments in which 10Gy5 cells were
stably transfected with the dominant negative c-Jun TAM-67 (Domann et al. 1994b). 10Gy5 cells overexpressing Jun B still form tumors in athymic nude mice. There is some evidence that c-Jun can complex with other transactivating factors besides AP-1 proteins and lead to gene transcription. For example, c-Jun has been shown to be able to complex with ATF2 and transactivate an AP-1/CRE like promoter element (Newell et al. 1994, Heckert et al. 1996). c-Jun and c-Fos have also dimerize at the leucine zipper with the NF-kappa B subunit p65 and lead to enhanced DNA binding and transactivation from both the NF-kappa B and AP-1 cis-elements (Stein et al. 1993). The results of this tumor study suggest that the differences between benign 308 and malignant 10Gy5 cells that affect the malignant phenotype of these cells involve other genetic changes besides AP-1 transactivation, such as c-Jun/ATF2 or AP-1/ NF-kappa B transcriptional activity.
VI. CONCLUSIONS

Differential AP-1 regulation in malignant 10Gy5 and benign 308 cells.

There is evidence to suggest that AP-1 transactivation is an important step in the multistep processes of malignant conversion in mouse skin. There is also a correlation with increased AP-1 activity and metastasis. AP-1 may be playing a role in transcriptional regulation of proteases which in turn may be involved in invasion and metastasis by degrading the basement membrane and underlying stroma.

Initial promoter studies of collagenase (Angel et al. 1987) and stromelysin (Matrisian et al. 1986b) mapped TRE elements in these promoter regions and suggested transcriptional induction of these genes by AP-1. More recent studies have tied together AP-1 transactivation, protease expression, and MAPK signal transduction. Gum et al. has shown that with 92 kDa type IV collagenase (MMP-9) abolishing the AP-1 motif in its promoter region, and also expressing a mutated c-Jun protein, leads to decreased MMP-9 transcription (Gum et al. 1997). With MMP-9, AP-1 activity may be regulated by JNK and ERK as inhibiting either of these two kinases reduces MMP-9 transcription (Gum et al. 1997). Another correlation with AP-1 signaling and protease expression involves studies with two NIH 3T3 fibroblast cell lines that are transfected with activated ras. Both of these cell lines had an invasive phenotype and overexpress either uPA or cathepsin L (Silberman et al. 1997). uPA expression appeared to require c-Jun and ERK/JNK.
activities, while cathepsin L expression required ERK activity and suppression of functional c-Jun and JNK kinase (Silberman et al. 1997).

AP-1 activity appears to be important for malignant conversion and tumor formation. Expression of a dominant negative c-Jun TAM-67 in malignant keratinocytes reduced AP-1 transactivation and reversed the development of squamous cell carcinomas (Domann et al. 1994b). The 308/10Gy5 mouse keratinocyte cell lines have been previously used as a model for studying the molecular mechanisms of malignant conversion of mouse skin (Domann et al. 1994a, Domann et al. 1994b). 10Gy5 was one of the malignant cell lines that had been transfected with TAM-67 to examine the effect of functional c-Jun on AP-1 transactivation and tumor formation (Domann et al. 1994b). Since increased AP-1 activity is observed in 10Gy5 compared to its parental 308 cells, and suppression of malignancy with reduced AP-1 transcription was observed, AP-1 activity has been correlated with malignant conversion in these cells (Domann et al. 1994a, Domann et al. 1994b). As a result of these studies, it was of interest to examine mechanisms involved in the differential AP-1 regulation in benign 308 and malignant 10Gy5 cells.

Potential mechanisms of AP-1 regulation to account for the increase in AP-1 activity in malignant 10Gy5 cells are discussed in Chapter 3. Three differences that may influence AP-1 regulation in benign 308 and malignant 10Gy5 cells were observed. Oxidation-reduction regulation of AP-1 appeared to be different in the two cell lines. There is evidence in the literature that AP-1 proteins have enhanced TRE DNA binding capability when they are reduced (Abate et al. 1990, Okuno et al. 1993). AP-1 proteins in
308 cells were not fully reduced in comparison to 10GyS cells when examined by a TRE gel mobility shift assay of protein treated with a reducing reagent (Figure 11). However, it can be concluded that redox regulation may not play a major role in the differential AP-1 regulation in these cells since 308 treated with increasing concentrations of the reducing agent DTT did not show as much AP-1 DNA binding as seen in 10GyS cells in the absence or presence of DTT (Figure 10).

The other two differences in AP-1 regulation seen in 10GyS and 308 cells involved Jun B protein. Jun B protein appeared to be either underphosphorylated in 308 or hyperphosphorylated in 10GyS cells (Figure 12). Phosphorylation of the Jun family members has been shown to influence both positively and negatively AP-1 DNA binding and transactivating capabilities. C-terminal phosphorylation of Jun proteins can inhibit AP-1 DNA binding and AP-1 transactivation (Au et al. 1994, Boyle et al. 1991, De Groot et al. 1993, Nikolakaki et al. 1993). For c-Jun, it has been shown that N-terminal phosphorylation at serines 63 and 73 can positively enhance AP-1 transactivation (Smeal et al. 1992). There is evidence for a kinase docking site between amino acids 30 and 79 of c-Jun that is recognized by JNK for serines 63 and 73 phosphorylation (Hibi et al. 1993, Derijard et al. 1994, Kallunki et al. 1996). While Jun B has a JNK docking site similar to c-Jun, there are some modifications in surrounding residues resulting in reduced AP-1 transactivation and the absence of phosphopeptides for serines 63 and 73 in tryptic mapping experiments (Kallunki et al. 1996). Site-directed mutagenesis of Jun B and c-Jun demonstrated that a proline residue and an arginine residue surrounding the docking site
of the phosphoacceptor region can confer AP-1 transactivation and phosphorylation of N-terminal serines (Kallunki et al., 1996).

A third difference observed in the two cell lines is that Jun B steady state protein levels were reduced in malignant 10Gy5 cells in comparison to benign 308 cells (Figure 6, Figure 15). There is evidence in the literature to suggest that Jun B protein may play a role as a negative regulator of cell proliferation and malignancy. Jun B is induced in both differentiated myeloid and keratinocyte cells (Lord et al., 1990, Datta et al., 1991, Gandarillas and Watt, 1995). Reducing Jun B protein levels through antisense studies in fibroblasts, breast cancer cells, and neuronal cells, showed an inverse correlation with Jun B expression and cell proliferation (Schlingensiepen et al., 1993). This effect of Jun B on cell growth was also observed when Jun B was overexpressed in fibroblast cells (Schutte et al., 1989). Jun B has also been shown to have decreased AP-1 transactivating capabilities from a single TRE element in comparison to c-Jun (Chiu et al., 1989, Schutte et al., 1989). Recent studies suggest that elevated Jun B levels can lead to reduced AP-1 transactivation and growth suppression in NIH 3T3 cells is a result of signal transduction through PKCζ and the ERK signal pathway (Ueda et al., 1996). Based on these data, it is possible that the increased AP-1 transactivation in 10Gy5 cells and its malignant phenotype in comparison to benign 308 cells may be a result of decreased Jun B protein levels.

Differences in AP-1 transactivation in benign 308 and malignant 10Gy5 cells could be a result of alterations in Jun B phosphorylation, AP-1 oxidation-reduction regulation, and/or changes in overall steady state Jun B protein levels (Figure 27). There
Figure 27: Proposed model for differential AP-1 regulation in benign 308 and malignant 10Gy5 cells. Differences in Jun B protein phosphorylation and/or Jun B protein expression levels may be involved in AP-1 regulation in these cells. There could also be additional genetic changes that are presently unknown that may lead to the increased AP-1 transactivation in 10Gy5 cells.
could also be additional genetic changes that could influence the differential AP-1 and Jun B regulation in 308 and 10Gy5 cells, perhaps through modifications of a MAP kinase signal transduction pathway.

A potential mechanism for the differences in AP-1 transactivation could involve alterations in Jun B phosphorylation. Jun B in 10Gy5 could be mutated at sequences surrounding the phosphoacceptor region at the N-terminus of the protein resulting in a MAPK, such as JNK, being able to phosphorylate Jun B causing enhanced AP-1 transactivation. It would be of interest to examine amino acids 30 through 79 of Jun B in 308 and 10Gy5 cells by tryptic phosphopeptide mapping. If differences were observed, site-directed mutagenesis of Jun B in 308 could be done to verify whether the specific mutations confer increased AP-1 transactivation. Upstream signal transduction could also be examined to determine whether a MAPK family member is involved in phosphorylating Jun B in these mouse keratinocyte cells. Redox regulation is another post-translational mechanism that could be a factor involved in the differential AP-1 regulation observed in these cells as the AP-1 proteins in malignant 10Gy5 cells are more fully reduced in comparison to 308 cells.

Finally, another possible mechanism for the correlation with AP-1 elevation and malignant conversion of benign 308 to malignant 10Gy5 cells could involve the difference in overall Jun B steady state protein levels. Decreased Jun B protein levels in 10Gy5 cells may influence AP-1 transactivation. In turn, this could lead to increased cell proliferation and tumorigenesis.
Effect of Jun B expression on AP-1 transactivation and tumorigenesis.

In order to test the hypothesis that Jun B protein levels modulate AP-1 activity and malignancy in 308 and 10Gy5 mouse keratinocytes, transfection studies were performed with either a Jun B antisense phosphorothioate oligonucleotide in benign 308 cells or a Jun B expression plasmid in malignant 10Gy5. Transient transfection of 308 cells with a Jun B antisense oligonucleotide increased AP-1 transactivation in these cells (Figure 20). 10Gy5 cells stably overexpressing Jun B also showed an affect on AP-1 transactivation in response to Jun B protein (Figure 23). Intermediate Jun B expressing 10Gy5 clones, Jun B C6 and Jun B C7, showed reduced AP-1 transcriptional activity, but the high expressing Jun B 10Gy5 clones did not have decreased AP-1 transactivation as determined by in vitro CAT assays.

There are several potential explanations for this AP-1 transactivation data with 10Gy5 cells overexpressing Jun B protein. Jun B has been shown to have weak transcriptional capabilites from a single TRE element (Chiu et al. 1989). One possibility for the results in the AP-1 CAT assay experiments with the 10Gy5-Jun B clones is that there is an accumulation of Jun B low affinity AP-1 DNA binding complexes. Another plausible explanation is that by altering Jun B protein levels in the cells, there are changes in the composition of the AP-1 complex. There is evidence to indicate that specific AP-1 family members dimerizing with each other can influence transactivation. Increasing expression of individual AP-1 family members can have varying affects on AP-1 transcriptional activity in an osteosarcoma cell line (McCabe et al. 1996) and also change
the specific AP-1 complexes that can bind to the TRE in immortalized human keratinocytes (Rosl et al. 1997). A third possibility is that the AP-1 transcriptional response in an in vitro CAT reporter assay may be sequence-dependent, with various gene promoters or promoter regions giving different transactivation, as discussed above (McCabe et al. 1996, Hadman et al. 1993). In addition, post-translational modifications of AP-1 proteins may influence AP-1 activity. Comparison of 308 and 10Gy5 cells indicated that there were differences in both AP-1 oxidation-reduction regulation and phosphorylation. It is possible that by increasing Jun B protein in 10Gy5 there are alterations in the redox potential of these cells or the availability of certain kinases.

Future experiments could examine the specific composition of the AP-1 complex in the 10Gy5 Jun B overexpressing clones. As sequence differences can influence transactivation in in vitro reporter assays, different AP-1 target sequences could be examined in these 10Gy5 stable Jun B expressing cell lines. It is also possible that the Jun B expressing clones have different amounts of phosphorylated Jun B protein in the cells or different kinase activities which could affect AP-1 activity and tumorigenesis. N-terminal phosphorylation of Jun B in the clones and the role of upstream kinase activation in AP-1 signal transduction pathways could be examined.

Studies with the 10Gy5 Jun B stably overexpressing cell lines showed reduced cell growth, increased latency of tumor formation, and a decrease in tumor size (Figure 24, Figure 25, Figure 26). While these results suggest that Jun B overexpression in keratinocyte cells is not sufficient to reverse malignancy, Jun B protein may play a role in regulating cell growth and tumor development of mouse skin (Figure 28). Jun B protein
Figure 28: Potential mechanisms for reduced tumor development by Jun B overexpression.
may be affecting tumor cell growth through downregulating genes important for cell proliferation and metastasis, perhaps by sequestering other AP-1 family members such as c-Fos from the AP-1 complex. Double immunoprecipitation experiments with 308 cells (Figure 14) showed specific dimerization of Jun B protein with c-Fos, suggesting that a sequestering mechanism may take place. Jun B may also induce genes involved in regulating cell differentiation or apoptosis. It would be of interest in future experiments to examine cell proliferation of these Jun B overexpressing 10Gy5 cells by \(^3\)H thymidine incorporation. It would also be interesting to look at expression of genes that may play a role in tumor development such as proteases or differentiation markers.

It is important to discuss the differences in the 10Gy5 tumor studies previously done with dominant negative c-Jun TAM-67 overexpression and the 10Gy5 tumor studies discussed above with Jun B overexpression. Domann et al. published data showing suppression of the malignant phenotype by TAM-67 expression in 10Gy5 (Domann et al. 1994b), perhaps as a result of reduced AP-1 transactivation. However, tumor studies with Jun B expression in 10Gy5 cells indicated that while tumor development was impeded by Jun B overexpression, tumor formation was not inhibited (Figure 25, 26). c-Jun is known to be able to dimerize with other transcription factor besides AP-1 such as ATF-2 to induce transcription from AP-1/CRE promoter element and NF-kappa B subunit p65 to enhance DNA binding and transactivation from the NF-kappa B and AP-1 cis-elements (Stein et al. 1993, Newell et al. 1994, Heckert et al. 1996). It would be of interest in the future to examine other transcription factors in the 308/10Gy5 mouse keratinocyte system.
such as c-Jun/ATF2 complexes or AP-1/ NF-kappa B complexes and their role in regulating malignancy in these cell lines.

In summary, skin carcinogenesis is a complex multistep process involving many genetic alterations. The benign 308 mouse keratinocyte cell line and its malignant variant 10Gy5 provide a useful system to examine molecular mechanisms involved in regulating malignant conversion. AP-1 transactivation may play an important role in this process. The results of the experiments discussed in this dissertation support the hypothesis that the individual AP-1 family members and the specific composition of the AP-1 complex may be critical in regulating gene transcription. Jun B protein may be a potential negative regulator of AP-1 transactivation as well as cell growth and tumor development, providing a useful target for manipulating skin carcinogenesis.
APPENDIX: ANIMAL SUBJECTS APPROVAL

THE UNIVERSITY OF
ARIZONA

Verification of Review
By The Institutional Animal Care and Use Committee (IACUC)

Final Approval Granted

PHS Assurance No A-3248-01 - USDA No 86-3

TITLE: PROTOCOL CONTROL # 95-133

"Radiation Induced Skin Tumor and Oncogene Activation"

PRINCIPAL INVESTIGATOR/DEPARTMENT

G. Tim Bowden - Radiation Oncology
Elizabeth Joseloff - Graduate Student - Dissertation Study

SUBMISSION DATE July 19, 1995
APPROVAL DATE September 28, 1995

GRANTING AGENCY

NIH/NCI

The University of Arizona Institutional Animal Care and Use Committee reviews all sections of proposals relating to animal care and use. The above named proposal has been granted Final Approval according to the review policies of the IACUC.

NOTES

*** Full approval of this control number is valid through* September 27, 1998

* When project or grant periods extend past the above noted expiration date, the Principal Investigator will submit a new protocol proposal for full review. Following IACUC review, a new Protocol Control Number and Expiration Date will be assigned.

*** Continued approval for this project was confirmed October 22, 1997

*** Revisions (if any), are listed below.

Michael A. Cusanovich, Ph.D
Vice President for Research

DATE October 22, 1997
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