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IDENTIFICATION OF TARGET GENES OF PEA3, AN ETS FAMILY TRANSCRIPTION FACTOR.

By

JASON ELLISON, B.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

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IDENTIFICATION OF TARGET GENES OF PEA3, AN ETS FAMILY TRANSCRIPTION FACTOR.

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AUTHOR: Jason Ellison, B. Sc. (University of Western Ontario)

SUPERVISOR: Professor J. A. Hassell

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ABSTRACT

PEA3 is a transcription factor of the ETS family, which has been implicated in mammalian development, tumorigenesis, and tumor progression. An attempt has been made to identify genes whose transcription is regulated by this factor in order to help elucidate its role in these processes.

Cell lines, designated RP2, RP10 and RP11, were generated in which *PEA3* overexpression can be induced at both the mRNA and protein levels. *PEA3* overexpression in these cell lines is accompanied by activation of transcription of known PEA3 target genes such as *MMP-9* and *MMP-14*. Control cell lines, identical to these except for the lack of inducible *PEA3*, show no such activation of PEA3 target genes under the same conditions.

In order to identify PEA3 target genes mRNA isolated from RP10 cells before and after induction of *PEA3* overexpression was compared by Differential Display-Reverse Transcription PCR. A number of mRNAs were identified that are coordinately upregulated with *PEA3*. These include the mRNA that encodes Activated Leukocyte Cell Adhesion Molecule (ALCAM), which is a strong candidate PEA3 target. Activation of *ALCAM* transcription occurs in RP10 cells upon induction of *PEA3* overexpression, but not in control cell lines. Similarly, activation of *ALCAM* transcription occurs in serum starved wild-type MEF cell lines upon induction of *PEA3* expression by the addition of EGF, but not in *PEA3*-null MEF cell lines under the same conditions. Finally, PEA3 is able to activate the expression of reporter genes downstream from fragments of the *ALCAM* promoter that contain consensus PEA3-binding sites.

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CONTRIBUTIONS BY OTHERS

Figure 3.3, Figure 3.5A, Figure 3.10, and Figure 4,8. Michael Laing, a graduate student in this laboratory, constructed the antisense *PEA3* riboprobe used in these RNase protection assays.

Figure 3.6. Dr. Richard Tozer, a post-doctoral fellow in this laboratory, using protein samples that I isolated, performed the Western blot shown in this figure.

Figure 4.8 and Figure 4.9. Laura Hastings, a technician in this laboratory generated the wild type and *PEA3*-null mouse embryo fibroblast cell lines used in these experiments.

Figure 4.11 and Figure 4.12. Trevor Sheppard, a graduate student in this laboratory, constructed the 3xPEA3Luc vector used as a positive control for these luciferase assays. I generated all ALCAM promoter vectors.

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CHAPTER 1

INTRODUCTION

1.1 Cancer: Its Nature and Causes.

Cancer is a disease characterized by the uncontrolled proliferation of a population of cells within a multicellular organism. The replication and behavior of cells is normally tightly controlled so that the activity of each individual cell meets the needs of the organism as a whole. In cancer, cells escape these controls and replicate continuously forming tumors. Such cancerous cells often metastasize to distant sites where they are able to form secondary tumors. Ultimately, these tumors interfere with the normal functioning of tissues in which they form, leading to deleterious effects to the organism (Cooper, 1990).

Cancer has occurred in humans throughout history. Written descriptions of tumors from ancient India date from approximately 2000 BC. However, only recently has any progress been made in elucidating the causes of this disease. The British physician Percival Pott made one of the earliest important discoveries in 1775. Pott noticed that the incidence of scrotal cancer among chimney sweeps was much higher than in the general population. He suggested that this might have been due to exposure to soot and poor hygiene among the chimney sweeps. His discoveries led the chimney sweep guilds of

Europe to rule that their members bathe daily, after which this cancer virtually disappeared (Diamandopoulos, 1996).

It was not until 1915 that Pott's suggestion that exposure to soot could cause cancer was directly confirmed. Japanese researchers Yamagiwa and Ichikawa demonstrated that repeated application of coal tar to the skin of rabbits induced skin cancer after a long period of latency (reviewed by Miller, 1970). This was the first direct demonstration that prolonged exposure to chemical substances in the environment could lead to cancer. A great number of chemical carcinogens have since been discovered, including many polycyclic aromatic hydrocarbons, aromatic amines and heavy metal containing compounds. Interestingly, many of these compounds are not carcinogenic by themselves, but are converted into carcinogens by the metabolism of the host organism (reviewed by Shields and Harris, 1993).

Cancers can also be caused by exposure to various forms of radiation. For example, prolonged exposure to sunlight has long been known to be associated with an increased incidence of various skin cancers, particularly amongst fair skinned people. Moreover, shortly after the discoveries of X-rays and ionizing radiation, elevated levels of various cancers were observed in people who worked with these forms of radiation (reviewed by Hal, 1993). As with chemical carcinogens, these observations were confirmed using experimental animals. For example, it was demonstrated that prolonged

exposure to ultraviolet (UV) light could cause skin tumors in mice, and increase the rate at which chemically induced tumors formed (Findlay, 1928).

All of these various carcinogenic agents are capable of causing damage to DNA. For example, exposing germ cells from the fruit fly *Drosophila melanogaster* to X-rays causes chromosomal breaks and rearrangements, leading to the production of heritable mutations (Muller, 1927). Similar effects are observed upon treating these cells with chemical carcinogens (Aurbach et. al., 1947). Furthermore, it has been shown that UV irradiation can damage DNA by causing the dimerization of the pyrimidine rings of adjacent pyrimidine residues (Beukers and Berends, 1960). Inability to repair this type of damage is associated with the disease Xeroderma Pigmentosum, which is characterized by extreme sensitivity to sunlight and multiple skin tumors (Cleaver, 1968). Carcinogens induce mutations that disrupt the regulation of cellular growth, leading to continuous inappropriate cell division.

1.1.1 The Progressive Nature of Cancer and the Theory of Clonal Evolution of Cancer.

The experimental induction of tumors in animals has demonstrated that tumor development occurs in stages. Upon repeated application of carcinogen, tumors initially form as benign growths, but subsequently undergo irreversible qualitative changes that lead to an increase in the aggressiveness of the tumor. These changes include the faster

growth, increased invasiveness, reduced antigenicity, resistance to various cytotoxic drugs (Foulds, 1958). A similar progression has been observed in naturally occurring tumors in humans. In many cases, this progression correlates with the presence of chromosomal abnormalities in the tumor cells. These include chromosomal translocations and duplications, amplifications or deletion of regions of chromosomes and loss of heterozygosity (LOH) (Nowell, 1989).

For example, skin cancer can be induced in mice by treatment with the carcinogen dimethylbenzanthracene (DMBA), followed by repeated application of the tumor promoter 12-O-tetradecanoyl phorbol-13-acetate (TPA). These tumors arise as benign papillomas, but some progress to the increasingly more malignant squamous cell carcinomas and spindle cell carcinomas. Papilloma cells arising in this manner show trisomies of chromosomes 6 and 7, while the more malignant cells also show additional abnormalities, including LOH of chromosomes 4, 6, 7 and 11 (Kemp et al., 1994).

Naturally occurring melanomas also progress through well-defined stages of increasing malignancy, from a benign melanocytic nevus to malignant melanoma (Clark et al., 1985). Abnormalities of the short arm of chromosome 9 and the long arm of chromosome 16 are associated with the cells of early stage melanoma, while alterations of chromosomes 1, 6 and 7 are associated with progression of the disease (Nowell, 1989).

Observations of this nature have given rise to the theory of clonal evolution of tumor cell populations. This theory postulates that tumors are initiated by a mutation

occurring in a single cell that gives it a growth advantage over neighboring normal cells. Generally, this initial mutation is insufficient to give rise to a tumor. However, mutations continue in this population due to genetic instability associated with its increased rate of mitosis. Occasionally, these will give a further selective growth advantage, which leads to the formation of progressively more aggressive tumor cells (Nowell, 1976).

1.2 Oncogenes, Proto-oncogenes, and the Regulation of Cell Division.

What sorts of genes are mutated in cancerous cells, and how do these mutations contribute to the loss of control of cellular growth? To understand this we must first understand the mechanisms by which the division of animal cells occurs and is controlled. When animal cells are dividing, they cycle through four distinct phases. These are the gap 1 (G1), DNA synthesis (S), gap 2 (G2), and mitosis (M) phases. During the G1 phase, cells are diploid (2N) in DNA content, are highly metabolically active and increase in size. During S phase DNA is replicated resulting in cells becoming tetraploid (4N) in DNA content. Cells then enters the G2 phase, which is defined as the interval of time between DNA synthesis and the final stage of the cell cycle, M phase. During M phase cells undergo mitosis to divide into two diploid daughter cells (Pardee et al., 1978).

Cells can also enter a state of quiescence, referred to as G0, in which they exit the cell cycle, do not divide for long periods of time and do not increase in size. Cells may

also become terminally differentiated, undergoing irrevocable morphological and physiological changes, which permanently prevents them from reentering the cell cycle. All quiescent or terminally differentiated somatic cells are 2N in DNA content. After dividing, cells may undergo another round of division or become quiescent or terminally differentiated at some point in G1. This decision depends of the presence or absence of appropriate growth factors (Pardee et al., 1978; Cooper, 1990).

1.2.1. Growth Factors and Growth Factor Receptors.

Growth factors are proteins that are secreted by or bound to the membrane of the cells that produce them. Cells require receptor molecules that are able to bind to these growth factors in order to respond to them (Cross and Dexter, 1991; Porter and Vaillancourt, 1998). The binding of growth factors to their receptors triggers changes in cellular biochemistry, which alter the activities of various proteins (Cantley et al., 1991; Porter and Vaillancourt, 1998). Ultimately, this leads to changes is gene expression. Genes whose products are required for cellular division or differentiation are turned on, while those whose products are inhibitory to these processes are turned off.

Growth factor receptors are membrane bound proteins that consist of three domains: an extracellular ligand-binding domain, a transmembrane domain, and an intracellular domain that has an enzymatic activity responsible for beginning the process of signal transduction (Cross and Dexter, 1991; Porter and Vaillancourt, 1998). Often

these receptors are protein tyrosine kinases, molecules that transfer phosphate groups from adenosine triphosphate (ATP) to tyrosine residues in substrate proteins, thereby altering the activities of these proteins. Binding of a growth factor to its receptor leads to receptor dimerization. Receptor molecules form dimers with identical or closely related receptor molecules. Upon dimerization, tyrosine residues within the kinase domains of these receptors become phosphorylated, causing a conformational change in these domains resulting in enhanced kinase activity. Phosphorylation of other tyrosine residues in the cytoplasmic domains of these receptors leads to recruitment of target molecules to the receptor, which also become phosphorylated (Cantley et al, 1991, Porter and Vaillancourt, 1998). These target molecules contain Src-homology 2 (SH2) or protein tyrosine binding (PTB) domains, modules that recognize and bind phosphotyrosine residues in growth factor receptors and other proteins within the context of specific amino acid sequences (Pawson and Gish, 1992: Pawson and Scott, 1997). These molecules are typically enzymes whose activity may be regulated by phosphorylation (Cantley et al., 1991; Porter and Vaillancourt, 1998).

The signals emanating from many growth factor receptor tyrosine kinases are very similar. The prototypical growth factor receptor tyrosine kinase is the platelet-derived growth factor (PDGF) receptor. This receptor will be used as an example to demonstrate the process of growth factor receptor tyrosine kinase signaling and introduce some of the molecules involved in this signaling.

1.2.2 Growth Factor Signaling Through the PDGF Receptor.

PDGF is a potent stimulator of growth of a variety of cells, including fibroblasts, smooth muscle cells and endothelial cells. It consists of a dimer of A and B subunits, with both homodimeric (AA, BB) and heterodimeric (AB) forms existing (Heldin et al., 1998). These molecules bind to and induce dimerization of two receptor molecules, the α- and β-receptors. PDGF-AA induces αα-receptor dimers, PDGF-AB αα- and αβ-receptor dimers and PDGF-BB all three possible combinations. Signaling through these various receptor combinations produce similar, though not identical results, as each recruits different combinations of signaling molecules (Heldin et al., 1998).

Dimerization and phosphorylation of PDGFR molecules leads to the recruitment of a number of intracellular signaling molecules, including phospholipase C-γ (PLC-γ), phosphotidylinositol-3'-kinase (PI3'K), Ras-interacting proteins (such as Grb-2, Shc and Ras GTPase activating protein (GAP)), non-receptor tyrosine kinases (such as Src) and protein phosphotyrosine phosphatases (such as SHP-2) (reviewed by Heldin et al., 1998). The manner in which each of these types of molecules acts to transduce signals from the PDGFR will be discussed briefly.

1.2.2.1 Signaling Through Phospholipase C-γ.

The phospholipases are a family of phosphodiesterases that metabolize phospholipids. Members of the phospholipase C family act on phosphotidylinositol-4,5-bisphosphate (PIP_2) molecules present in the plasma membrane, cleaving them to form diacyl glycerol (DAG) and inositol trisphosphate (IP_3) (Berridge, 1993). The γ-isoform

of phospholipase C contains two SH2 domains through which it is recruited by the activated PDGFR. Phosphorylation of PLC-γ by the receptor leads to activation of its phosphodiesterase activity and production of DAG and IP_3 (Berridge, 1993; Heldin et al., 1998). IP_3 binds to and opens calcium channels in the endoplasmic reticulum, causing an influx of Ca^{2+} into the cytoplasm. DAG interacts with, and activates, members of the protein kinase C (PKC) family, serine/threonine kinases that phosphorylate serine and threonine residues in a number of proteins, altering their activities. As PKC also requires Ca^{2+} for activity, the release of Ca^{2+} from the endoplasmic reticulum by IP_3 also contributes to PKC activation (Berridge, 1993). PKC and Ca^{2+} activate Na^+/H^+ exchangers in a number of cell types, leading to the import of Na^+ and the export of H^+, and alkalization of the cytoplasm (Ma et al., 1994). Thus, activation of PLC-γ by the PDGFR leads to a number of changes in cellular biochemistry, including increased Ca^{2+} concentration and pH as well as changes in the phosphorylation status and activities of a number of proteins.

1.2.2.2 Signaling Through Phosphotidylinositol 3'-kinase.

PI3'-kinases are enzymes that phosphorylate inositol containing phospholipids, such as PIP_2 at the 3' position of their inositol ring (Carpenter and Cantley, 1990). PDGFR can interact with and activate certain members of this family. These proteins are composed of two subunits, p85, a regulatory subunit and p110, the catalytic subunit. The p85 subunit contains two SH2 domains through which it interacts with activated PDGFR. This interaction leads to the activation of the p110 catalytic subunit (Margolis, 1992; Heldin et al., 1998). The preferred substrate for PI3'-kinase is PIP_2, which it

phosphorylates to generate phosphotidylinositol-3,4,5-trisphosphate (PIP_3). PIP_3 interacts with and activates a number of serine/threonine kinases, including Akt (PKB) and members of the PKC family (Klippel et al., 1997; Heldin et al., 1998). PI3'-kinase activity also contributes to activation of Na^+/H^+ exchangers and the alkalization of the cytoplasm in response to PDGF (Ma et al., 1994).

1.2.2.3 Signaling Through Ras.

Many cellular processes, including growth and differentiation, control of the cytoskeleton and transportation of molecules between different parts of the cell, are controlled by members of a superfamily of small, plasma membrane associated GTPases (Bourne et al., 1991; Boguski and McCormick, 1993). Of these molecules, members of the Ras family (H-Ras, N-Ras, K-RasA, and K-RasB) are particularly important in growth factor signaling. Small GTPases are able to bind GTP or GDP but are active only in the GTP-bound form. As their name implies, these molecules have weak GTPase activity, hydrolyzing GTP to GDP, leading to inactivation of the protein (Bourne et al., 1991). This weak activity is enhanced by interaction of the GTPase with specific GTPase-activating proteins (GAPs). Reactivation of the GTPase is carried out by their interaction with guanine-nucleotide exchange factors (GEFs), which cause GDP bound by the GTPase to be replaced by GTP (Boguski and McCormick, 1993).

Ras proteins are important in transducing a number of signals, including the response to growth factors and cellular stress. In the active state, Ras is able to activate a number of protein kinases. For example, when activated in response to growth factors, Ras interacts with the serine/threonine kinase Raf, recruiting it to the plasma membrane

where it becomes active (Leevers et al., 1994). Raf then phosphorylates and activates the dual-specificity kinase MEK, which is capable of phosphorylating proteins on serine, threonine and tyrosine residues (Dent et al., 1992). MEK phosphorylates and activates two serine/threonine kinases, the extracellularly regulated kinases (ERK) 1 and 2 (Crews et al., 1992). Upon phosphorylation, ERK-1 and -2 are relocalized from the cytoplasm to the nucleus where they are able to phosphorylate and alter the activity of various transcription factors, leading to changes in gene expression (Wilkinson and Millar, 1998). Ras is capable of activating other similar kinase cascades, which always involve at least three kinases acting upon one another. In response to cellular stress, such as exposure to UV light, a different kinase cascade is initiated through Ras, which culminates in the activation of members of the Jun N-terminal kinase (JNK) family. At least five similar, independent kinase cascade pathways exist in mammalian cells. These are called mitogen activated protein (MAP) kinase cascades, and the final kinases activated by these pathways make up the family of MAP kinases (reviewed by Schaeffer and Weber, 1999).

While Ras is activated by growth factor receptors, these molecules do not interact directly with Ras. The PDGFR activates Ras via two adapter molecules, Grb-2 and Shc (Heldin et al., 1998). Grb-2 is a small molecule, consisting of an SH2 domain and two SH3 domains, modules that bind proline-rich sequences in other proteins (Pawson and Gish, 1992). Through its SH3 domains Grb-2 forms a complex with the protein Sos-1, a GEF for Ras. The activated PDGFR recruits the Grb-2/Sos-1 complex to the plasma membrane through the SH2 domain of Grb-2, where it is brought into close proximity to Ras molecules that it is then able to activate (Margolis, 1992; Boguski and McCormick, 1993).

Shc is another SH2 domain containing protein that is recruited to the activated PDGFR. Upon binding the PDGFR, Shc is phosphorylated, creating a binding site for Grb-2. Again, this leads to recruitment of the Grb-2/Sos-1 complex to the plasma membrane, where it is able to activate Ras (Rozakis-Adcock et al., 1992; Heldin et al., 1998).

The GAP for Ras also contains two SH2 domains and is recruited by the PDGFβ receptor, bringing it into position to inactivate Ras (Heldin et al., 1998). Thus, the stimulation of Ras by PDGF is tightly regulated, with a balance of positive and negative signals generated. This balance is a common feature of growth factor receptor-mediated signaling that ensures that cells normally proliferate in a controlled manner.

1.2.2.4 Signaling Through Non-Receptor Tyrosine Kinases.

Tyrosine kinases exist that, unlike growth factor receptors, do not contain transmembrane or extracellular domains. The prototype for these non-receptor tyrosine kinases is c-Src. Phosphorylation of c-Src at a carboxy-terminal site by another non-receptor tyrosine kinase, c-Src kinase (Csk), creates a binding site for an SH2 domain in c-Src, causing c-Src to fold up on itself in an inactive conformation (Nada et al., 1991; Schwartzberg, 1998). This SH2 domain can also bind to activated PDGFR causing its displacement from the carboxy-terminus of c-Src, allowing c-Src to enter an active conformation. Phosphorylation by the PDGFR also contributes to the activation of c-Src (Heldin et al., 1998; Schwartzberg, 1998). Phosphorylation by c-Src regulates the activities of a number of important signaling molecules. For example, Raf can be activated by c-Src as well as by interaction with Ras (Stokoe and McCormick, 1997).

1.2.2.5 Signaling Through Protein Phosphotyrosine Phosphatases.

Certain protein phosphotyrosine phosphatases contain SH2 domains. One of these is SH2-containing phosphatase-2 (SHP-2), which can be phosphorylated and activated by the PDGFR (Lechleider et al., 1993; Heldin et al., 1998). SHP-2 may act as a negative regulator of PDGF-mediated signaling, dephosphorylating and inactivating the receptor and its substrates. However, SHP-2 may also contribute to the transduction of a positive signal by the PDGFR. Phosphorylation of SHP-2 by the PDGFR creates a binding site for the SH2 domain of Grb-2. Therefore, SHP-2 can act in a manner similar to Shc, recruiting Grb-2 to the plasma membrane and leading to Ras activation (Li et al., 1994). Also, SHP-2 may dephosphorylate the carboxy-terminal regulatory site of c-Src, leading to its activation (Peng and Cartwright, 1995; Heldin et al., 1998). These opposing activities of SHP-2 demonstrate the fine control of the level of the signal that is generated by the PDGFR in normal cells.

In summary, growth factors generate intracellular signals by binding to receptors in the plasma membranes of their target cells. Upon binding of their ligand these receptors activate multiple, interdependent, tightly controlled signaling pathways, which ultimately lead to changes in gene expression. While the PDGFR has been used as an example here, all growth factor receptors generate signals by similar mechanisms. The ability of a growth factor to generate a signal, and the nature and intensity of that signal depends on the presence of a receptor for that growth factor in the target cell, and the expression levels of the signaling molecules that receptor uses to transduce the signal. Cancer cells are able to divide in the absence of growth factors. Mutations occurring in

genes whose products are involved in growth factor signaling that prevent these molecules from being regulated properly contribute to the uncontrolled proliferation of these cells.

1.2.3 RNA Tumor Viruses and Oncogenes.

Much of our understanding of how mutations in genes whose products are involved in growth factor signaling contribute to carcinogenesis comes from studies on retroviruses known as the RNA tumor viruses. Upon infection of host cells, a DNA copy of the retroviral genome, the provirus, is produced and is integrated into the genome of the host. Transcription of the proviral DNA by the host cell generates progeny viral genomes and mRNAs, which are translated into proteins encoded by the viral genome (reviewed by Cooper, 1990).

Two types of RNA tumor viruses exist (Cooper, 1990). Acutely transforming viruses rapidly induce tumors in infected animals. The prototype for this type of virus is the Rous sarcoma virus (RSV), which rapidly induces tumors in chickens (Rous, 1911). Weakly oncogenic viruses induce tumors after a latency of several months. The prototype for this type of virus is the avian leukosis virus (ALV), which induces B-cell lymphomas in chickens (Cooper, 1990). Both RSV and ALV are able to infect and replicate in cultured cells, but only RSV transforms these cells. That is, in confers upon them properties of cancer cells, such as loss of contact inhibition of cell division, the ability to grow in semi-solid media and to form tumors when injected into immunodeficient mice (Freedman and Shin, 1974).

RSV and ALV are closely related viruses, infecting the same host cells and replicating using a similar mechanism. However, the genome of RSV is approximately 1.5 kilobases longer that that of ASV (Duesberg and Vogt, 1972; Duesberg and Vogt, 1973; Cooper, 1990). It was hypothesized that this extra RNA contained the sequences required for the transforming ability of RSV. Consistent with this, mutant RSV strains that lack transforming activity have been isolated, and have genomes of similar size to ASV, suggesting they have lost the extra sequences required for transformation (Martin and Duesberg, 1972). These sequences were shown to be homologous to sequences found in the chicken genome (Stehelin et al., 1976).

The extra sequences in RSV encode a mutant version of the non-receptor kinase c-Src (see Section 1.2.2.4). The viral version of the *src* gene, *v-src*, is mutated such that the sequences encoding the carboxy-terminal 19 amino acid residues of the cellular form of Src, c-Src, have been replaced by sequences encoding a new set of 12 amino acids (Takeya and Hanafusa, 1983). Since v-Src lacks the carboxy-terminal tyrosine phosphorylated by Csk it cannot by inactivated by this kinase, and is therefore constitutively active (Schwartzberg, 1998). As well, *v-src* is expressed in RSV infected cells at 10 to 100-fold higher levels than that at which *c-src* is normally expressed (Cooper, 1990). *v-src* is an oncogene, a dominant gene whose product contributes to the loss of regulation of cellular growth, and the formation of tumors. The normal version of this gene *c-src* is referred to as a proto-oncogene.

Many acutely transforming retroviruses exist, as do many retroviral oncogenes (reviewed by Cooper, 1990). These oncogenes arise by recombination events between the proviral forms of non-transforming viruses and proto-oncogene sequences within the

host cell genome (Takeya and Hanafusa, 1983; Cooper, 1990). In general, the products of proto-oncogenes, such as c-Src, are involved in growth factor mediated signaling. Sequences at the 3' or 5' ends of proto-oncogenes are frequently replaced with viral sequences during recombination events, leading to the loss of important regulatory elements. As well point mutations frequently occur in viral oncogenes that may also contribute to increased loss of regulation of their products. Therefore, upon infection with viruses carrying oncogenes, host cells express high levels of molecules involved in transmitting growth factor mediated signals, which cannot be properly regulated. This leads to continuous transmission of the signal and inappropriate continuous cellular division (Cooper, 1990).

1.2.4 Oncogenes and Proto-Oncogenes in Spontaneously Occurring Tumors.

The discovery of viral oncogenes led researchers to ask whether similar mutant genes could be found in the cells of spontaneously arising tumors. To address this question, cultured cells were transfected with DNA derived from various tumor cells. These DNA preparations were able to induce transformation of the recipient cells, indicating that these preparations contained oncogenes (Krontiris and Cooper, 1981). Analysis of cells transformed by DNAs derived from human bladder or lung carcinoma cell lines indicated that the sequences responsible for transformation contained the *H-ras* and *K-ras* genes respectively (Der et al., 1982). *H-ras* and *K-ras* were originally identified as the transforming oncogenes of the Harvey and Kirsten sarcoma viruses. When compared to the *ras* genes of normal cells, it was found that both the tumor cell-derived and viral transforming *ras* genes contain point mutations that result in

constitutive activation of the Ras proteins (Tabin et al., 1982). Therefore, mutations arising in spontaneous human cancers give rise to oncogenes similar to those found in transforming viruses.

A number of oncogenes have been identified in tumor cells. Some of these have also been identified in transforming viruses, while others do not have viral counterparts. Like viral oncogenes, the oncogenes of spontaneously arising cancers encode abnormal versions of proteins involved in transducing signals from growth factors, which cannot be properly regulated. In some cases, increased levels of expression of non-mutated proto-oncogenes can contribute to tumorigenesis. For example, the *c-myc* proto-oncogene is amplified in a number of human cancers, leading to elevated expression of this gene (Collins and Groudine, 1982; Cooper, 1990). c-Myc is a transcription factor, and a target of a number of growth factor-mediated signaling pathways (Bouchard et al., 1998). The products of proto-oncogenes can be involved in any step of a growth factor-mediated signaling cascade, from the growth factor itself, to the transcription factors ultimately responsible for altering gene expression in response to these factors (reviewed by Cooper, 1990).

1.3 Tumor Suppressor Genes.

While it is clear that activating mutations in proto-oncogenes can contribute to the formation of cancers, the behavior of many familial cancers cannot be explained simply by the occurrence of mutations of these types. Oncogenes behave in a dominant manner. The introduction of an oncogene into a cell by a virus can lead to tumorigenesis in spite of the presence of two normal alleles of this gene. However, certain familial cancers can

be best explained as arising from recessive, inactivating mutations in growth regulatory genes, with affected individuals inheriting one normal and one mutant copy of a gene of this type. Tumors then arise from cells in which the second copy of this gene is similarly mutated (Knudson, 1971). Growth regulatory genes whose inactivation contributes to carcinogenesis are called tumor suppressor genes.

1.3.1 Retinoblastoma and the *rb* Tumor Suppressor Gene.

Retinoblastoma is a childhood cancer of the eye in which tumors arise from retinoblasts during the first five years of age. This disease occurs in both familial and sporadic forms. In familial cases, multiple tumors may occur, often in both eyes. In general, only one tumor occurs in sporadic cases, with an age of onset later than that observed in familial cases. A statistical analysis comparing the frequencies and ages of onset of familial and sporadic retinoblastoma suggests that this disease is caused by two mutational events, affecting both alleles of the same growth regulating gene (Knudson, 1971). In familial retinoblastoma, one mutant allele is inherited, with a somatic mutation occurring in the other allele, while in sporadic cases somatic mutations occur in both alleles.

The retinoblastoma susceptibility (*rb*) gene is on human chromosome 13. Consistent with this disease arising from the loss of functional copies of the *rb* gene, deletions of the region of chromosome 13 that contains this gene are frequently observed in both familial and sporadic cases of retinoblastoma (Sparkes et al., 1983). This is consistent with this disease arising from the loss of functional copies of this gene. The *rb*

gene has been identified and cloned (Lee et al., 1987). It is expressed in all normal cell types and encodes a protein, pRb, that is a member of a family of structurally related proteins, also including p107 (Ewen et al., 1991) and p130 (Li et al., 1993; Hannon et al., 1993). These proteins are involved in the control of cellular growth in many cell types (reviewed by Grana et al., 1998).

The activity of pRb is determined by its phosphorylation state, which changes as cells pass through the cell cycle. When cells are in G0 or G1, pRb is in a hypophosphorylated state. At the G1/S transition, pRb becomes hyperphosphorylated and remains in this state until M phase, when it reverts to the hypophosphorylated state (DeCaprio et al., 1989). Cyclin proteins and cyclin dependant kinases (Cdks) control the phosphorylation state of pRb. The cyclins are a family of proteins whose level of expression fluctuates in a cell cycle dependant manner. Cyclins associate with and activate specific Cdks (Sidle et al., 1996). The D-type cyclins (cyclins D1, D2 and D3) are expressed early G1 phase of the cell cycle, and interact with and activate Cdk-4 and Cdk-6 (Bates et al., 1994). These Cdks are then able to phosphorylate pRB, converting it to the hyperphosphorylated state (Ewen et al., 1993; Dowdy et al., 1993). The hyperphosphorylated state is maintained from late G1 to M phase by cyclin/Cdk complexes present in these phases (Sidle et al., 1996).

In the hypophosphorylated state pRb forms a complex with members of the E2F family of proteins. There are five members of this family, which dimerize with the

proteins DP-1 or DP-2 to form active transcription factors (Wu et al., 1995). Hypophosphorylated pRb binds to dimers of this type containing E2F-1, -2 and -3, and inhibits the ability of these dimers to activate transcription (Chelliban et al., 1991: Hiebert et al., 1992; Wu et al., 1995). Upon becoming hyperphosphorylated, pRb is unable to bind E2F-contaning dimers, and E2F proteins are then able to activate transcription of genes whose products are required for DNA replication and cell division (Nevins, 1992). Growth factors cause pRb hyperphosphorylation and E2F activation by stimulating the expression of D-type cyclins (Grana and Reddy, 1995). Deregulated expression of the D-type cyclins can accelerate the rate of cell division and contribute to tumorigenesis (Zuelle et al., 1993; Grana and Reddy, 1995).

1.3.2 The p53 Tumor Suppressor Gene.

Li-Fraumeni syndrome (LFS) is another familial cancer syndrome, characterized by the occurrence of a wide variety of tumor types, including breast carcinomas, soft tissue sarcomas, brain tumors, osteosarcoma, leukemia and adrenocortical carcinoma. Like retinoblastoma, the occurrence of these tumors is best explained by the inheritance of one mutant allele of a tumor suppressor gene, and subsequent somatic mutations of the other allele of this gene. In LFS these mutations occur in the gene encoding the tumor suppressor p53 (Malkin et al., 1990). p53 is the most frequently mutated gene in human cancer, with mutations found in over 50% of human tumors, in virtually all tumor types

(Amundson et al., 1998). These mutations are clustered in highly conserved regions of the *p53* gene that are important for the proper function of p53 (Holstein et al., 1991; Levine et al., 1991).

The p53 protein is important in the cellular response to DNA damage, acting to protect cells from mutations. p53 is a transcription factor that binds to specific DNA sequences as a tetramer, and activates the transcription of genes downstream from these sequences (Raycroft et al., 1990). Normal cells express low levels of p53, but levels of this protein increase in response to DNA damage due to stabilization of the p53 protein (Kastan et al. 1991). The transcription of three classes of genes is activated by p53. These encode cell cycle inhibitors, DNA repair proteins and proteins involved in induction of apoptosis (reviewed by Amundson et al., 1998). An example of a p53 target involved in cell cycle regulation in the Cdk inhibitor p21 (WAF1/Cip-1), which inhibits Cdk-2 and Cdk-4 and causes the cell cycle to arrest in G1 (Harper et al., 1993; El-Deiry et al., 1993). During this cell cycle arrest, p53 may induce the expression of DNA repair genes, leading to repair of damaged DNA. Alternatively, in certain cell types, p53 leads to the induction of apoptotic cell death and the elimination of cells with damaged DNA (Amundson et al., 1998).

A number of other tumor suppressor genes exist, whose mutation can contribute to the formation on cancers. Cancer is a progressive disease with tumors gradually

becoming more aggressive (Section 1.1.1). This occurs as tumor cells accumulate multiple mutations, activating oncogenes and inactivating tumor suppressor genes.

1.4 Invasion and Metastasis of Tumor Cells.

As cancers become more aggressive, the probability that they will metastasize increases. That is, tumor cells spread from the primary tumor and establish secondary tumors at various sites throughout the body. This process is largely responsible for the lethality of cancers, because once it occurs the disease becomes virtually incurable. Metastasis occurs in steps, with tumor cells initially escaping from the primary tumor and invading surrounding tissue. These cells then enter the vasculature, where they are carried to distant sites. At these sites, they escape the vasculature and invade the surrounding tissue, forming secondary tumors (Meyer and Hart, 1998).

Similar to loss of control of proliferation in cancer cells, metastasis is ultimately caused by changes in gene expression. The levels of expression of cell adhesion molecules, proteolytic enzymes, angiogenic factors and cytoskeletal elements are altered as tumor cells progress to a more invasive state. Moreover, tumor cells may secrete proteins which induce changes in the expression of these molecules in neighboring non-tumor cells (Woodhouse et al., 1997; Meyer and Hart, 1998). Some of the proteins involved in the metastatic process will be introduced below.

1.4.1 Proteolytic Enzymes.

A number of proteotytic enzymes contribute to tumor metastasis. These enzymes break down components of the extracellular matrix (ECM). Proteolysis of the ECM surrounding tumor cells must occur if these cells are to invade this matrix as they pass into or out of the vasculature. This process is also required for the vascularization of tumors, permitting the invasion of endothelial cells into the tumor mass. Proteases may also contribute to tumor growth by causing the release of growth factors sequestered within the ECM (Woodhouse et al, 1997; Meyer and Hart, 1998). Certain serine proteases and the matrix metalloprotienases (MMPs) are particularly important in these processes.

1.4.1.1 Serine Proteases.

The serine proteases are defined by the presence of a conserved serine residue at their active site. A member of this family of enzymes that is particularly important in tumor cell metastasis is urokinase-type plasminogen activator (uPA) (Blasi, 1993; Meyer and Hart, 1998). uPA is produced and secreted by cells as an inactive pro-enzyme, or zymogen, pro-uPA, that can bind to a receptor (uPAR) present on the extracellular face of the plasma membrane. Upon binding, pro-uPA can be cleaved by another serine protease, plasmin, generating active uPA. uPA can in turn cleave the zymogen plasminogen to generate the active protease plasmin which is able to activate a number of

zymogens, including many pro-MMPs (Blasi, 1993). Four inhibitors, the plasminogen activator inhibitors (PAI) -1, -2, and -3, and protein nexin-1 regulate the activity of uPA by binding the uPA/uPAR complex, causing its internalization by the cell (Blasi, 1993; Meyer and Hart, 1998). uPA then undergoes partially proteolysis and is released by uPAR, which then returns to the cell surface (Blasi, 1993).

This system of uPA activation by membrane associated uPAR allows for concentration of proteolytic activity at various points on the cell surface, enabling directional proteolysis for cell migration and invasion (Pollanan et al., 1988, Blasi, 1993). In invasive tumors, uPA and uPAR are found on the surface of cells at the invasive edge of the tumor. Interestingly, stromal cells surrounding the tumor are often the source of this uPA (Pyke et al, 1991). This suggests that tumor cells can interact with nearby non-tumor cells, causing them to express and secrete proteases and contribute to the invasiveness of the tumor (Meyer and Hart, 1998).

High levels of uPA or uPAR are poor prognostic indicators in a number of cancer types, such as breast, colorectal and gastric cancers (Duggan et al., 1995; Meyer and Hart, 1998). Also, transfection of tumor cell-derived cell lines with uPA or uPAR can increase the ability of these cells to invade reconstituted ECM and form metastatic tumors when injected into experimental animals (Achbarou, 1994; Xing and Rabbani, 1996).

1.4.1.2 The Matrix Metalloproteinases and Their Inhibitors.

The MMPs are a family of zinc-dependant proteases, capable of cleaving many ECM components (Coussens and Werb, 1996; Woodhouse et al., 1997; Meyer and Hart, 1998; Westermark and Kahari, 1999). Like serine proteases, cells secrete MMPs as inactive zymogens, which are cleaved by serine proteases such as plasmin, or other MMP molecules to give rise to activated MMPs. This cleavage results in the removal of an inhibitory propeptide domain from the amino-terminus of the pro-MMP. The propeptide domain contains a cysteine residue that interacts with a zinc ion at the active site of the MMP molecule, interfering with its activity (Coussens and Werb, 1996). The tissue inhibitors of metalloproteinases (TIMPs) inhibit the MMPs. There are four members of this family, TIMP-1, TIMP-2, TIMP-3, and TIMP-4. These molecules bind to the active sites of MMP molecules and prevent their activity (Coussens and Werb, 1996; Westermark and Kahari, 1999). Proteolysis and inactivation of the TIMPs can be carried out by a number of proteases (Itah and Nagase, 1995; Coussens and Werb, 1996).

The simplest MMP is MMP-7, which consists of only a catalytic domain and the inhibitory prodomain. The other MMPs contain domains involved in the recognition of substrate molecules (Coussens and Werb, 1996). The MMPs can be grouped into four general classes. The collagenases (MMP-1, MMP-8 and MMP-13) cleave triple helical regions of fibrillar collagen molecules. The stromelysins (MMP-3, MMP-7, MMP-10 and MMP-12) cleave fibronectin and proteoglycans. The gelatinases (MMP-2 and MMP-

9) cleave degraded collagen, fibronectin, elastin and certain types of native collagen. Finally, the membrane-type MMPs (MMP-14, MMP-15, MMP-16 and MMP-17) are defined by the presence of a transmembrane domain, through which they are bound to the plasma membrane (Woodhouse et al., 1997; Westermark and Kahari, 1999).

The membrane-type MMPs provide another means by which proteolytic activity can be concentrated at the surface of cells. In addition to their ability to proteolyze ECM components, these proteases can also activate other MMPs. For example, MMP-14 can form a ternary complex with TIMP-2 and pro-MMP-2. Formation of this complex leads to cleavage of pro-MMP-2 by MMP-14, and the generation of active MMP-2, which can remain bound in the ternary complex, localizing proteolytic activity to the cell surface (Will et al., 1996). Again, this concentration of proteolytic activity can contribute to the ability of cells to invade the ECM surrounding them.

The expression of MMPs in tumors and tumor-derived cell lines is associated with increased metastatic and invasive abilities, which can be inhibited by the presence of TIMPs (Coussens and Werb, 1996; Meyer and Hart, 1998). In tumors, both tumor cells and stromal cells surrounding them produce MMPs. For example, in mammary carcinomas, only MMP-7 and MMP-13 are expressed by tumor cells. However, MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-12, MMP-13 and MMP-14 are expressed by various tumor associated cells including stromal fibroblasts, macrophages and endothelial cells (Heppner et al., 1996). Tumor cells induce this expression of MMPs by either

direct cell-cell contact or by secretion of an MMP-inducing factor (Coussens and Werb, 1996). For example the tumor cell-derived collagenase-stimulatory factor (TCSF) is present on the surface of many tumor cell types. This protein can stimulate expression of MMP-1, MMP-2 and MMP-3 in normal fibroblasts (Kataoka et al., 1993).

1.4.2 Cell Adhesion Molecules.

In order for a cell to metastasize, it must first detach from neighboring cells in the primary tumor. This requires the loss of interaction of this cell and neighboring cells. It must then migrate through the ECM, pulling itself along ECM molecules. After entering the vasculature, it must adhere to the endothelium at a distant site. Here, it infiltrates the surrounding tissue, invading and pulling itself through the ECM. These processes require the interaction of the cell with ECM components and endothelial cells, which occur through cell adhesion molecules (CAMs) present on the extracellular surface of cells. Changes in the expression levels of these molecules can alter the ability of tumor cells to undergo metastasis (Albeda, 1993; Meyer and Hart, 1998). There are three major classes of CAMs, the cadherins, the integrins and members of the immunoglobulin superfamily of proteins.

1.4.2.1 The Cadherins.

The cadherins are transmembrane glycoproteins that mediate homotypic cell-cell interactions. Cadherin molecules bind molecules of the same type present on the surface of neighboring cells in a calcium dependant manner (Takeichi, 1991; Albeda, 1993; Meyer and Hart, 1998). This family includes the E-cadherins, found in epithelial cells, the N-cadherins, found in neural and muscle cells and the P-cadherins, found in placental and epithelial cells (Takeichi, 1991; Albeda, 1993). The cytoplasmic domain of cadherin molecules forms a complex with three proteins, α-, β- and γ-catenin, through which they are linked to cytoskeletal elements (Ozawa et al., 1989).

The loss of cadherin expression correlates with invasiveness in many tumor types, including breast, prostate, stomach, bladder, colorectal and pancreatic tumors (reviewed by Meyer and Hart, 1998). This is likely the result of a weakening of the affinity of the cells of these tumors for each other. Similarly, the treatment of cultured epithelial cells with antibodies directed against E-cadherin confers invasive properties on these cells (Behrens et al., 1989). Conversely, transfection of invasive tumor cell lines with E-cadherin can reduce invasive activity (Vleminckx et al., 1991).

As well as mediating cell-cell interactions, cadherins also have a role in regulating cellular proliferation. The cadherin associated protein β-catenin can interact with and activate transcription factors of the T cell factor-lymphoid enhancer factor (Tcf-Lef) family (Behrens et al., 1993). Cadherins provide one mechanism of regulating β-catenin. Binding of β-catenin by cadherins prevents its interaction with Tcf-Lef (Orsulic et al.,

1999). Therefore, loss of cadherin expression increases the activity of Tcf-Lef proteins, which are able to activate transcription of genes whose products stimulate cellular proliferation or inhibit apoptosis. Activating mutations in β-catenin have been shown to contribute to tumorigenesis (Morin et al., 1997).

1.4.2.2 The Integrins.

The integrins are a family of CAMs that mediate adhesion of cells to various ECM components or other CAMs. These proteins are heterodimers of an α- and β-subunit. At least 14 α-subunits and 8 β-subunits exist, giving rise to over 20 heterodimers, each of which interacts with specific ligands (Hynes, 1992; Albeda, 1993; Meyer and Hart, 1998). Integrins also act in the transduction of extracellular signals. In this manner they can influence such functions as cell migration, differentiaton and apoptosis (Hynes, 1992; Meyer and Hart, 1998).

Expression of various integrins can either inhibit or promote metastasis. For example, breast cancer cells that do not express $\alpha_2\beta_1$ integrin become much less invasive upon transfection with this integrin (Zutter et al., 1995). Decreased expression of a number of integrins has been observed in many metastatic tumor types (reviewed by Albeda, 1993; Meyer and Hart, 1998). Conversely, the metastatic ability of many tumors correlates with expression of certain integrins. For example, the levels of integrins, such as the $\alpha_v\beta_3$ integrin, increase as melanoma becomes more invasive (Albeda et al., 1990). The ability of cells to interact with ECM components surrounding them can cause them to be held in place and interfere with their ability to migrate. However, these interactions are also essential for the ability of cells to migrate, as they are required for cells to pull

themselves through the ECM. The effects of integrins on the ability of a cell to invade the ECM depend on cell type, the composition of the ECM and the level of expression of the various integrins (Palecek et al., 1997; Meyer and Hart, 1998).

1.4.2.3 Cell Adhesion Molecules of the Immunoglobulin Superfamily.

Members of the immunoglobulin superfamily of proteins are defined by the presence of immunoglobulin-like domains. Many CAMs that contain domains of this type are involved in both homophilic and heterophilic interactions with a wide variety of cell surface proteins. This family of CAMs includes N-CAM, ICAM-1, ICAM-2 and V-CAM (Albeda, 1993; Meyer and Hart, 1998). Elevated levels of a number of these proteins are associated with increased metastatic behavior in tumor cells. For example, metastatic ability in melanoma correlates with ICAM-1 expression (Natali et al., 1990).

Immunoglobulin superfamily members are thought to be involved in the interaction of tumor cells that have entered the vasculature with endothelial cells prior to extravasion. Endothelial cells express many members of this family, which may interact with CAMs on the surface of tumor cells allowing them to adhere to the endothelium prior to extravasion (Meyer and Hart, 1998). Also, many immunoglobulin superfamily members can bind to cell surface molecules present on the surface of circulating leukocytes, allowing interaction of leukocytes and tumor cells in the vasculature (Springer, 1990). Interaction of immunoglobulin superfamily members on the surface of endothelial cells with leukocytes also occurs prior to these cells leaving the vasculature. Leukocytes may carry tumor cells along as they exit the bloodstream and infiltrate tissues (Albeda, 1993; Meyer and Hart, 1998).

1.5 Transcription and Transcription Factors.

As has been discussed, proto-oncogenes and tumor suppressor genes ultimately act to regulate cellular growth by altering the transcription of various genes. This occurs through the regulation of the activity of various transcription factors. For example, cells respond to growth factors by rapidly increasing the levels of transcription of a group of genes, the immediate early genes. This occurs by the activation of pre-existing transcription factors. Many of the products of the immediate early genes are also transcription factors that go on to activate transcription of their various target genes (Cooper, 1990).

1.5.1 Control of Transcription by Promoter and Enhancer Elements.

RNA polymerase II carries out the transcription of all messenger RNAs (mRNAs) in eukaryotic cells. This enzyme is unable recognize appropriate start sites for transcription by itself, and therefore requires certain DNA binding proteins to direct it to these sites. Some of these transcription factors, the general transcription factors, are utilized in the transcription of all genes, while others are only involved in the transcription of specific target genes (Dynan and Tijan, 1985). Many transcription factors bind to DNA sites in the promoter region of a gene sequences a short distance upstream from the start site of transcription. These promoter regions contain elements required for basal levels of transcription that are recognized by the general transcriptional

machinery, and modulator elements that are recognized by specific factors that can enhance or inhibit transcription (Dynan and Tijan, 1985; Maniatis et al., 1987).

There are two types of basal promoter elements, TATA-containing and TATA-less promoters. The first contains the consensus sequence TATAAA, 25 to 30 base pairs upstream from the start site of transcription (Dynan and Tijan, 1985; Hernandez, 1993). The TATA box is bound by the general transcription factor TFIID, a complex of the TATA-binding protein (TBP), which recognizes the TATA box, and a number of TBP associated factors (TAFs). After binding to the TATA box, TFIID acts as a site of nucleation for the formation of a pre-initiation complex containing RNA polymerase II and other basal transcription factors (including TFIIA, TFIIB, TFIIE, TFIIF and TFIIH) (Hernandez, 1993). The exact combination of these factors required for basal levels of transcription varies from one promoter to the next (Parvin et al., 1992).

TATA-less promoters contain initiator (Inr) elements, DNA sequences that overlap the start site of transcription (Smale and Baltimore, 1989). Initiator elements can independently direct the formation of pre-initiation complexes containing RNA polymerase II and various transcription factors, allowing basal levels of transcription to occur from these sites. They are also found on some TATA-containing promoters and contribute to initiation of transcription from these promoters as well. The consensus sequence of the Inr is relatively weak. A number of families of initiators exist, which are recognized by different proteins that can act as nucleation sites for the formation of the

pre-initiation complex. However, in all cases TBP must be present for formation of these complexes (Hernandez, 1993). In some promoters, TFIID is able to directly interact with the Inr (Wang and van Dyke, 1993).

In addition to the basal transcription factors, a number of proteins exist that are able to modulate the levels of transcription of various genes, either activating or repressing this transcription. These proteins bind to specific DNA sequences found either in the promoter region of their target genes or in enhancer elements (Dynan and Tijan, 1985; Maniatis et al., 1987). Enhancers are DNA elements that contribute to the activation of transcription of genes that may be a long distance upstream or downstream. Transcriptional activation by an enhancer is independent of the orientation of the target gene relative to the enhancer. Frequently, enhancers are involved in conferring tissue specific expression on the genes they regulate (Serfling et al., 1985).

1.5.2 Structure and Mechanism of Action of Activators of Transcription.

Activators of transcription contain two independent functional domains, a DNA-binding domain and a transcriptional activation domain. The DNA-binding domain contains secondary structure elements through which it is able to make contact with specific nucleotide sequences that it is able to bind (Johnson and McKnight, 1989). Helix-turn-helix motifs and zinc fingers are common examples of these structures. Dimerization of many transcription factors is required for DNA binding. Structural

elements such as leucine zippers, through which proteins are able to dimerize, are frequently found in the DNA binding domains of transcriptional activators (Landschultz et al., 1988; Johnson and McKnight, 1989).

Transcriptional activation domains act to increase levels of transcription by stabilizing or aiding the formation of the pre-initiation complex. They do this by interacting with the TAF components of TFIID, either directly or through bridging proteins. A number of different classes of activation domains exist, usually defined by the frequent occurrence of specific amino acid residues. Glutamine rich, proline rich and isoleucine rich and acidic activation domains exist (Tijan, 1996).

1.5.3 Regulation of Transcription Factors.

The activity of transcriptional activators may be regulated in response to many stimuli, including growth factors and DNA damage (Sections 1.2 and 1.3). Transcription factors can be regulated in two ways: by altering the concentration of a transcription factor in the nucleus, or the activity of the transcription factor (Calkhoven and Ab, 1996).

A simple way that the concentration of a transcription factor can be regulated is through transcription of its mRNA, which may occur through the regulation of other transcription factors. Often, many transcription factors contribute to regulation of the transcription of their own genes. Degradation of the mRNA for a transcription factor may also play a role regulating its concentration. The mRNAs encoding many

transcription factors whose expression is increased in response to extracellular stimuli contain sequences that stimulate their degradation, causing the levels of these mRNAs, and the proteins they encode, to decrease rapidly upon the removal of these stimuli (Calkhoven and Ab, 1996).

The concentration of a transcription factor can also be controlled through regulation of the translation of its mRNA. For example, insulin or growth factor stimulation lead to an increase in the translation of many mRNAs, including a number encoding transcription factors (Lin et al., 1994; Pause et al., 1994). This occurs through regulation of the eukaryotic translation initiation factor-4E (eIF-4E), a component of a complex called eIF-4F, which complex recognizes and binds the 5-methylguanosine cap at the 5'-end of mRNAs. The 4E-binding proteins 4E-BP1 (PHAS-1) and 4E-BP2 can bind and sequester eIF-4E in an inactive complex. Growth factors or insulin stimulation causes the phosphorylation of 4E-BP1 and 4E-BP2 by ERK-1 and ERK-2, preventing their inhibition of eIF-4E and stimulating cap-dependant translation. Higher eIF-4E activity primarily facilitates the translation of mRNAs containing complex secondary structures in their 5'-untranslated region, which frequently occur in mRNAs encoding transcription factors (Proud, 1994; Calkhoven and Ab, 1996).

Both the activity and concentration of a transcription factor can be altered by changes in the splicing of its mRNA, a process that may be regulated. Differential splicing leads to the production of multiple mRNA isoforms whose stability and

efficiency of translation may differ. Moreover, they may encode transcription factors with different activities or intracellular localization. In fact, in some cases differential splicing of the same primary transcript can give rise to both transcriptional activators and repressors with the same DNA binding specificity (Foulkes and Sassone-Corsi, 1992; Calkhoven and Ab, 1996). In a similar manner, the selection of alternative start sites for translation of mRNAs encoding transcription factors can yield proteins with differing activities. This occurs through regulation of the translation initiation factor eIF-2, which recognizes initiation sites for translation (Calkhoven and Ab, 1996).

The phosphorylation of transcription factors by various protein kinases can also alter the nuclear concentration or activity of transcription factors. Transcription factors may be sequestered in inactive complexes with anchor proteins in the cytoplasm. Phosphorylation or dephosphorylation of the transcription factor may disrupt this association, allowing it to translocate to the nucleus. Moreover, phosphorylation may modulate the ability of a transcription factor to bind DNA or to activate transcription (Hill and Treisman, 1995; Calkhoven and Ab, 1996).

Finally, the activity of transcription factors may be regulated by interactions with other proteins (Calkhoven and Ab, 1996). Transcription factors frequently bind DNA as dimers or multimers with DNA binding proteins of similar or different types, which provides a variety of possible complexes that can be formed. Also, the interaction of transcription factors with non-DNA-binding proteins may be important for their activity.

For example, transcription factors that interact with the basal transcriptional machinery through bridging proteins require the presence of these proteins for activity (Tijan, 1996). Thus, the activity of transcription factors may vary depending on the nature and levels of its protein partners present at a given time.

1.5.4 The Regulation of the AP-1 Family of Transcription Factors.

The AP-1 proteins serve as a good example to demonstrate the regulation of transcription factors by a variety of different methods. These transcription factors are dimers of Jun and Fos proteins, which bind to common DNA motifs, called AP-1 sites or TPA-responsive elements (TREs) (Angel and Karin, 1991). The ability of AP-1 proteins to stimulate transcription from these sites is increased in response to a number of stimuli, including growth factors, cytokines and UV irradiation (Angel and Karin, 1991; Karin, 1996).

AP-1 activity is regulated by protein-protein interactions. Jun and Fos proteins contain leucine zippers within their DNA-binding domains, through which they are able to dimerize, a process required for binding of DNA by these proteins (Smeal et al., 1989; Angel and Karin, 1991). Members of the Jun family (c-Jun, JunB and JunD) can homodimerize or form heterodimers, containing two different family members. These complexes are somewhat unstable, and bind to DNA and activate transcription relatively weakly (Smeal et al., 1989; Angel and Karin, 1991). Members of the Fos family (c-Fos,

FosB, Fra-1, Fra-2) are unable to dimerize with each other, instead, forming heterodimers with Jun proteins. These dimers are more stable and able to bind DNA more strongly than Jun-Jun homodimers (Smeal et al., 1989; Angel and Karin, 1991). While c-Fos and FosB contain potent transactivation domains, Fra-1 and Fra-2 do not. Therefore, dimers of c-Fos and FosB with Jun proteins are stronger transcriptional activators than Jun-Jun homodimers, while dimers of Fra-1 or Fra-2 with Jun are in most cases weaker (Suzuki et al., 1991). The activity of AP-1 proteins at any given time depends on the concentrations of the various monomers in the nucleus. c-Jun can also dimerize with another transcription factor, activating transcription factor-2 (ATF-2) (van Dam et al., 1993). These dimers bind DNA sequences that differ from those bound by Jun-Fos dimers by one nucleotide. Such a sequence is found in the *c-jun* promoter, allowing c-Jun to contribute to regulation of its own transcription.

AP-1 activity is also regulated by phosphorylation. In response to various cellular stresses, members of the JNK family of protein kinases are activated (Section 1.2.2.3). These kinases phosphorylate sites in the amino-terminal transcriptional activation domain of c-Jun, increasing its activity (Derijard et al., 1994). This phosphorylation allows c-Jun to interact with the CREB binding protein (CBP), an adapter protein through which c-Jun can interact with the basal transcription machinery and activate transcription (Karin, 1996). The JNKs can also phosphorylate ATF-2 in a similar manner, increasing its ability to activate transcription (Gupta et al., 1995). Therefore, activation of the JNKs

leads to an increase in both c-Jun levels and activity. In contrast, phosphorylation of c-Jun at a cluster of sites in its DNA binding domain can inhibit c-Jun activity by interfering with its ability to bind DNA (Boyle et al., 1991). Phosphorylation of these sites inhibits the binding of c-Jun homodimers but not c-Jun-c-Fos heterodimers (Karin, 1996). These sites can be phosphorylated by the kinases ERK-1 and ERK-2, which are activated in response to growth factor stimulation (section 1.2.2.3) (Minden et al., 1994). Like c-Jun, the ability of c-Fos to activate transcription is increased by phosphorylation by a MAP kinase. However, neither the ERKs nor JNKs phosphorylate c-Fos. A third MAP kinase, the Fos-regulating kinase (FRK) activates c-Fos by phosphorylating its transcriptional activation domain in response to growth factors and Ras activation (Deng and Karin, 1994).

The *c-fos* promoter contains a number of elements allowing induction of *c-fos* transcription in response to a variety of stimuli (Karin, 1996). One of these is the serum response element (SRE), which mediates *c-fos* induction in response to serum, growth factors, or the products of many oncogenes. At the SRE a ternary complex formed, including the ubiquitous serum response factor (SRF) and a ternary complex factor (TCF), which cannot bind the *c-fos* SRE by itself (Treisman, 1994). The TCFs, which include the proteins Elk-1, SAP-1a and SAP-2, are members of the Ets family of transcription factors (section 1.5). Growth factors and the products of oncogenes stimulate *c-fos* transcription through activation of ERK-1 and ERK-2, which

phosphorylate the TCFs and increase their ability to activate transcription (Treisman, 1994; Price et al., 1995; Wasylyk et al, 1998).

In summary, growth factors or cellular stress stimulate AP-1 activity by increasing levels of *c-fos* and *c-jun* transcription and increasing the activities of the c-Fos and c-Jun proteins. Both *c-fos* and *c-jun* mRNA contain elements in their 3'-untranslated regions that make these mRNAs unstable, causing their levels to rapidly decrease in the absence of these stimuli (Zubiaga et al., 1995; Calkhoven and Ab, 1996).

1.5.5 Oncogenic Transcription Factors.

As has been discussed, transcription factors are the final targets of the signals generated by growth factors or the products of oncogenes. The importance of transcription factors in cell growth and tumorigenesis is demonstrated by the existence of oncogenes encoding transcription factors. Once again, the components of AP-1 serve as a good example. Avian sarcoma virus 17 (ASV17) and FBJ murine sarcoma virus (FBJ-MuSV) are acutely transforming retroviruses that encode oncogenic versions of *c-jun* and *c-fos* respectively (Van Beveren et al., 1983; Maki et al., 1987). The *v-jun* and *v-fos* oncogenes are capable of transforming cells in culture and inducing tumors in animal models (Bos et al., 1990; Angel and Karin, 1991).

There are a number of differences between the *c-jun* and *v-jun* sequences. The 3'-untranslated region of the *c-jun* mRNA is not present in *v-jun*. Also, the protein encoded

by *v-jun* differs from c-Jun, containing a substitution of two amino acid residues in the DNA binding domain and a deletion of 27 amino acids near its amino terminus (Nishimura and Vogt, 1988). This protein is able to form homodimers, and heterodimers with Fos proteins, and bind to and activate transcription from TRE sequences in the same manner as c-Jun (Angel and Karin, 1991). One of the amino acid substitutions in the DNA binding domain of v-Jun results in the loss of a site of phosphorylation through which c-Jun activity can be negatively regulated by ERKs and other kinases (section 1.4.4.) (Boyle et al., 1991; Minden, 1994). Mutation of this site in c-Jun interferes with the ability of these kinases to phosphorylate its DNA binding domain and inhibit c-Jun activity. However, simply introducing this mutation into c-Jun is insufficient to confer upon it transforming activity as potent as that of v-Jun, although its transforming ability is increased relative to that of wild-type c-Jun (Angel and Karin, 1991). The 27 amino acid deletion in the amino terminus of c-Jun also contributes to the transforming ability of v-Jun. However, the loss of the 3'-untranslated region of the *c-jun* mRNA it the most important factor in *v-jun* induced transformation as this region contains sequences that lead to the rapid turnover of *c-jun* mRNA (section 1.4.4.) (Bos et al., 1990). The *v-jun* mRNA is more stable than that of *c-jun* and the most important change in oncogenic activation of *c-jun* is simply overexpression of the gene (Angel and Karin, 1991).

Comparison of the *v-fos* and *c-fos* sequences indicates that *v-fos* has undergone a 104 base pair, out-of-frame deletion, resulting in the substitution of the carboxy-terminal

48 amino acid residues of c-Fos with 49 unrelated amino acid residues (Van Beveren et al., 1983). However, this is not responsible for the transforming activity of *v-fos*. Like *v-jun* loss of sequences encoding the 3'-untranslated region in the *v-fos* mRNA increase the stability of this mRNA and are responsible for the transforming activity of *v-fos* by leading to elevated levels of its expression (Miller et al., 1984; Angel and Karin, 1991).

1.6 The Ets Family of Transcription Factors.

The Ets proteins are a family of transcription factors, defined by the presence of a conserved DNA-binding domain, the ETS domain. This domain is approximately 85 amino acids long, and contains three highly conserved tryptophan residues spaced 17 to 18 amino acids apart (Karim et al., 1990). Ets proteins are usually activators of transcription, but some, such as Ets-2 repressor factor (ERF), act to repress transcription (Sgouras et al., 1995). Ets proteins have been found in every metazoan species studied, including human, mouse, chicken, *Xenopus*, sea urchin and *Drosophila* (Lautenberger et al., 1992). Outside of the ETS domain the amino acid sequences of the various Ets proteins are widely divergent. These proteins have been grouped into sub-families based on sequence similarity within the ETS domain, the position of the ETS domain within the protein, and the presence of other conserved domains outside of the ETS domain (Graves and Petersen, 1998; Wasylyk et al., 1998). The various Ets sub-families and their members are shown in Table 1.1. In this table the various Ets sub-families and their

members are shown. In cases where proteins have been given multiple names, each name is shown, separated by a slash. In cases where differential splicing of a single *ets* gene gives rise to multiple, functionally distinct proteins, the names of these isoforms are shown in brackets. The data shown was summarized from Graves and Petersen (1998) and Laudet et. al (1999).

The secondary structure of the ETS domain has been determined. It consists of three α-helices, which interact with DNA, packed against a four-stranded antiparallel β-sheet, a structure known as the winged helix-turn-helix motif (Donaldson et al., 1994). The conserved tryptophan residues form an important part of the hydrophobic core of this motif and are required for its correct packing. All Ets proteins bind to DNA sequences that contain a core sequence of GGA(A/T) (Karim et al., 1990; Graves and Petersen, 1998). Individual Ets proteins recognize DNA sequences 9 to 15 base pairs in length containing this core sequence. However, the consensus DNA binding sites for most Ets proteins show few invariant positions outside the core region. Although there is some selectivity for preferred sequences, most Ets proteins are capable of binding to similar DNA sequences *in vitro* (Graves and Petersen, 1997; Bowman and Hassell, unpublished data).

1.6.1 Regulation and Specificity of Ets Proteins.

Transgenic mice have been generated that overexpress specific *ets* genes in various tissues. The overexpression of different *ets* genes results in different phenotypes

Table 1.1 The Ets Protein Sub-Families and Their Members.

Ets Subfamily	Protein Name(s)
ETS	Ets-1 Ets-2 Pnt (Pnt P1, Pnt P2)
ER71	ER71/ETV2
GABP/ELG	GABPα/E4TF-1/NRF-2α ELG
PEA3	PEA3/E1A-F/ETV4 ER81/ETV1 ERM/ETV5
ERG	ERG Fli-1/ERG-B Ets-6 FEV
ERF	ERF PE-1/ETV3/PEP-1
ELK	Elk-1 SAP-1 (SAP-1a, SAP-1b) Net/SAP-2/ERP Lin-1
ETS-4	Ets-4
ELF	Elf-1 NERF (NERF-1, NERF-2) E74 (E74A, E74B) MEF
ESE	ESE-1/ERT/ESX
YAN	Yan TEL/ETV6
SPI	SPI-1/PU.1 SPI-B/SPI-2

in these mice. Similarly, knock out mice have been generated in which various *ets* genes have been disrupted by homologous recombination (reviewed by Graves and Pedersen, 1997). Again, the loss of expression of different *ets* genes results in different phenotypes. Therefore, in spite of the fact that Ets proteins bind similar DNA sequences, their functions are not redundant, and each has different effects on gene expression. Regions outside the ETS domain are responsible for conferring biological specificity on these proteins (Graves and Petersen, 1997).

Many Ets proteins contain domains that inhibit their own ability to bind DNA or activate transcription. For example, an auto-inhibitory region in Ets-1 contains an α-helix that physically interacts with the ETS domain of this protein and interferes with its binding to DNA (Petersen et al., 1995). The sequences of the auto-inhibitory domains of various Ets proteins are widely divergent. Due to this, the means of relieving auto-inhibition differs between Ets proteins, contributing to their biological specificity (Graves and Petersen, 1998). Ets proteins are regulated by the same mechanisms as other transcription factors (section 1.5.3.), with phosphorylation and interactions with other proteins being particularly important. Many Ets proteins are activated by Ras via MAP kinase signaling pathways (Yang et al., 1996; Wasylyk et al., 1998). For example, the activities of the TCFs are regulated by interaction with SRF and phosphorylation by MAP kinases (Section 1.5.4). The transcription of a number of genes, whose promoters contain Ras-responsive elements (RREs), is stimulated by activated Ras. The SRE is one example of a RRE. Other RREs, the prototype of which is found in the polyomavirus enhancer, contain adjacent binding sites for Ets and AP-1 proteins, which act synergistically to activate transcription from these sites. Often, Ets proteins are incapable

of activating transcription from RREs in the absence of AP-1. RREs are found in the promoters of a number of genes, including those of uPA and members of the MMP and TIMP families (section 1.4.1.) (Logan et al., 1996; Westermark and Kahari, 1999). RREs of this type can respond differently to various Ets proteins. For example, Ets-2 cooperates with AP-1 to activate transcription of the *MMP-1* and *MMP-3* genes, while Erg activates transcription of the *MMP-1* gene, but not the *MMP-3* gene. In fact, the presence of Erg interferes with the activation of *MMP-3* transcription by Ets-2 (Buttice et al., 1996). AP-1 transcription factors can synergize with many Ets proteins. Since many Ets proteins and AP-1 dimers exist, a large number of different combinations of these proteins are possible. In this manner, specificity is conferred upon Ets proteins as these various combinations may have differing effects on different promoters (Graves and Petersen, 1998; Wasylyk et al., 1998).

The interactions of Ets proteins with other transcription factors can confer tissue specific activities upon them. For example, many Ets proteins can interact with the transcription factor Pax-5, which is expressed specifically in B-lymphocytes. Pax-5 has been shown to interact with Fli-1, Ets-1, GABPα, Net and Elk-1, but not SAP-1. Ets proteins synergize with Pax-5 to activate transcription from the *mb-1* promoter, leading to B-cell specific expression of this gene. In the absence of Pax-5 no Ets protein can activate transcription from this promoter (Fitzsimmons et al., 1996).

The interactions of Ets proteins with other transcription factors can be very specific. For example, the promoter for the prolactin gene, which is expressed in the cells of the pituitary gland, contains a RRE composed of an Ets binding site and a binding site for Pit-1, a pituitary specific transcription factor. In this case, Ets-1 is the only Ets

protein known to activate transcription from the prolactin RRE. Neither Ets-1 nor Pit-1 can activate transcription of the prolactin gene in the others absence (Bradford et al., 1996).

A number of interactions of Ets proteins with other transcription factors are known. Different combinations of these proteins can regulate the transcription of various genes. The ability of an Ets protein to activate transcription from a given promoter depends on a number of factors. These include the combinations of Ets proteins and other transcription factors required at this promoter, the presence of these partners, the presence of other Ets proteins and the phosphorylation state of the Ets protein (Graves and Petersen, 1998). Due to these factors, the activity of Ets proteins can be tightly regulated and highly specific.

1.6.2 Ets Proteins and Tumorigeneisis.

Signaling pathways that are activated by growth factors and the products of oncogenes regulate many Ets proteins. For example, phosphorylation by various MAP kinases in response to Ras activation leads to the activation of many Ets proteins. Therefore, it is not surprising that the loss of regulation of certain of these proteins can contribute to tumorigenesis. In fact, the first Ets protein to be discovered was the product of a viral oncogene. The avian erythroblastosis virus E26 is a transforming retrovirus that causes erythroblastosis in chickens. The genome of this virus contains a fusion of the viral *gag* gene with portions of the *c-myb* and *ets-1* genes (Nunn et al., 1983; Leprince et al., 1983). This *gag/v-myb/v-ets* oncogene is responsible for the erythroid leukemogenicity of the E26 virus.

A number of *ets* genes are involved in the formation of Ewing's sarcoma, a childhood bone tumor, and peripheral neuroepitheliomas (PNETs). These tumors are characterized by the presence of chromosomal translocations that fuse the 5'-half of the *EWS* gene to the 3'-end of these *ets* genes (Dittmer and Nordheim, 1998). The proteins encoded by these fusions consist of EWS sequences fused to an ETS domain. The function of the EWS protein is unknown, but in the context of these fusion proteins the EWS sequences act as a strong transcriptional activator (Bailly et al., 1994). In 85% of Ewing's sarcomas, EWS is fused to Fli-1, while in 10% it is fused to Erg (Delattre et al., 1992; Sorensen et al., 1994). In rare cases EWS has been found to be fused to ETV-1 (ER81), E1A-F (PEA3) and FEV (Jeon et al., 1995; Kaneko et al., 1996; Peter et al., 1997). The fusion of these Ets proteins to EWS leads to the deletion of sequences required for their proper regulation and specificity, and results in constitutively active Ets proteins that activate transcription of similar genes, giving rise to a single disease phenotype.

Similar chromosomal translocations have been observed in certain types of human leukemia. Chromosomal translocations associated with acute myeloid leukemia give rise to genes whose products are fusions of the ETS domain of Erg with the EWS related protein FUS (TLS) or the transcription factor MN1 (Panagopoulos et al., 1994; Buijs et al. 1995). Like EWS-Ets fusions, these proteins lack regions required for proper regulation and specificity of action by Erg.

Certain weakly transforming retroviruses can cause erythroleukemias by inducing overexpression of *ets* genes. Infection with these retroviruses leads to integration of proviral sequences into the host cell genome (section 1.2.4), where they can

act as enhancers, inappropriately activating the transcription of nearby genes. In 75% of erythroleukemias induced by the Friend murine leukemia virus, the site of proviral insertion is near the *fli-1* gene, leading to overexpression of this gene (Ben-David et al., 1991). Similarly 95% of erythroleukemias induced by the Friend spleen focus-forming virus are caused by proviral insertion, leading to overexpression of *spi-1/PU-1* (Moreau-Gachelin et al., 1988).

The overexpression of some *ets* genes has been associated with breast cancer, including members of the PEA3 sub-family. For example, *PEA3* expression has been shown to correlate with *HER2/Neu* expression in breast cancers (Benz et al., 1997). HER2/Neu is a receptor tyrosine kinase related to the epidermal growth factor receptor expressed in 20 to 30% of human breast cancers and associated with increased incidence of metastasis and poor prognosis (Singleton and Strickler, 1992; Hynes and Stern, 1994). *PEA3* expression also correlates with increasing metastatic potential of breast cancer cells (Section 1.7.2) (Benz et al., 1997). Other *ets* genes, such as *ESX/ESE-1* are also overexpressed in *HER2/Neu* positive breast cancers and are thought to contribute to the increased invasive ability of these tumors (Chang et al., 1997).

1.6.3 Ets Proteins and Tumor Metastasis and Angiogenesis.

The expression of *ets* genes in cancer cells often correlates with their metastatic potential. For example, Ets-1 expression in breast cancer cells and gastric adenocarcinomas correlates with their grade of invasiveness (Nakayama et al., 1996; Dittmer and Nordheim, 1998). Various Ets proteins, including Ets-1, Ets-2, Erg and PEA3 can activate transcription of genes encoding uPA and members of the MMP and

TIMP families (reviewed by Dittmer and Nordheim, 1998). These proteins are involved in the breakdown of extracellular matrix components, which is necessary for tumor cell metastasis and vascularization of tumors (section 1.4.1).

Ets-1 may play a very important role in the vascularization and metastasis of tumors. Tumor angiogenesis is stimulated by secretion of vascular endothelial growth factor (VEGF) by tumor cells (Klagsbrun and Soker, 1993). VEGF induces *ets-1* expression in endothelial cells, which can then induce expression of matrix degrading proteases required for invasion of the tumor by these cells (Wernert et al., 1992; Dittmer and Nordheim, 1998). Ets-1 may also contribute to the metastatic phenotype by upregulating the expression of parathyroid hormone-related protein (PTHrP) (Dittmer et al., 1994). Many metastatic tumors secrete PTHrP, which acts to increase their invasive behavior (Dittmer and Nordheim, 1998). Finally, Ets-1 activates expression of the *MET* proto-oncogene, which encodes the hepatocyte growth factor (HGF) receptor. Expression of *MET* is sufficient to confer an invasive phenotype upon cells in the presence of HGF (Giordano et al., 1993). MET activation leads to increased expression of *ets-1*, creating a positive feedback loop, in which both *MET* and *ets-1* expression remains elevated and an invasive phenotype maintained (Gambarotta et al., 1996; Dittmer and Nordheim, 1998).

1.7 PEA3 and the PEA3 Subfamily of Ets Proteins.

The polyomavirus enhancer activator 3 (PEA3) was first described as a DNA-binding activity in mouse 3T6 cell nuclear extracts that binds and protects the sequence AGGAAG in the polyomavirus enhancer (Martin et al., 1988). Proteins binding to this

element contribute to activation of replication and transcription of the polyomavirus DNA (Mueller et al., 1988; Muller et al., 1988). Reiterated copies of the PEA3 binding site were used as a probe to screen a cDNA library in order to isolate cDNAs encoding proteins that can bind this motif. A cDNA encoding a novel Ets protein, PEA3 (E1AF, ETV-4), was isolated (Xin et al., 1992). PEA3 can bind and activate transcription of reporter genes downstream of reiterated copies of the AGGAAG motif. The PEA3 subfamily of Ets proteins is made up of PEA3 and two other members, ER81 (ETV-1) and ERM (Brown and McKnight, 1992; Monte et al., 1994). These three proteins share approximately 95% amino acid identity within their ETS domains and an overall amino acid identity of approximately 50%.

1.7.1 Regulation of Proteins of the PEA3 Subfamily.

Members of the PEA3 subfamily of Ets Proteins are targets of signal transduction pathways that act through Ras. Ras activation leads to phosphorylation of ER81 and ERM by the ERK family of kinases, activating these proteins (Janknecht, 1996; Janknecht et al., 1996). In response to Ras activation, PEA3 is activated by phosphorylation by members of both the ERK and JNK family of kinases (O'Hagan et al., 1996). Expression of constitutively active kinases from both the ERK and JNK pathways leads to stimulation of PEA3 activity. Also, expression of dominant negative mutant proteins from both pathways interferes with the ability of activated Ras to stimulate PEA3 activity. Kinases of both the ERK and JNK families can phosphorylate PEA3 *in vitro* (Cox and Hassell, unpublished data). However, the sites of phosphorylation of

PEA3 by these kinases *in vivo* are not known, nor is the mechanism by which these kinases stimulate PEA3 activity.

PEA3 activity can also be stimulated by expression of an activated form of HER2/Neu, a receptor tyrosine kinase of the epidermal growth factor receptor family (O'Hagan and Hassell, 1998). This is consistent with the ability of Ras to activate PEA3, as HER2/Neu is known to signal through Ras (Hynes and Stern, 1994). Dominant negative Ras mutants completely block the activation of PEA3 by HER2/Neu, while dominant negative kinases of the ERK and JNK pathways partially block PEA3 activation (O'Hagan and Hassell, 1998). Therefore, PEA3 is a potential target of growth factor-mediated signaling pathways acting through receptor tyrosine kinases and Ras.

1.7.2 The Role of PEA3 in Tumorigenesis and Tumor Cell Invasion and Metastasis.

PEA3 is often overexpressed in the cells of breast cancers relative to its expression in normal mammary epithelial cells (section 1.6.2). It is overexpressed in 93% of *HER2/Neu* expressing breast tumors (Benz et. al, 1997). Expression of *HER2/Neu* is linked with an increased invasive and metastatic propensity of these tumors, and therefore, poor prognosis for the cancer patient (Singleton and Strickler, 1992; Hynes and Stern, 1994). Transgenic mice in which high levels of expression of the rat *neu* gene is directed to the mammary epithelium develop mammary tumors that subsequently metastasize to the lung (Guy et. al, 1992). Both the mammary tumors and the lung metastases of these mice overexpress *PEA3* relative to normal tissue adjacent to these tumors (Trimble et al., 1993). PEA3 is able to activate transcription from its own

promoter as well as that of *HER2/Neu*, suggesting that a positive feedback loop, similar to that involving Ets-1 and MET (section 1.6.3), may exist in *HER2/Neu* expressing mammary tumors (Benz et al., 1997). This loop would maintain *PEA3* and *HER2/Neu* expression at elevated levels, contributing to the maintenance of the cancer phenotype.

PEA3 may contribute to the metastatic nature of the tumors in which it is expressed. PEA3 is able to activate transcription of a number of genes whose products are involved in breakdown of the ECM, including *MMP-1*, *MMP-3*, *MMP-9* (Higashino et al, 1997; Xin and Hassell, unpublished data), *MMP-7* (Xin and Hassell, unpublished data, H. Crawford, personal communication), *MMP-14* (Habelhah et al., 1999) and *TIMP-1* (Edwards et al., 1992). PEA3 can also activate transcription of the vimentin gene, whose product is a cytoskeletal protein that plays a key role in cellular motility (Chen et al., 1996). Transfection of non-invasive breast cancer cell lines with *PEA3* is sufficient to confer an invasive phenotype upon these cells, giving them the ability to penetrate reconstituted ECM and to form invasive tumors in immunocompromised mice (Kaya et al., 1996). Elevated levels of expression of *MMP-9* accompany this phenotype. Conversely, the invasive activity of squamous cell carcinoma-derived cell lines that express high levels of *PEA3* can be reduced by transfection with antisense *PEA3*. This reduction is accompanied by a reduction in the expression levels of *MMP-1*, *MMP-3* and *MMP-9* (Hida et al., 1997).

1.7.3 The Role of PEA3 in Normal Tissues During Development and in the Adult.

In the adult mouse *PEA3* in expressed constitutively only in the brain and the epididymis (Xin et al., 1992). However, during development of the mouse, *PEA3* is

expressed in a number of cell types, often at times when these cells are proliferating and migrating (Chotteau-Lelievre et al., 1997; Laing, 1998). Often, this expression overlaps with the expression of the other members of the PEA3 subfamily, particularly that of *ERM*. Mice in which the *PEA3* gene has been disrupted develop normally, possibly due to redundancy of PEA3 function in development with another Ets protein or proteins (Laing, 1998). However, adult male *PEA3*-null mice are sterile, consistent with *PEA3* expression in the epididymis. Interestingly, this sterility is not due to morphological changes in the epididymis or problems in spermatogenesis, and the sperm of *PEA3*-null mice are functional in *in vitro* fertilization assays (Laing, 1998). The sterility of *PEA3*-null males may have a neurological basis. Members of the PEA3 subfamily play roles in guiding motor and sensory neurons to appropriate targets. During chick development, *PEA3* and *ER81* are selectively expressed in pools of motor neurons that innervate specific muscles and the sensory neurons that provide feedback from the same muscles. Expression of these genes defines the target muscles that these neurons are to innervate (Lin et al., 1998). It has been hypothesized that PEA3 plays a similar role in the innervation of the smooth muscle of the penis by sympathetic and parasympathetic neurons and that disruption of this process in *PEA3*-null mice may give rise to erectile dysfunction.

The mammary glands of *PEA3*-null mice are morphologically different from those of wild-type mice. The ducts of the mammary glands of *PEA3*-null mice show a much simpler branching pattern compared to ducts of wild-type mice (MacNeil and Hassell, unpublished data; Laing, 1998). This branching defect does not impair

mammary gland function in these mice, but may reflect an impaired ability of ductal epithelial cells to infiltrate the surrounding tissue during mammary gland development.

PEA3 also plays a role in muscle regeneration in the adult. In damaged muscle, a population of myoblasts, called satellite cells, becomes activated, proliferating and migrating to the site of damage, where they differentiate into muscle cells (Campion, 1984). *PEA3* is expressed in activated satellite cells during proliferation and the early stages of differentiation (Taylor et al., 1997). Transfection of satellite cells in culture with dominant inhibitory mutants of *PEA3* inhibits the ability of satellite cells to undergo differentiation, suggesting the importance of PEA3 in this process. However, it is possible that dominant inhibitory *PEA3* may cause this effect by interfering with the activity of other Ets transcription factors.

1.8 Experimental Rational.

Growth factors stimulate cellular proliferation by creating intercellular signals that ultimately alter the activity of certain transcription factors, causing changes in gene expression, with transcription of genes required for cell division upregulated, and that of genes inhibitory to this process downregulated (Section 1.2.1). In normal cells, the signals generated by growth factors in normal cells are tightly controlled in order to prevent inappropriate cell division. Cancers arise due to the accumulation of mutations in genes involved in the proper regulation of these signals, inactivating molecules that control cell division or giving rise to signaling molecules whose activity cannot be properly regulated. Also, inactivating mutations of molecules involved in repairing DNA damage or eliminating cells in which this damage has occurred may contribute to the

formation of cancers (Sections 1.2.3 and 1.3). The net effect of these mutations is that cells divide in the absence of appropriate signals.

As tumor cells accumulate mutations, they become more aggressive, with the potential to invade the surrounding tissue and metastasize increasing (section 1.4). This is accompanied by further changes in gene expression. Expression of genes encoding proteolytic enzymes that degrade the ECM surrounding tumor cells is increased and the levels of expression of cell adhesion molecules is altered, disrupting the normal interactions of tumor cells with neighboring cells and with components of the ECM on which they grow.

Members of the Ets family of transcription factors have been shown to play important roles in these processes (section 1.6). In particular, we are interested in PEA3, a member of this family (section 1.7). Signaling pathways involving Ras and the receptor tyrosine kinase HER2/Neu activate PEA3 (O'Hagan et al., 1996; O'Hagan and Hassell, 1998). *PEA3* is overexpressed in many cases of human breast cancer, including 93% of breast cancers that express *HER2/Neu* (Benz et al., 1997). Expression of *HER2/Neu* and *PEA3* in these cancers correlates with increased invasive and metastatic potential. *PEA3* is also overexpressed in the tumors of transgenic mice that express high levels of the rat *neu* gene in their mammary epithelium (Trimble et al., 1993). PEA3 activates transcription of a number of genes whose products increase the motility and invasive activity of cells, including members of the MMP family of proteases (Section 1.7.2). Moreover, expression of *PEA3* in non-invasive breast cancer cell lines is sufficient to confer an invasive phenotype upon them (Kaya et al., 1996).

Finally, *PEA3* is expressed in a number of tissues during the development of the mouse (section 1.7.3). The cells of these tissues are often proliferating and migrating at the times when *PEA3* is expressed (Chotteau-Lelievre et al., 1997). This is consistent with the ability of PEA3 to contribute to the invasive ability of tumor cells. PEA3 also plays a role in the targeting of neurons to appropriate tissues during development (Lin et al., 1998).

It seems, therefore, that PEA3 plays important roles in tumorigenesis and tumor progression, as well as mammalian development. To better understand the nature of these roles, the identification of genes whose expression is regulated by PEA3 would be beneficial. To this end, cell lines have been generated in which *PEA3* overexpression can be induced, allowing the isolation of mRNA populations from cells that differ only in the expression level of *PEA3*. These mRNA populations have been compared using differential display reverse transcription PCR (DDRT-PCR) in the hopes of identifying PEA3 target genes whose expression level would be expected to change in response to *PEA3* overexpression.

CHAPTER 2

MATERIALS AND METHODS

2.1 Mammalian Cell Culture.

Cells were grown on 10 cm tissue culture plates under an atmosphere of 5% CO_2 in a humidified Water Jacketed™ cell culture incubator (Forma-Scientific). Rat-1 and MCF-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL) and 1X penicillin/streptomycin (Gibco-BRL) at 37^{0}C. Mouse embryo fibroblast cell lines were grown in DMEM supplemented with 10% calf serum (Gibco-BRL) and 1X penicillin/streptomycin at 37^{0}C. RP2, RP10, RP11, LAP1 and LAP6 cells were propagated in DMEM supplemented with 10% FBS, 1X penicillin/streptomycin and 400 µg/ml geneticin (G418) (Gibco-BRL) at 32^{0}C.

2.2 Calcium Phosphate Transfections.

All transfections were carried out at 37^{0}C. Transfections were carried out on cells in 10% DMEM supplemented with 10% FBS and 1X penicillin/streptomycin. Cells to be transfected were plated at a density of $5x10^5$ cells/10 cm tissue culture plate 16 hours before transfection. The DNA to be transfected was mixed and calf thymus DNA added to bring the total amount of DNA to 25 µg. DNA was precipitated by addition of 1/10 volume of 3 M sodium acetate and 2.5 volumes of absolute ethanol. Pellets were resuspended in 450 µl of sterile water, to which 50 µl of 2.5 M $CaCl_2$ was added. The DNA/$CaCl_2$ solution was added dropwise to 500 µl of 2X HEPES buffered saline (HBS)

(280 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4, pH 7.15) with bubbling of the HBS. Calcium phosphate precipitation was allowed to occur for 20 minutes. The precipitate was then added to the cells. Transfections of MCF-7 cells were carried out for 16 hours. Transfections of Rat-1, LAP1 and LAP6 cells were carried out for 8 hours. The medium was then removed from the transfected cells. Cells were washed twice with 1X phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4,3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4) and refed with DMEM supplemented with 10% FBS and 1X penicillin/streptomycin.

2.3 Cloning Methodology.

Vector and insert DNA were digested with the appropriate restriction enzymes. Digestions were carried out using 1 unit of restriction enzyme in the appropriate 1X digestion buffer supplied with the enzyme under conditions suggested by the manufacturer. If digestion with multiple enzymes requiring different buffers was carried out, extraction with one volume of 1:1 phenol:chloroform was carried out after the first digestion. DNA was then precipitated with 1/10 volume sodium acetate and 2.5 volumes absolute ethanol. DNA was resuspended in the appropriate 1X buffer for the second digestion.

If dephosphorylation of vector DNA was required, extraction with one volume of 1:1 phenol:chloroform was carried out after restriction endonuclease digestion. Vector DNA was precipitated with 1/10 volume sodium acetate and 2.5 volumes absolute ethanol. DNA was resuspended in 18 µl H_2O, to which was added 2 µl 10X CIAP buffer

and 1 unit CIAP (Calf intestinal alkaline phosphatase) (Boeringer Mannheim). Dephosphorylation was allowed to continue for 30 minutes at 37^0C.

After appropriate enzymatic manipulations of vector and insert DNA, 6X gel loading buffer (95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol) was added to a final concentration of 1X. DNA was then electrophoresed through a 1 to 1.5% agarose gel containing 2 μg/ml ethidium bromide in 1X TAE buffer (40 mM tris-acetate, 2 mM EDTA, pH 8.5) at 100 V. Vector and insert bands were excised from the gel. DNA was extracted from agarose gel slices using either the Geneclean II (Bio 101) or QUIQuick gel (Quigen) gel extraction kits according to manufacturers specifications.

Vector and insert DNA were mixed in the desired ratio for ligation. Water was added to bring the total volume to 16 μl. To this 4 μl of 5X ligation buffer and 1 U of T4 DNA ligase (Gibco-BRL) was added. Ligations were carried out at 16^0C for 16 hours. Appropriate competent *E. coli* strains were transformed by adding 2 μl of ligation mix to 100 μl of competent *E. coli* in 20 mM β-mercaptoethanol in a Falcon 2059 tube. Cells were incubated on ice for 20 minutes. Cells were then heat shocked for 30 s at 42^0C and placed on ice for 2 minutes. To the transformed cells was added 500 μl SOC medium (2% bactotryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose). Cells were incubated at 37^0C with shaking for 1 hour. Cells were then plated on LB-agar plates containing 100 μg/ml ampicillin and incubated at 37 ^{0}C for 16 hours. Isolated colonies of LB-ampicillin plates were picked and inoculated into LB broth (1% bactotryptone, 0.5% yeast extract, 1% NaCl, pH 7.0) containing 100 μg/ml ampicillin. For small scale plasmid preparations 2 ml LB-ampicillin was used, while for large scale plasmid preparations 500 ml was used. Cells were grown with shaking at

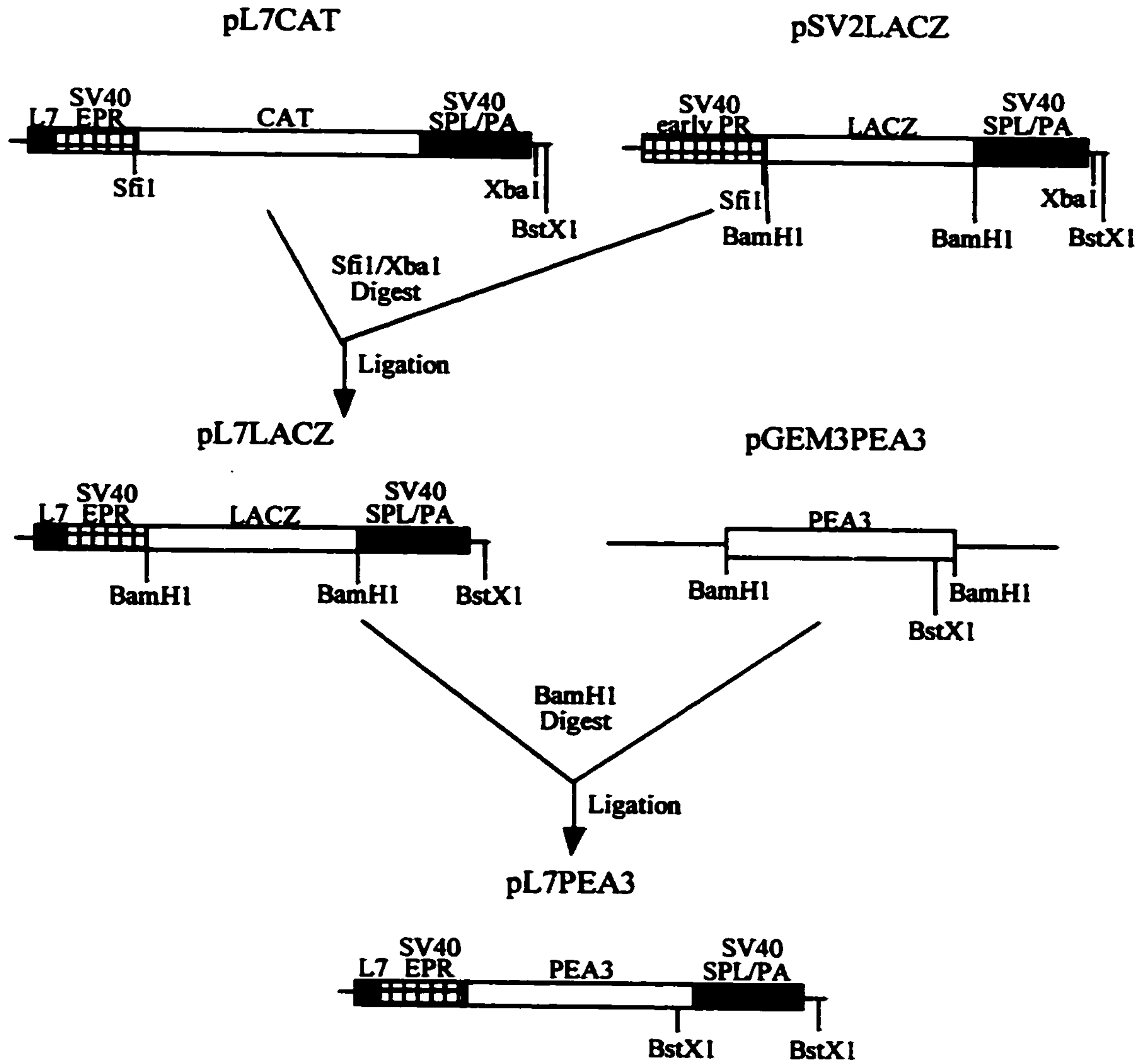
37^0C for 16 hours. Small scale plasmid preparations were carried out according to the protocol of Sambrook et. al (1989). Large scale plasmid preparations were carried out using the Quigen plasmid maxi kit according to manufacturers specifications. Plasmid DNA was digested with appropriate restriction endonucleases and agarose gel electrophoresis was carried out to determine if ligations were successful.

2.4 Construction of pL7PEA3.

The construction of the plasmid pL7PEA3 (Section 3.1) is outlined in Figure 2.1. The plasmid pL7CAT was obtained from the laboratory of T. Shenk. The plasmid pSV2LACZ encodes the *LACZ* gene under the control of the SV40 early promoter, and contains the SV40 splice donor and acceptor sites, and polyadenylation signal, which are also present in pL7CAT. pL7CAT was digested with SfiI and XbaI (Gibco-BRL) to remove the *CAT* gene and SV40 splice sites and polyadenylation signal. The *LACZ* gene, splice sites and polyadenylation signal were excised from pSV2LACZ and ligated into the pL7 vector backbone to yield pL7LACZ, which encodes *LACZ* under the control of an enhancerless SV40 early promoter and seven *lac* operator sequences. The plasmid pGEM3PEA3, which contains the entire *PEA3* cDNA flanked by two BamHI sites (Keserovic and Hassell, unpublished) was obtained. The *LACZ* gene was excised from backbone. This yielded pL7PEA3, encoding the *PEA3* cDNA under the control of the enhancerless SV40 promoter and seven *lac* operator sequences. The ability of cells to inducibly express *PEA3* from this construct was tested by transiently co-transfecting HeLa cells with pL7PEA3, pHβALAP267 and pPyORI4XPEA3 (Section 3.1) and testing these cells for inducible CAT activity.

Figure 2.1 Construction of pL7PEA3.

The vector pL7PEA3 encodes the mouse *PEA3* cDNA under the control of an enhancerless SV40 early promoter (SV40 EPR) and seven *lac* operator sequences (L7). To create this vector the *CAT* gene and SV40 splice sites and polyadenylation signals were excised from pL7CAT, which encodes *CAT* under the control of these promoter sequences, by cleavage with the restriction endonucleases Sfi1 and Xba1. The *LACZ* gene and SV40 splice sites and polyadenylation signals were removed from the vector pSV2LACZ by excision with these endonucleases, and ligated into the pL7 vector backbone to create pL7LACZ. The *LACZ* gene was removed from this vector by excision with BamH1. The *PEA3* cDNA was removed from the vector pGEM3PEA3 by excision with BamH1, and ligated into the pL7 vector backbone, generating pL7PEA3.


pL7CAT
pSV2LACZ
L7
SV40
EPR
CAT
SV40
SPL/PA
SfiI
XbaI
BstXI
SV40
early PR
LACZ
SV40
SPL/PA
SfiI
BamHI
BamHI
XbaI
BstXI
SfiI/XbaI
Digest
Ligation
pL7LACZ
pGEM3PEA3
L7
SV40
EPR
LACZ
SV40
SPL/PA
BamHI
BamHI
BstXI
PEA3
BamHI
BamHI
BstXI
BamHI
Digest
Ligation
pL7PEA3
L7
SV40
EPR
PEA3
SV40
SPL/PA
BstXI
BstXI

2.5 Generation of RP2, RP10 and RP11 Cell Lines.

The plasmids pHβALAP267 and pL1-3neo (Section 3.1) were obtained from the laboratory of T. Shenk. The reporter plasmid pPyORI4XPEA3 (Xin et. al, 1992) was obtained from the Hassell laboratory. Rat-1 cells were co-transfected with 5 μg each of pHβALAP267, pL7PEA3, pL1-3neo and pPyORI4XPEA3. Cells were grown at 37 ^{0}C for 48 hours after transfection. At this time cells were transferred to 32 ^{0}C. After 16 hours at this temperature, the medium was removed and replaced with DMEM/FBS containing 400 μg/ml G418. Selection with G418 was carried out until F418 resistant colonies were apparent and could be easily picked. G418 containing medium was replaced every 48 hours. Individual colonies were picked and transferred to a single well of a 96 well tissue culture dish, and selection was continued in G418 containing medium. Each cell line was expanded until such time as there were sufficient cells for screening of these lines to be carried out (three near confluent 10 cm plates for each cell line). Screening of the cell lines was carried out by comparing the CAT activities of extracts prepared from each cell line grown at either 32 ^{0}C or 39.5 ^{0}C. Cells were grown at these temperatures for 48 hours in G418-free DMEM/FBS. Extracts were prepared from one 10 cm plate of each cell line grown under both conditions and CAT assays performed (Section 2.7). Extracts prepared from three cell lines, designated RP2, RP10 and RP11, showed higher CAT activity at 32 ^{0}C than at 39.5 ^{0}C.

2.6 Generation of LAP1 and LAP6 Cell Lines.

Rat-1 cells were transfected with 5 μg each of pHβALAP267 and pL1-3neo. Cells were grown at 37 ^{0}C for 48 hours after transfection. At this time cells were transferred to

32 ^{0}C. After 16 hours at this temperature, the medium was removed and replaced with DMEM/FBS containing 400 µg/ml G418. Selection with G418 was carried out until G418 resistant colonies were apparent and could be easily picked. G418 containing medium was replaced every 48 hours. Individual colonies were picked and transferred to a single well of a 96 well tissue culture dish. Selection with G418-containing medium was continued. Each cell line was expanded until such time as there were sufficient cells for screening of these cell lines to be carried out (three near confluent 10 cm plates for each cell line). Each cell line was transfected with 5 µg of pL7CAT. After transfection cells were grown for 48 hours at either 32 ^{0}C or 39.5 ^{0}C in DMEM/FBS containing no G418. Extracts were prepared from one 10 cm plate of each cell line grown under both conditions and CAT assays performed (Section 2.7). Extracts from two cell lines, designated LAP1 and LAP6, showed higher CAT activity at 32 ^{0}C than at 39.5 ^{0}C.

2.7 CAT Assays.

CAT assays were performed according to the protocol of Sambrook et. al, 1989. The medium was removed from plates of cells to be assayed and cells washed three times with ice cold PBS. Cells were removed from plates by scraping with a rubber policeman and collected in 1 ml of cold PBS. Cells were pelleted by centrifugation and resuspended in 100 µl of 250 mM tris-Cl, pH 7.8. Cells were lysed by three cycles of freezing with dry ice and thawing at 37 ^{0}C, and debris was removed by centrifugation. Extracts were incubated at 37 ^{0}C for 1 hour with 500 mM tris-Cl, pH 7.8, 7.5 µCi/ml ^{14}C-labeled chloramphenicol (Amersham) and 500 µg/ml acetyl-CoA (Gibco-BRL) in a volume of 130 µl. After this incubation, 1 ml of ethyl acetate was added to the samples.

After vigorous vortexing, the mixtures were centrifuged for 5 min. and 900 μl of the organic phase was removed. Ethyl acetate was evaporated in a Savant Speedvac Plus AR. Reaction products were resuspended in 25 μl ethyl acetate and spotted on a 25 mm silica gel thin layer chromatography plate. Thin layer chromatography was carried out using chloroform:methanol (95:5) as a solvent. Chromatography plates were dried and exposed to X-ray film.

2.8 Isolation of RNA from Mammalian Cells.

Cells were lysed and RNA prepared using the Trizol RNA isolation reagent (Gibco-BRL) according to manufacturers specifications. RNA pellets were resuspended in 1X DNase buffer (10 mM tris-Cl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$) and incubated with 10 units of RNase-free DNase I (Boeringer Mannheim) for 30 min. at 37 ^{0}C. The RNA preparations were extracted with 1 volume of phenol:chloroform (1:1) and precipitated with 1/10 volume NaOAc and 2.5 volumes absolute ethanol. The integrity of the RNA samples was checked by electrophoresis on a denaturing agarose gel. RNA was denatured by incubation with 1X MOPS buffer (200 mM MOPS, 50 mM NaOAc, 10 mM EDTA), 6.5% formaldehyde and 50% formamide for 15 min. at 55 ^{0}C. Agarose gel loading buffer was added and ethidium bromide was added to a final concentration of 15 μM. Samples were electrophoresed at 100 V on a 1.2% agarose gel containing 1% formaldehyde in 1X MOPS buffer.

2.9 Reverse Transcription-Polymerase Chain Reaction (RT-PCR).

Reverse transcription was carried out according to the specifications of Gibco-BRL. RNA samples to be reverse transcribed were mixed with 1X RT buffer (25 mM tris-Cl, pH 8.3, 37.6 mM KCl, 1.5 mM $MgCl_2$, 5 mM DTT), 20 μM dNTPs (Pharmacia), 30 U RNAGuard (Amersham) and 1 μM oligo-dT. The RNA was denatured by heating to 65 ^{0}C for 5 min. Samples were transferred to a 37 ^{0}C water bath and 1 U of Superscript II reverse transcriptase (Gibco-BRL) added. The samples were incubated at 37 ^{0}C for 1 hour. The reaction was stopped by incubation at 95 ^{0}C for 5 min.

PCR (Mullis and Falloona, 1987) was carried out on 2 μl of the RT reaction mixture. This was mixed with 1X PCR buffer (100 mM tris-Cl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$), 20 μM dNTPs (Pharmacia), 1 μM 5'- and 3'-primers, and 1 U Taq polymerase (Gibco-BRL). Thirty cycles of 95 ^{0}C for 30 sec., 55 ^{0}C for 1 min., and 72 ^{0}C for 1 min. were carried out in a GeneAmp PCR System 9600 (Perkin-Elmer). PCR products were electrophoresed through a 1% agarose gel and purified from gel slices using the GeneClean II (Bio 101) or QuiQuick (Quigen) gel extraction kits. All primers used for reverse transcription or PCR were manufactured by the Institute for Molecular Biology and Biotechnology Central Facility (McMaster University).

2.10 Generation of Antisense Riboprobes.

Antisense riboprobes were generated according to the protocol of Gilman (1988). Transcription of antisense riboprobes was carried out by mixing 250 ng of linearized template DNA with 1X transcription buffer (40 mM tris-Cl. pH 8.0, 8 mM $MgCl_2$, 2 mM

spermidine, 50 mM NaCl), 10 mM DTT, 400 µM each ATP, CTP and GTP (Pharmacia), 2 mCi/ml [α-^{32}P]UTP (3000 Ci/mmol, Amersham) and 10 U T7 or T3 RNA polymerase. The transcription mixtures were incubated at 37 ^{0}C for 1 hour. Reactions were stopped by the addition of 1 U RNase-free DNase I (Boeringer Mannheim) and incubation at 37 ^{0}C for 15 min. After the addition of 20 µg of yeast tRNA as a carrier, extraction with 1:1 phenol:chloroform was carried out. RNA was precipitated with 500 mM NH_4OAc and 75% ethanol, resuspended in 10 µl RNA loading buffer (95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol), and purified by electrophoresis through a 6% urea/acrylamide gel. Full length riboprobes were localized by autoradiography, excised from the gel and eluted into 1 ml of 500 mM NH_4OAc, 1 mM EDTA, 0.1% SDS.

The antisense mouse *PEA3* riboprobe plasmid construct used in this study was generated by Micheal Liang in the Hassell lab. The rat *GAPDH* riboprobe construct was generated by carrying out RT-PCR on RP10 cell RNA. RT-PCR was carried out using the primers 5'-CTCCCTCACAATTCCATCCCAGACC-3' and 5'-GGGTGCAGCGAAC TTTATTGATGGT-5' to amplify a 95 base pair cDNA fragment of rat *GAPDH*, which was subcloned into the pCR2.1 vector (Invitrogen). The rat *G71* (*ALCAM*) riboprobe construct was generated by subcloning the *G71* DDRT-PCR product (Sections 2.16 and 4.2.2) into the pCR2.1 vector. The mouse *ALCAM* riboprobe construct was generated by carrying out RT-PCR on mouse brain RNA using the primers 5'-TGG CATCTAAGGTGTCCCCTTCTTG-3' and 5'-TTGCCATCTGGGAAACTGTCTCTTG-3'. The resulting 499 base pair cDNA was subcloned into the pCR2.1 vector. The rat *ICAM-1* riboprobe was generated by RT-PCR carried out on RP10 cell RNA using the primers 5-'GGGTTGGAGACTAACTGGATGAAAG-3' and 5'-TGACCTCGGAGACA

TTCTTGAACAG-3' to generate a 442 base pair cDNA fragment. The rat *N-CAM* riboprobe was generated by RT-PCR using RP10 cell RNA with the primers 5'-AGTGGCTGGCAAGAAACAATTCTGC-3' and 5'-AATGTTGGCGTTGTAGATG-3' to generate a 420 base pair cDNA fragment. These cDNAs were also subcloned into the pCR2.1 vector. A number of isolates of the rat *GAPDH*, *G71* (*ALCAM*), *ICAM-1*, *N-CAM*, and mouse *ALCAM* constructs were sequenced to ensure the cDNAs were in the antisense orientation relative to the T7 promoter of the pCR2.1 vector. Sequencing was carried out by the Institute for Molecular Biology and Biotechnology Central Facility (McMaster University).

2.11 Ribonuclease Protection Assays.

Ribonuclease protection assays were carried out according to the protocol of Gilman (1988). The RNA to be analyzed was mixed with $5x10^4$ cpm of each riboprobe used in the assay, and precipitated with 1/10 volume of NH_4OAc and 2.5 volumes of absolute ethanol. The pellets were resuspended in 30 µl of hybridization buffer (40 mM PIPES, pH 6.4, 400 mM NaCl, 1 mM EDTA, 80% formamide) and the RNA was denatured by boiling for 5 min. Hybridizations were carried out for 16 hours at 45 ^{0}C for 16 hours. To each hybridization mixture, 350 µl of digestion buffer (10 mM tris-Cl, pH 7.5, 300 mM NaCl, 5 mM EDTA, 2 µg/ml RNase T1 (Boeringer Mannheim)) was added, and digestions were carried out at 30 ^{0}C for 1 hour. The digestions were stopped by the addition of 10 µl of 20% SDS and 2.5 µl of 20 mg/ml proteinase K (Boeringer Mannheim) and incubation at 37 ^{0}C for 15 min. The samples were extracted with 1:1 phenol:chloroform, and 1 µg of carrier yeast tRNA was added. The digested RNA was

precipitated with 2.5 volumes of absolute ethanol and resuspended in 10 µl RNA loading buffer. The samples were electrophoresed on a 6% denaturing urea/polyacrylamide gel in 1X TBE buffer (90 mM tris-borate, 2 mM EDTA) at 55 W for 2.5 hours. The gels were dried using a Model 583 gel dryer (Bio-Rad) and analyzed by autoradiography.

2.12 Generation of Radiolabelled cDNA Probes.

Radiolabelled cDNA probes were generated according to the protocol of Tabor and Struhl (1988). The cDNA to be labeled was excised from the vector in which it was propagated using the appropriate restriction endonucleases, and purified by electrophoresis through a 1% agarose gel (Section 2.3). Approximately 100 ng of the cDNA was combined with 5 µg of random hexanucleotides (Boeringer Mannheim) and denatured by boiling for 5 min. To this was added 50 mM each of dATP, dGTP, and dTTP (Pharmacia), 1X Klenow fragment buffer (50 mM tris-Cl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 50 µg/ml BSA), 2 mCi/ml [α-^{32}P]dCTP (3000 Ci/mmol, Amersham) and 5 U Klenow fragment (Gibco-BRL). Labeling was carried out at room temperature for 1 hour, and the resulting cDNA probes purified by gel exclusion chromatography on a Sephadex G50 (Pharmacia) column.

The rat *GAPDH* cDNA used as a probe in this study was obtained as described in Section 2.10. The rat *MMP-9* cDNA was generated by RT-PCR carried out on RNA isolated from RP10 cells induced to overexpress *PEA3* for 8 hours, using the primers 5'-GCTCTAGGCTACAGCTTTG-3' and 5'-GTGAACGAATGGCCTTTAGTGTCTC-3'. The resulting 256 base pair cDNA fragment was subcloned into the pCR2.1 vector (Invitrogen). Similarly, the rat *MMP-14* cDNA was obtained by RT-PCR carried out on

induced RP10 cell RNA, using the primers 5'-TTCCAGGAGCAATTCCCTC-3' and 5'-ATCTCATTATGCTGCCACTTGAGGC-3'. The resulting 533 base pair cDNA fragment was also subcloned into pCR2.1. The 456 base pair *G71* (*ALCAM*) cDNA probe used in the screening of a rat brain cDNA library (Section 2.18) was generated by DDRT-PCR (Sections 2.16 and 4.2.2). The 210 base pair *G71* (*ALCAM*) cDNA fragment used to screen a mouse genomic library (Section 2.19) was generated by RT-PCR carried out on mouse brain RNA using the primers 5'-TGGCATCTAAGGTGTCCCCTTCTTG-3' and 5'-GGAATGCAATAAATACTGGGGACCC-3', and subcloned into pCR2.1.

2.13 Northern Blots.

Northern blot analyses were carried out according to the protocol of Selden (1988). The RNA to be analyzed was mixed with 1X MOPS electrophoresis buffer (20 mM MOPS, 5 mM NaOAc, 100 μM EDTA), 6.5% formaldehyde and 50% formamide, in a volume of 50 μl. The RNA was denatured by incubation at 55 ^{0}C for 15 min., and 10 μl of RNA loading buffer added. The samples were electrophoresed on a 1.2% agarose gel containing 1% formaldehyde in 1X MOPS buffer at 100 V for 3 hours. The gel was rinsed three times with DEPC-treated water and equilibrated with 10X SSC (1.5 M NaCl, 150 mM Na Citrate, pH 7.0) for 45 min. The RNA was transferred to a Genescreen Plus nylon membrane (NEN Life Sciences) by capillary action using 20X SSC (3 M NaCl, 300 mM Na Citrate, pH 7.0) overnight. The RNA was cross-linked to the membrane using a UV Stratalinker 2400 (Stratagene). Membranes were prehybridized in 10 mls of 50% formamide, 5X SSPE (750 mM NaCl, 50 mM NaH_2PO_4, 5 mM EDTA) 5X Denhardt's Solution and 100 μg/ml salmon sperm DNA for 4 hours at 42 ^{0}C. To this was

added 1×10^7 cpm of cDNA probe that had been denatured by boiling for 10 min. Hybridizations were carried out for 16 hours. The filters were washed once with 2X SSC, 0.5% SDS for 15 min. at room temperature and twice with 1X SSC, 0.1% SDS at 65 ^{0}C for 1 hour. The filters were then analyzed by autoradiography.

2.14 Southern Blots.

Southern blot analyses were carried out according to the protocol of Sambrook et. al. (1989). The DNA to be analyzed was digested using the appropriate restriction endonucleases and electrophoresed on a 1% agarose gel in TAE buffer. The DNA was denatured by soaking the gel in 1.5 M NaCl, 0.5 M NaOH for 45 min. The gel was then neutralized by soaking in 1M tris-Cl, pH 7.4, 1.5 M NaCl for 45 min. The DNA was transferred to a Genescreen Plus nylon membrane (NEN Life Sciences) by capillary action using 10X SSC overnight. The DNA was cross-linked to the membrane using a DNA Stratalinker 2400 (Stratagene). Prehybridization, hybridization with the cDNA probe and washing of the filters was performed as described in Section 2.13.

2.15 Preparation of Nuclear Extracts and Western Blotting.

Nuclear extracts were prepared from RP10 cells as has been previously described (Lee et. al, 1998). The extracts were electrophoresed through a 10% SDS/polyacrylamide gel. The gel was equilibrated with transfer buffer (20 mM tris-Cl, pH 8.0, 150 mM glycine, 20% methanol), and proteins were electrophoretically transferred to a nitrocellulose membrane at 15 V overnight in transfer buffer. The membrane was dried and incubated with blocking buffer (10 mM tris-Cl, pH 7.3, 150

mM NaCl, 0.05% Tween 20, 5% skim milk, 0.05% Na Azide) at room temperature for 2 hours with agitation. The membrane was then incubated with a mixture of the anti-PEA3 monoclonal antibodies MP13 and MP16 for 2 hours at room temperature. The primary antibodies were removed and the membrane rinsed with TBS-T (10 mM tris-Cl, pH 7.3, 150 mM NaCl, 0.05% Tween 20) and reblocked for 2 hours at room temperature. The membrane was then incubated with a horse radish peroxidase conjugated goat anti-mouse secondary antibody in blocking buffer for 2 hours at room temperature. The western blots were developed by chemiluminescence using a BM Chemiluminescence kit (Boeringer Mannheim) according to manufacturers specifications.

2.16 Differential Display Reverse Transcription PCR (DDRT-PCR).

DDRT-PCR was carried out according to the protocol of Liang and Pardee (1992). Reverse transcriptions were carried out on the RNA samples to be analyzed as was described in Section 2.9, except only 200 μg of RNA was used and one of the primers 5'-T_{12}MA-3', 5'-T_{12}MC-3', 5'-T_{12}MG-3', or 5'-T_{12}MT-3' (M represents A, C or G) was used instead of oligo-dT. PCR was carried out on 2 μl of the RT reaction mixture. This was mixed with 1X PCR buffer (100 mM tris-Cl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$), 2 μM dNTPs (Pharmacia), 200 nM 5'-primer, 1 μM 3'-primer, 50 μCi/ml [α^{32}P]dCTP (3000 Ci/mmol, Amersham) and 1 U Taq polymerase (Gibco-BRL). Forty cycles of 95^{0}C for 30 sec., 40 ^{0}C for 2 min., and 72 ^{0}C for 30 sec. were carried out in a GeneAmp PCR System 9600 (Perkin Elmer). For each sample the 3'-primer used for reverse transcription was used in combination with one of five 5'-primers, AP6 (5'-GCAATCGATG-3'), AP7 (5'-CCGAAGGAAT-3'), AP8 (5'-GGATTGTGCG-3'), AP9

(5'-CGTGGCAATA-3') or AP10 (5'-TAGCAAGTGC-3'). The samples were electrophoresed on a 6% urea/polyacrylamide gel in 1X TBE buffer at 55 W for 3 hours. Gels were dried using a Model 583 gel dryer (Bio-Rad) and analyzed by autoradiography. Bands of interest were excised from the gel and the DNA eluted by boiling for 15 min. in 500 mM NH_4OAc, 1 mM EDTA, 0.1% SDS. cDNAs were reamplified by PCR, using the same primers and conditions described above, and electrophoresed on a 1.2% agarose gel to ensure a product of the expected size was obtained. The cDNAs were excised and eluted from the gel, subcloned into the pCR2.1 vector (Invitrogen), and sequenced. Sequences were compared to the GenBank database to determine whether the cDNAs were novel or had been previously isolated.

2.17 Reverse Northern Blotting.

Reverse northern blotting was carried out according to the protocol of Zhang et. al (1996). cDNAs were excised from the vectors in which they were propagated using the appropriate restriction endonucleases, and separated from the vector backbone by electrophoresis through a 1% agarose gel. Bands were excised from the gels and the cDNA eluted. Approximately 5 μg of each cDNA was denatured by boiling in 0.5 N NaOH for 5 min. The cDNAs were then neutralized with 3 M NaOAc, pH 5.0 and spotted on a Genescreen Plus II nylon membrane (NEN Life Sciences) using a Bio-Dot microfiltration apparatus (Bio-Rad). cDNAs were cross-linked to the membrane using a DNA Stratalinker 2400 (Stratagene). cDNA probes were prepared by reverse transcription of 10 μg of the RNA samples to be analyzed. Reverse transcriptions were carried out as described in Section 2.9, except 2 mCi/ml [α^{32}P]dCTP (3000 Ci/mmol,

Amersham) was substituted for cold dCTP. cDNA probes were purified by gel exclusion chromatography over a Sephadex G-50 column (Pharmacia). Prehybridization of the membrane, hybridization with the cDNA probes, and washing of the membranes was carried out as described in Section 2.13.

2.18 cDNA Library Screening.

A rat brain cDNA library in the λgt10 vector (Clontech) was screened in order to obtain a cDNA encoding *G71* (ALCAM) (Section 4.2.4). Screening of this library was carried out according to manufacturers specifications. Using the G71 cDNA obtained by differential display as a probe, $1x10^6$ plaques were screened. Plaque lifts were carried out using Hybond-N filters (Amersham). The G71 cDNA was labeled as described in Section 2.12 and prehybridization, hybridization with the G71 probe and washing of the filters was carried out as described in Section 2.13. Screening of $1x10^6$ plaques yielded one positive hybridization. This plaque was picked, the phage eluted and re-plated, and a secondary screening carried out as described above. Again, positive plaques were picked, eluted and re-plated and a tertiary screening carried out as described above. Positive plaques from the tertiary screen were picked, and the phage eluted and used to lyse 10 plates of C600hfl cells. Lambda DNA was purified from the lysate using a Lambda DNA Purification Kit (Stratagene) according to manufacturers specifications. The cDNA insert was amplified from 1 μg of lambda DNA by PCR, using the lambda specific primers 5'-AGCAAGTTCAGCCTGGTTAAGT-3' and 5'-AATACTCATAAAG AAGGTCCC-3', and the PCR conditions described in Section 2.10. A Southern Blot (Section 2.14) was carried out, using the G71 cDNA probe to confirm the PCR amplified

cDNA was able to hybridize to this probe. The PCR amplified cDNA was purified by electrophoresis through a 1% agarose gel, and after elution from the gel, was subcloned into the pCR2.1 vector (Invitrogen). Upon sequencing, it was discovered that the 967 base pair cDNA isolated in this manner contained the entire sequence of the G71 probe used to screen the library at its 3'-end, as well as 511 base pairs if 5'-sequence, confirming its identity as a longer G71 cDNA. Comparison of this sequence to the GenBank database showed that G71 is the rat homologue of Activated Leukocyte Cell Adhesion Molecule (ALCAM) (Section 4.2.4).

2.19 Genomic Library Screening.

A genomic library derived from the SV129 mouse strain in the lambda FixII vector (Stratagene) was screened in order to obtain the ALCAM promoter sequences (Section 4.2.7). Screening of this library was carried out according to manufacturers specifications. Using a cDNA probe containing the 210 5'-most base pairs of the mouse ALCAM cDNA (Section 2.12), 1x106 plaques were screened. Plaque lifts were carried out using Hybond-N filters (Amersham). The ALCAM cDNA probe was labeled as described in Section 2.12. Prehybridization, hybridization with the cDNA probe and washing of the filters was carried out as described in Section 2.13. Screening of $1x10^6$ plaques yielded five positive hybridizations. Secondary and tertiary screening of these positives was carried out as described in Section 2.18. Positive plaques from the tertiary screens were picked, and the phage eluted and used to lyse 10 plates of XL1-blue cells. Lambda DNA was purified using a Lambda DNA Purification Kit (Stratagene) according to manufacturers specifications. Digestion of each of the five lambda DNA isolates was

carried out using Bgl II, and the digested DNA electrophoresed on a 0.8% agarose gel. Southern blotting was carried out on this DNA as described in Section 2.14, using the ALCAM cDNA probe used to screen the library. A 1135 base pair DNA fragment, present in all five DNA isolates hybridized to this probe. This fragment was subcloned into the pBluescript II KS vector (Stratagene). Upon sequencing, it was discovered that this DNA fragment contained the 5'-end of the ALCAM cDNA and 862 base pairs of upstream sequences (Section 4.2.7).

2.20 Generation of Luciferase Reporter Constructs.

Fragments of the ALCAM promoter sequences were subcloned upstream of the luciferase reporter gene in the pGL3 basic vector (Promega). The sequences from -862 to +4 (ALCAM-862) relative to the 5'-end of the ALCAM cDNA were PCR amplified from the ALCAM promoter DNA, isolated as described in Section 2.19, using the primers 5'-AAGCTTGGGCCCACGGGGCCCGCTCGCTTG-3' and 5'-GAGCTCGATCTCAGAT CCTATGTCTTTGGG-3'. The sequences from -508 to +4 (ALCAM-508) relative to the 5-end of the ALCAM cDNA were PCR amplified from the ALCAM promoter DNA using the primers 5'-AAGCTTGGGCCCACGGGGCCCGCTCGCTTG-3' and 5'-GAGCTCGC TTACACACGCCTTCCCGTCCTC-3'. The sequences from -263 to +4 (ALCAM-263) relative to the 5'-end of the ALCAM cDNA were PCR amplified from the ALCAM promoter DNA using the primers 5'-AAGCTTGGGCCCACGGGGCCCGCTC GCTTG-3' and 5'-GAGCTCAAGAAACCGCGTCTACCTTGACTG-3'. These primers introduced a SacI site at the 5'-end and a HindIII site at the 3'-end of each ALCAM promoter fragment. After PCR amplification, each ALCAM promoter fragment was

purified by electrophoresis through a 1% agarose gel, and subcloned into the pCR2.1 vector (Invitrogen). The ALCAM promoter fragments were excised from this vector using SacI and HindIII, and subcloned between the SacI and HindIII sites of the pGL3 basic vector.

2.21 Luciferase Assays.

Luciferase assays were carried out using the Promega Luciferase Assay System and reagents, according to manufacturers specifications. The assays were done using a Lumat LB 9501 luminometer (Berthold).

CHAPTER 3

GENERATION AND CHARACTERIZATION OF CELL LINES IN WHICH PEA3 OVEREXPRESSION MAY BE INDUCED

3.1 Introduction.

The Ets family transcription factor PEA3 is thought to play an important role in carcinogenesis and tumor progression (section 1.7.2). Growth factor receptor tyrosine kinase-mediated signal transduction pathways can regulate PEA3. For example, the ability of PEA3 to activate transcription is increased by activated HER2/Neu or Ras (O'Hagan et al., 1996; O'Hagan and Hassell, 1998). Furthermore, *PEA3* expression is increased in the presence of activated HER2/Neu (Benz et al., 1997). *HER2/Neu* is overexpressed in 20% to 30% of human breast cancers, and in 95% of these cases, *PEA3* is also overexpressed (Hynes and Stern, 1994; Benz et al., 1997). Expression of both *HER2/Neu* and *PEA3* correlates with increased rates of metastasis and poor prognosis in such tumors (Benz et al., 1997). *PEA3* is also overexpressed in the tumors of transgenic mice that overexpress the rat *Neu* gene in their mammary epithelium (Trimble et al., 1993). Therefore, PEA3 may be an important downstream effector of HER2/Neu, and as such may contribute to the formation of tumors that express *HER2/Neu* or in conferring a metastatic phenotype upon these tumors. However, at this time the nature of this role is not well understood.

PEA3 overexpression in certain tumors and tumor-derived cell lines correlates with increased invasive and metastatic ability. The ability of PEA3 to activate transcription of a number of *MMP* genes is a likely contributing factor, as expression of

MMPs in a number of tumor types correlates with increased metastatic ability (Coussens and Werb, 1996; Westermark and Kahari, 1999). *PEA3* overexpression is sufficient to confer an invasive phenotype upon MCF-7 cells, a non-invasive breast cancer cell line, with increased expression of *MMP-9* accompanying this phenotype (Kaya et al, 1996). Whether the ability of PEA3 to activate transcription of *MMPs* is sufficient to explain the correlation of *PEA3* expression with the metastatic ability of tumors is not yet known.

The expression of *PEA3* is developmentally regulated with expression often occurring at sites where cells are proliferating and migrating (Chotteau-Lelievre et al., 1997; Laing, 1998). This is consistent with loss of regulation or improper expression of *PEA3* contributing to the formation of metastatic tumors. *PEA3*-null mice show no developmental abnormalities, possibly due to redundancy of *PEA3* function (Laing, 1998). However, *PEA3*-null male mice are sterile. The cause of this phenotype remains unknown, as does the role of *PEA3* in mammalian development.

To better understand the contribution of PEA3 to tumor formation, tumor progression and mammalian development, the identification of genes whose expression is regulated by PEA3 would be desirable. As a first step in identifying PEA3 target genes, cell lines were generated in which *PEA3* overexpression is inducible. An inducible system based on the activity of a mutant form of the *Escherichia coli lac* repressor protein has been used (Baim et al., 1991). The components of this system, used in the generation of *PEA3*-inducible cell lines are shown in Figure 3.1A. The effector molecule, *lacI* activator protein (LAP) 267 is a fusion of the *lac* repressor and the herpes simplex virus VP16 transcriptional activator, in which the VP16 transactivation domain has been inserted between amino acid residues 267 and 268 of the *lac* repressor protein.

Figure 3.1 Structures of the Plasmids Used in the Creation of *PEA3*-Inducible and Control Cell Lines.

The structures of all plasmids used in the construction of RP or LAP cell lines. pHβALAP267 encodes LAP267 under the control of the human β-actin promoter, allowing high expression of LAP267 in the presence of serum. pL1-3neo encodes the neomycin G418-resistance gene under the control of an enhancerless SV40 early promoter and 4 *lac* operators, allowing *neo* expression inducible by LAP267. pL7PEA3 encodes *PEA3* under the control of the enhancerless SV40 early promoter and 7 *lac* operators, allowing inducible expression of *PEA3*. pL7CAT encodes the CAT reporter gene under the control of the enhancerless SV40 early promoter and 7 *lac* operators, allowing inducible expression of CAT. pPyORI4xPEA3 encodes the CAT reporter gene under the control of the polyomavirus origin of replication and 4 PEA3 binding sites, allowing activation of CAT expression by PEA3. (B) Induction of gene expression by LAP267 in RP cell lines. RP cell lines have been stably cotransfected with pHβALAP267, pL1-3neo, pL7PEA3 and pPyORI4xPEA3. Upon activation of LAP267, expression of *neo* and *PEA3* is induced through the *lac* operator sequences upstream of these genes. *CAT* expression is then induced by PEA3, allowing easy detection of PEA3 activity. (C) Induction of gene expression by LAP267 in LAP cell lines. LAP cell lines have been stably cotransfected with pHβALAP267 and pL1-3neo. Upon activation of LAP267 expression of *neo* is induced through the *lac* operator sequences upstream of this gene. Transfection of LAP cells with pL7CAT allows rapid detection of inducible LAP267 activity.

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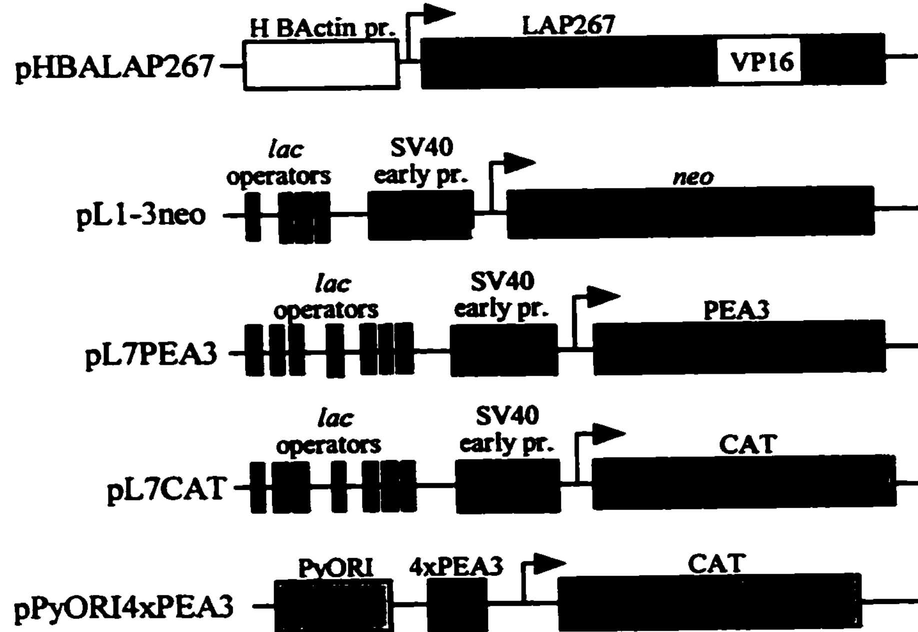

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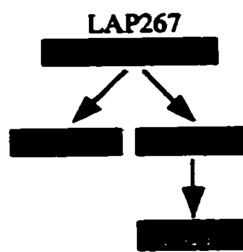

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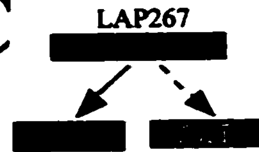


This results in the conversion of the *lac* repressor from a transcriptional repressor to an activator. LAP267 is a temperature sensitive protein, active at 32^{0}C but not at 39.5^{0}C. However, the activity of LAP267 can be rescued at 39.5^{0}C by the presence of isopropyl β-D-thiogalactopyranoside (IPTG). Thus, LAP267 can be activated both by decreasing the temperature at which cells expressing it are grown to 32^{0}C, or addition of IPTG to the cell culture medium. The plasmid pHβALAP267 encodes LAP267 under the control of the human β-actin promoter, allowing high expression of LAP267 in the presence of serum. As no mammalian homologue of the *lac* repressor exists, it is unlikely that LAP267 can activate transcription of endogenous genes in mammalian cells.

LAP267 retains the DNA-binding activity of the wild-type *lac* repressor, and is able to bind *lac* operator sequences and activate transcription of genes under the control of promoters containing these sequences. Therefore, the plasmid pL7PEA3 was constructed, in which the mouse *PEA3* cDNA was cloned downstream of an enhancerless SV40 early promoter, in turn downstream of seven *lac* operator sequences. This construct allows *PEA3* transcription to be induced by active LAP267. The plasmid pL1-3neo was also used in the construction of *PEA3*-inducible cell lines. This plasmid encodes the *neo* G418-resistance gene under the control of four *lac* operator sequences, and allows selection of stable cell lines expressing LAP267 (Labow et al., 1990). Finally, the plasmid pPyORI4xPEA3 encodes the chloramphenicol acetyl transferase (CAT) reporter gene under the control of four PEA3 binding motifs, to allow rapid detection of inducible PEA3 activity. Three cell lines, RP2, RP10 and RP11 cells were generated by stably transfecting Rat-1 cells with pHβALAP267, pL7PEA3, pL1-3neo and

pPyORI4xPEA3 (Figure 3.1B). *PEA3* overexpression can be induced in these cell lines at both the mRNA and protein levels. Furthermore, induction of *PEA3* overexpression coincides with increased levels of expression *MMP* genes, known to be PEA3 targets, in these cells.

Also, control cell lines, LAP1 and LAP6 cells have been generated, by stable transfection of Rat-1 cells with pHβALAP267 and pL1-3neo, which express *LAP267* but do not inducibly overexpress *PEA3* (Figure 3.1C). Inducible LAP267 activity can be detected in these cells by transfection with pL7CAT, a plasmid encoding the CAT reporter gene under the control of seven *lac* operator sequences (Figure 3.1A). These cell lines were generated to ensure that changes in gene expression observed in RP cell lines upon *PEA3* induction are not due to PEA3 independent activity of LAP267.

3.2 Results.

3.2.1 Generation of RP2, RP10 and RP11 Cell Lines.

Rat-1 cells were co-transfected with the plasmids pHβALAP267, pL7PEA3, pL1-3neo and pPyORI4xPEA3, and selected with G418 at 32^{0}C. Fifty G418 resistant cell lines, designated RP1 through RP50, were isolated and grown up. Each cell line was incubated for 48 hours at either 32^{0}C or 39.5^{0}C. Extracts were prepared from cells grown under both conditions and CAT assays performed. Three of the cell lines, RP2, RP10 and RP11, showed CAT activity at 32^{0}C but not at 39.5^{0}C. Further analysis was carried out on these lines.

3.2.2 RP2, RP10 and RP11 Cells Show Inducible CAT Activity.

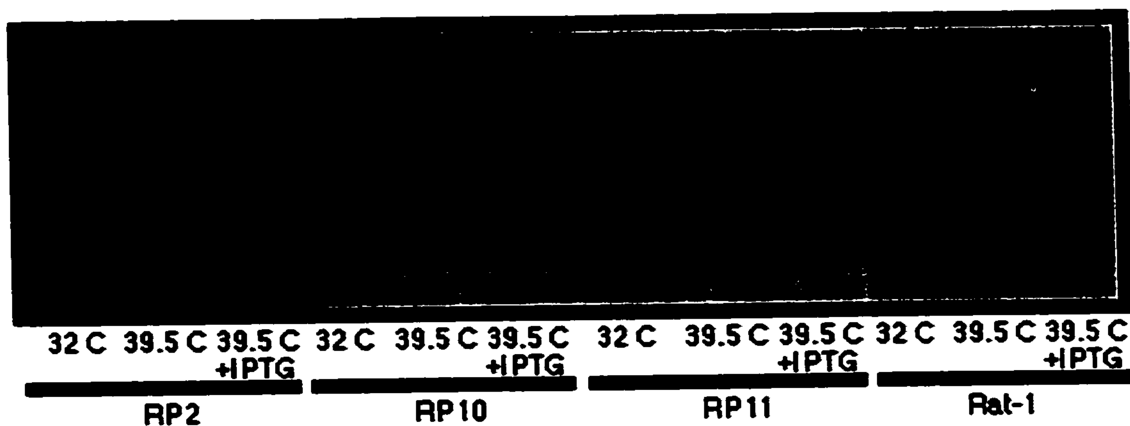
Experiments were carried out to ensure that the CAT activity observed in RP2, RP10 and RP11 cells was induced by both growth at 32^0C and addition of IPTG. The three cell lines were grown for 48 hours at either 32^0C, 39.5^0C or 39.5^0C in the presence of 5 mM IPTG. CAT assays were carried out on extracts prepared from each of the cell lines grown under all three conditions and CAT assays carried out (Figure 3.2A). All three cell lines show low CAT activity at 39.5^0C that is increased by lowering the temperature to 32^0C or the addition of IPTG at 39.5^0C. CAT activity is undetectable in Rat-1 cell grown under the same conditions. Elevated CAT activity is observed under conditions in which LAP267 is expected to be active. These results are consistent with LAP267 inducing *PEA3* expression and PEA3, in turn, activating CAT expression.

The specific CAT activity was determined for extracts from each cell line grown under all three conditions (Figure 3.2B). The specific CAT activity of extracts obtained from RP2 cells grown under all three conditions is approximately four times higher than that of RP10 and RP11 cells grown under the same conditions. However, the fold induction of CAT activity, by lowering temperature or addition of IPTG, is similar for each cell line, with CAT activity induced 19 to 21-fold in RP2 cells, 17 to 18-fold in RP10 cells and 12 to 17-fold in RP11 cells (Figure 3.2C).

Figure 3.2 Inducible CAT Activity of RP2, RP10 and RP11 Cells.

(A) CAT assays carried out on extracts of RP2, RP10, RP11 and Rat-1 cells grown under various conditions. Cells were grown for 48 hours at 32^0C, at 39.5^0C in the absence of IPTG or at 39.5^0C in the presence of 5 mM IPTG. CAT assays were performed on 50 µg of extracts from all cell lines under the various conditions, as indicated. In RP2, RP10 and RP11 cells elevated CAT activity is observed in extracts prepared from cells grown at 32^0C or at 39.5^0C in the presence of IPTG, relative to that observed in extracts prepared from cells grown at 39.5^0C in the absence of IPTG. CAT activity is undetectable in extracts prepared from Rat-1 cells grown under all conditions. (B) Specific CAT activity (%Acetylation/µg extract) of cell extracts. To ensure CAT assays were carried out under quantitative conditions, the experiments in (A) were repeated using 10 µg of extract. The per cent acetylation of chloramphenicol occurring during the CAT assays was quantified using a phosphorimager. CAT assays were carried out on three independently isolated samples under each condition tested. Error bars represent the standard error of the mean determined for these samples. (C) Fold induction of CAT activity by reduction in temperature or addition of IPTG. Fold inductions of CAT activity were determined by setting the specific CAT activity of each cell line at 39.5^0C to 1.

A



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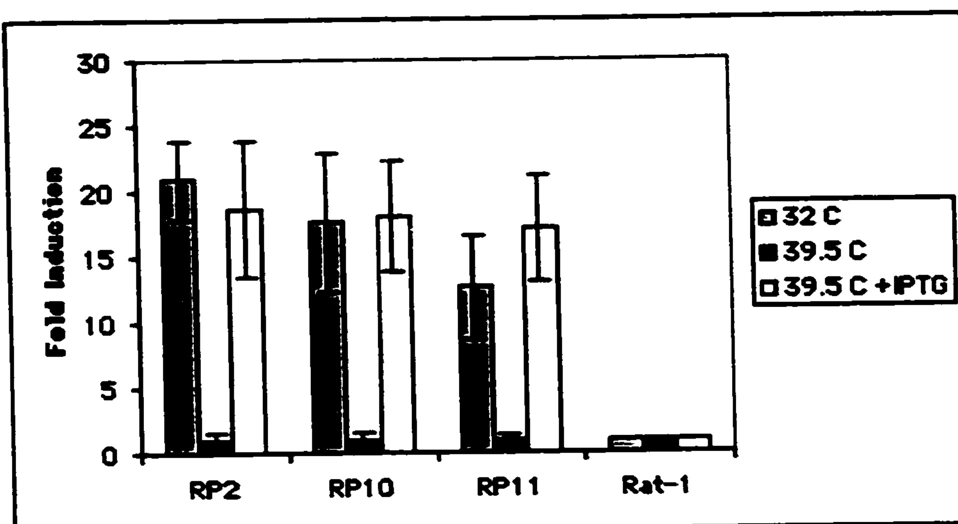

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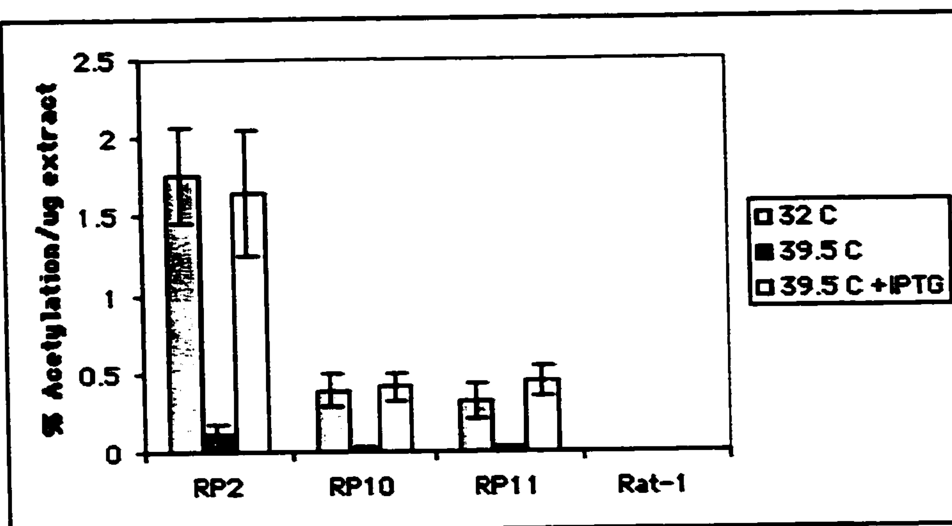


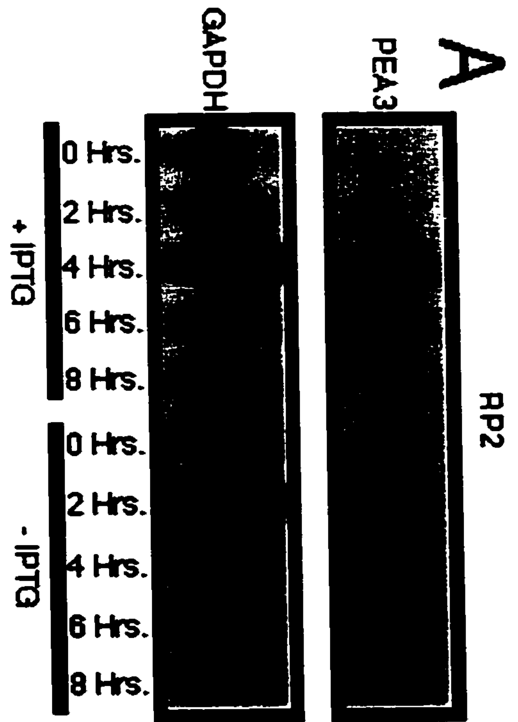
3.2.3 Overexpression of the *PEA3* mRNA Can be Induced in RP2, RP10 and RP11 Cells.

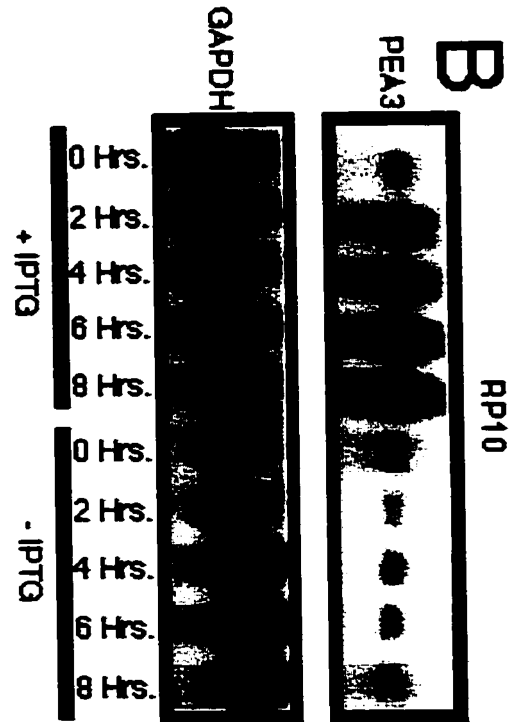
The inducible CAT activities of RP2, RP10 and RP11 cells infers that overexpression of *PEA3* can be induced in these cells. RNase protection experiments were carried out to determine whether levels of *PEA3* mRNA are increased under conditions of elevated CAT activity, and to determine the kinetics with which such a change may occur. RP2, RP10 and RP11 cells were grown for varying lengths of time at 39.5^{0}C in the presence or absence of 5 mM IPTG. RNA was isolated from these cells and analyzed for expression of PEA3 mRNA. The RNA samples were hybridized with an antisense *PEA3* riboprobe specific for mouse *PEA3*, expected to detect only *PEA3* mRNA expressed from the pL7PEA3 construct. The samples were also hybridized with an antisense rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) riboprobe, as an internal control for RNA loading. In all three cell lines PEA3 mRNA is rapidly induced in the presence of IPTG (Figures 3.3A, 3.3B and 3.3C). In the absence of IPTG, only low background expression of mouse *PEA3* from pL7PEA3 is observed, which remains unchanged at all time points tested.

The levels of *PEA3* and *GAPDH* expression in each cell line at various times after induction with IPTG has been quantified by phosphorimaging. For each cell line *PEA3* mRNA levels were normalized to *GAPDH* levels and the fold elevation of *PEA3* mRNA expression over that of uninduced cells determined (Figures 3.4A, 3.4B and 3.4C). In RP2 cells *PEA3* mRNA levels increase 8 to 10-fold relative to *GAPDH* in the presence of IPTG (Figure 3.4A). In these cells the induction of *PEA3* mRNA appears to be transient, reaching a peak at 4 to 6 hours post-induction and declining after this. In RP10 and RP11

Figure 3.3 Induction of *PEA3* mRNA in RP2, RP10 and RP11 Cells.

RNase protection assays were carried out on 10 μg of RNA isolated from RP2, RP10 or RP11 cell lines after 0 hours, 2 hours, 4 hours, 6 hours or 8 hours or growth at 39.5^{0}C, in the presence or absence of IPTG, a indicated. RNA samples were hybridized with antisense mouse *PEA3* and rat *GAPDH* riboprobes. *GAPDH* serves as an internal control for RNA loading. Protected species of approximately the expected sizes of 323 base pairs for *PEA3* and 95 base pairs for *GAPDH* are observed. In all three cases, *PEA3* mRNA levels become elevated in the presence of IPTG, but not in the absence of IPTG. (A) RNase protection carried out on 10 μg of RP2 RNA preparations. (B) RNase protection carried out on 10 μg of RP10 RNA preparations. (C) RNase protection carried out on 10 μg of RP11 preparations.


A
RP2
PEA3
GAPDH
0 Hrs.
2 Hrs.
4 Hrs.
6 Hrs.
8 Hrs.
0 Hrs.
2 Hrs.
4 Hrs.
6 Hrs.
8 Hrs.
+ IPTG
- IPTG


B
RP10
PEA3
GAPDH
0 Hrs.
2 Hrs.
4 Hrs.
6 Hrs.
8 Hrs.
0 Hrs.
2 Hrs.
4 Hrs.
6 Hrs.
8 Hrs.
+ IPTG
- IPTG

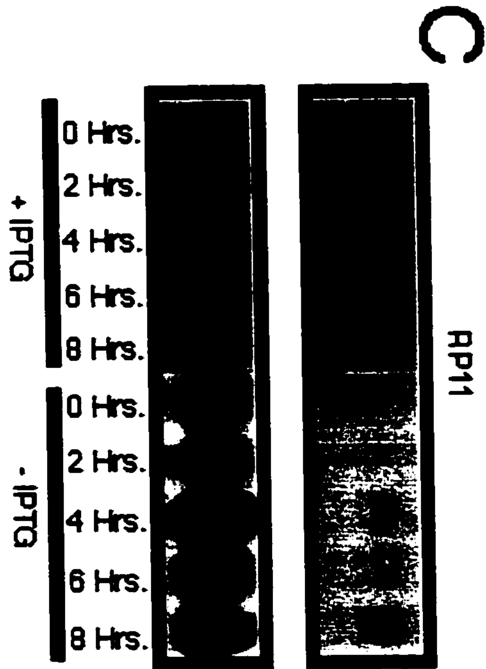
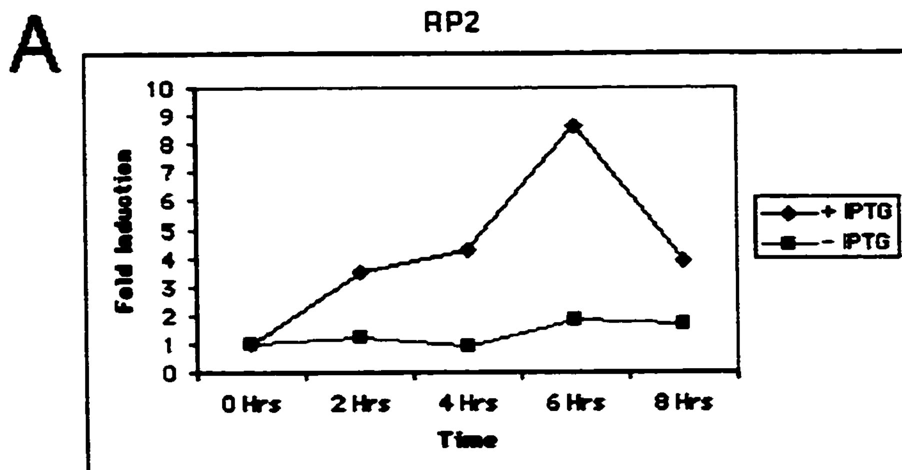

C
RP11
0 Hrs.
2 Hrs.
4 Hrs.
6 Hrs.
8 Hrs.
0 Hrs.
2 Hrs.
4 Hrs.
6 Hrs.
8 Hrs.
+ IPTG
- IPTG

Figure 3.4 Fold Induction of *PEA3* mRNA After Induction with IPTG in RP2, RP10 and RP11 Cell Lines.

PEA3 and *GAPDH* mRNA levels observed in experiments such as those shown in Figure 3.3 were quantified by phosphorimaging. The values obtained for *PEA3* mRNA were normalized to *GAPDH* levels. The fold induction of *PEA3* mRNA, both in the presence and absence of IPTG, was determined by setting the normalized *PEA3* level at 0 hours under both conditions to 1. (A) Fold induction of *PEA3* mRNA in RP2 cells in the presence or absence of IPTG. (B) Fold induction of *PEA3* mRNA in RP10 cells in the presence or absence of IPTG. (C) Fold induction of *PEA3* mRNA in RP11 cells in the presence or absence of IPTG.

A



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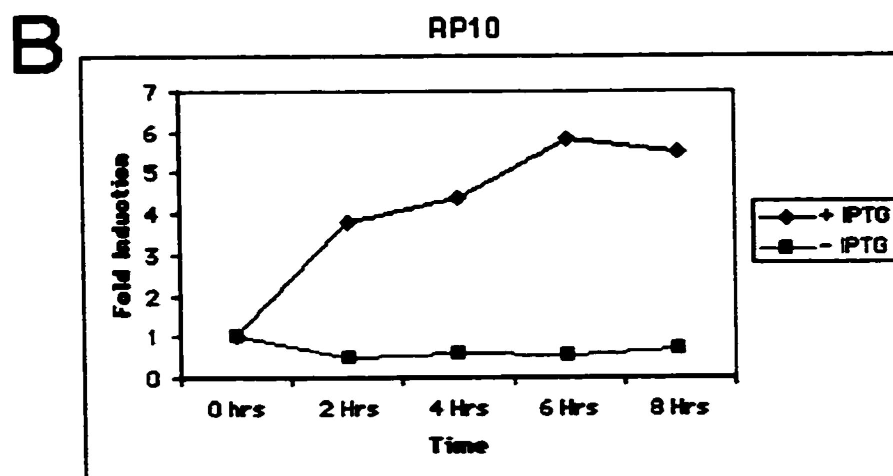

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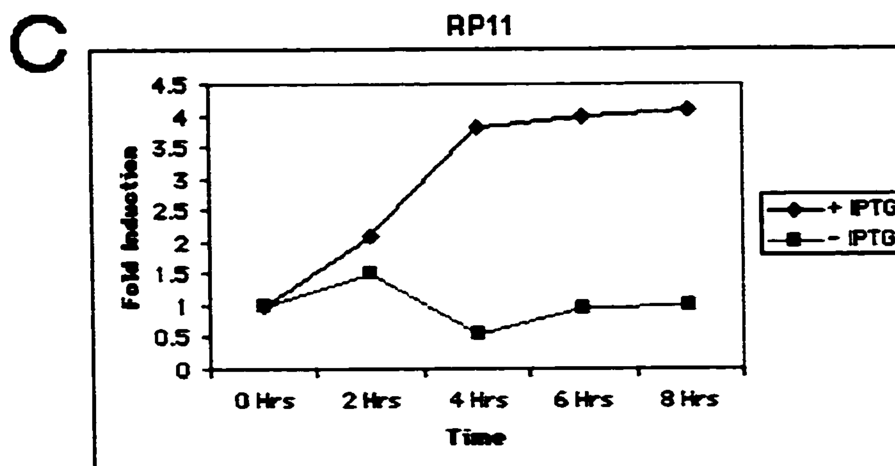


cells *PEA3* mRNA levels reach a maximum at approximately 6 hours post-induction and remain elevated thereafter. In RP10 cells *PEA3* mRNA levels are elevated approximately 5 to 6-fold relative to *GAPDH* mRNA levels (Figure 3.4B), while in RP11 cells this increase is approximately 4-fold (Figure 3.4C). RP10 and RP11 cells show lower background *PEA3* levels than RP2 cells. RP10 cells also show greater inducibility of *PEA3* mRNA when compared to RP11 cells. For these reasons, RP10 cells were selected as the best cell lines for the purpose of identifying PEA3 target genes. The majority of work performed toward this end has been carried out using these cells.

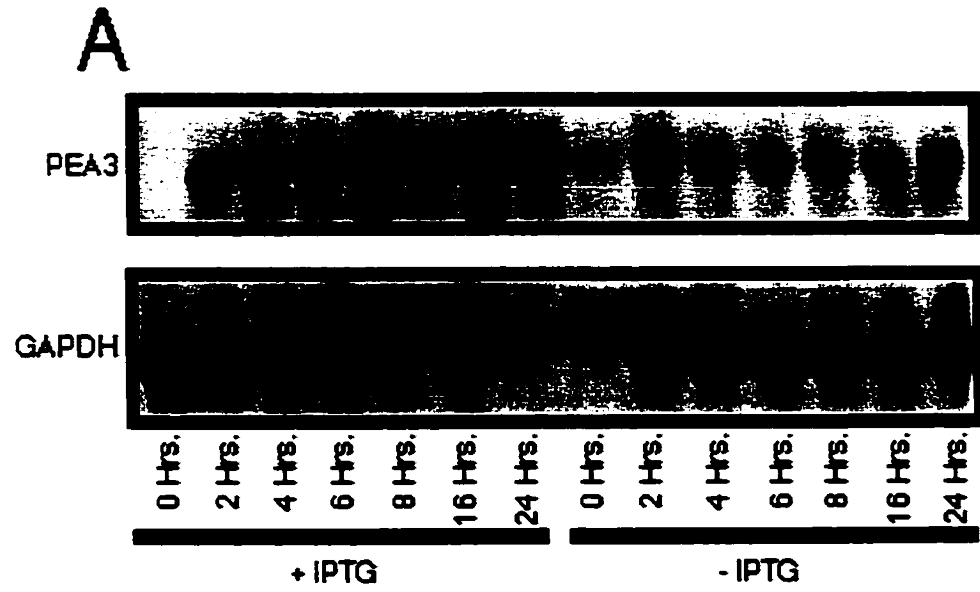
RNase protection experiments were carried out to determine how long *PEA3* mRNA levels remain elevated in RP10 cells after induction with IPTG. RP10 cells were again grown at 39.5^0 C in the presence and absence of IPTG for varying lengths of time, and RNA was prepared from these cells. RNase protection analysis shows that *PEA3* mRNA levels remain elevated in these cells up to 24 hours after the addition of IPTG (Figure 3.5B). Levels of expression of *PEA3* and *GAPDH* mRNA were determined by phosphorimaging. *PEA3* mRNA levels were normalized to *GAPDH* and the fold induction of *PEA3* mRNA relative to uninduced RP10 cells determined (Figure 3.5B). *PEA3* mRNA levels plateau by 6 hours after induction with IPTG and remain elevated for up to 24 hours post-induction. No elevation of *PEA3* mRNA levels occurs at any time in RP10 cells grown in the absence of IPTG.

3.2.4 PEA3 Protein Levels are Increased in RP10 Cells in Response to IPTG.

Western blot experiments were carried out to ensure that an increase in PEA3

Figure 3.5 Elevation of *PEA3* mRNA in RP10 Cells Up to 24 Hours After Induction with IPTG.

(A) RNase protection analysis of RP10 RNA at various times after induction with IPTG. RP10 cells were grown at 39.5^{0}C for varying periods of time in the presence or absence of 5 mM IPTG as indicated. RNase protection assays were carried out on 10 μg of RNA isolated from these cells. The RNA was hybridized to antisense mouse *PEA3* and rat *GAPDH* riboprobes. *GAPDH* serves as an internal control for RNA loading. *PEA3* mRNA levels rapidly become elevated in the presence of IPTG, and remain elevated for up to 24 hours after induction. No increase in *PEA3* mRNA levels is apparent in the absence of IPTG. (B) Fold induction of *PEA3* mRNA in RP10 cells. Levels of *PEA3* and *GAPDH* mRNA were quantified by phosphorimaging. *PEA3* mRNA levels were normalized to *GAPDH* levels. The fold induction of *PEA3* mRNA, both in the presence and absence of IPTG, was determine by setting the normalized *PEA3* level at 0 hours under both conditions to 1.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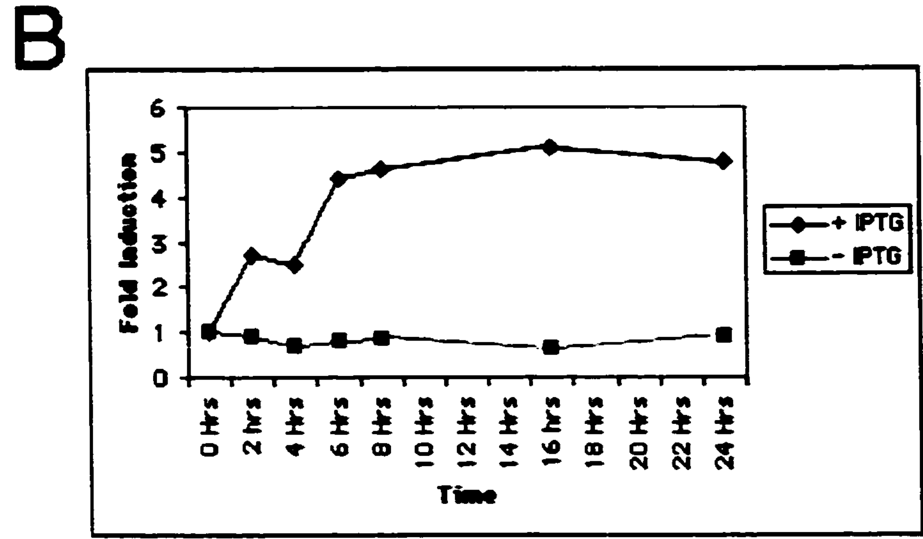
PEA3
GAPDH
0 Hrs.
2 Hrs.
4 Hrs.
6 Hrs.
8 Hrs.
16 Hrs.
24 Hrs.
0 Hrs.
2 Hrs.
4 Hrs.
6 Hrs.
8 Hrs.
16 Hrs.
24 Hrs.
+ IPTG
- IPTG


B
Fold Induction
Time
+ IPTG
- IPTG

protein levels similar to that of its RNA occurs in RP10 cells in the presence of IPTG. RP10 cells were grown at 39.5^{0}C in the presence or absence of IPTG for varying lengths of time. Nuclear extracts were prepared from these cells and western blots carried out using PEA3-specific monoclonal antibodies (Figure 3.6). These antibodies detect a protein in these extracts with the expected apparent molecular mass of 68 kDa, approximately that expected for PEA3. The level of PEA3 protein is elevated in RP10 cells at 39.5^{0}C in the presence of IPTG in a manner similar to that of *PEA3* mRNA. No increase in the level of PEA3 protein is observed in the absence of IPTG. Therefore, *PEA3* is inducible by IPTG at both the protein and mRNA levels in RP10 cells in the expected manner.

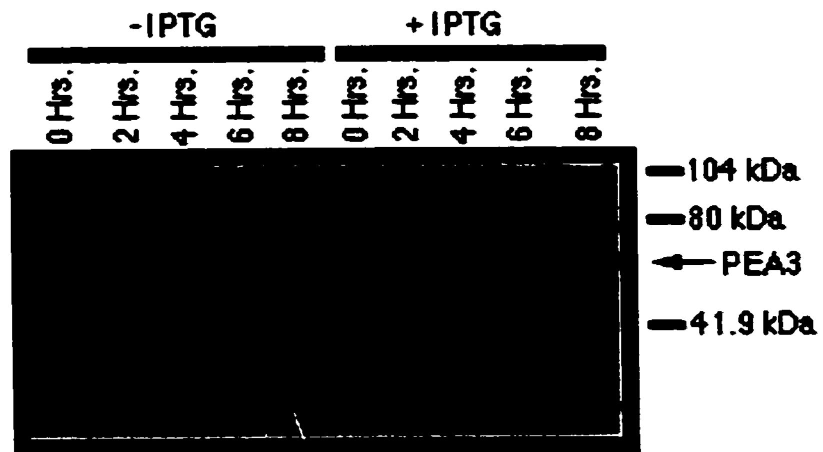
3.2.5 PEA3 Activates Transcription of Known Target Genes in RP10 Cells.

To demonstrate that PEA3 was functional in RP10 cells, the ability of PEA3 to activate transcription of known target genes in these cells has been tested. PEA3 is known to activate transcription of a number of *MMP* genes, including *MMP-9* and *MMP-14* (Higashino et al., 1995; Kaya et al., 1996; Habelhah et al., 1999). The effects of inducing *PEA3* overexpression on the levels of *MMP-9* and *MMP-14* mRNA were tested in RP10 cells. RP10 cells were grown at 39.5^{0}C for varying lengths of time in the presence and absence of IPTG. RNA was isolated from these cells and levels of *MMP-9* and *MMP-14* mRNA determined by northern blot analysis.

Hybridization of the RNA samples with a rat *MMP-9* cDNA probe demonstrated that upon induction with IPTG, levels of *MMP-9* mRNA rapidly become elevated in RP10 cells (Figure 3.7A). No elevation of *MMP-9* mRNA levels is observed in these

Figure 3.6 Induction of PEA3 Protein in RP10 Cells.

RP10 cells were grown for varying lengths of time in the presence of absence of 5 mM IPTG as indicated. Western blots using monoclonal antibodies directed against PEA3 were carried out on 75 μg of nuclear extracts prepared from these cells. As well as numerous background bands, these antibodies detect a band with an apparent molecular mass of approximately 68 kDa, that expected for PEA3 (Xin et. al, 1992). PEA3 protein levels rise in RP10 cells in the presence of IPTG but not in its absence.


-IPTG
+IPTG
0 Hrs.
2 Hrs.
4 Hrs.
6 Hrs.
8 Hrs.
0 Hrs.
2 Hrs.
4 Hrs.
6 Hrs.
8 Hrs.
104 kDa
80 kDa
PEA3
41.9 kDa

cells in the absence of IPTG. As before, RNA samples were hybridized with a rat *GAPDH* cDNA probe to control for RNA loading. *MMP-9* and *GAPDH* mRNA levels were quantified by phosphorimaging. *MMP-9* levels were normalized to *GAPDH* and the fold induction of *MMP-9* mRNA expression over that of uninduced RP10 cells determined (Figure 3.7B). *MMP-9* mRNA levels are increased 3 to 5-fold upon induction of *PEA3* overexpression. Overexpression of *MMP-9* occurs with kinetics closely mimicking those of *PEA3* overexpression, suggesting direct activation of *MMP-9* transcription by PEA3.

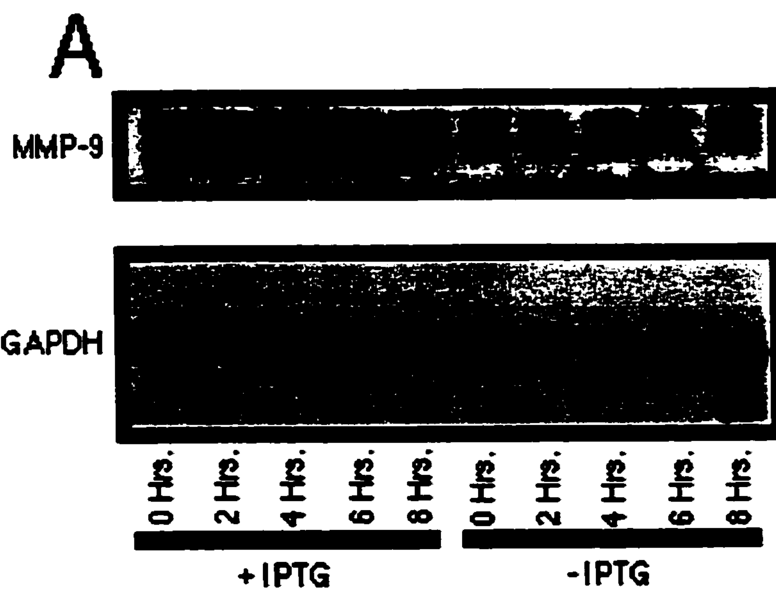
Similar northern blot analyses were carried out, in which RP10 cell RNA samples were hybridized to a rat *MMP-14* cDNA probe (Figure 3.8A). Induction of *PEA3* overexpression with IPTG leads to a modest increase in *MMP-14* mRNA levels, which is not observed in RP10 cells grown in the absence of IPTG. As before, *MMP-14* mRNA levels were normalized to *GAPDH* levels and the fold induction of *MMP-14* mRNA levels over those of uninduced RP10 cells determined (Figure 3.8B). Levels of *MMP-14* mRNA are elevated approximately 2-fold upon expression of *PEA3* overexpression. Like those of *MMP-9*, the kinetics of *MMP-14* overexpression closely matches that of *PEA3*, suggesting transcription of *MMP-14* is directly activated by PEA3.

3.2.6 Generation of LAP1 and LAP6 Cell Lines.

If RP10 cells are to be used to study changes in gene expression that result from *PEA3* overexpression, there must be some means to ensure that observed changes, such as elevated expression of *MMP-9* and *MMP-14*, are not the result of LAP267 directly effecting transcription of these genes. To this end, cell lines were generated that stably

Figure 3.7 Induction of *MMP-9* mRNA in RP10 Cells.

(A) Northern blot analysis showing *MMP-9* expression in RP10 cells at various times after induction with IPTG. RP10 cells were grown at 39.5^{0}C for varying lengths of time in the presence or absence of 5 mM IPTG as indicated. Northern blots were carried out using 30 μg of total RNA isolated from these cells. RNA was hybridized with a rat *MMP-9* cDNA probe, which detects two mRNA species of approximately the expected sizes for *MMP-9*, 3.2 kb and 2.5 kb (Masure et al., 1993). Blots were stripped and hybridized with a rat *GAPDH* cDNA probe as an internal control. *MMP-9* levels rise relative to *GAPDH* levels upon induction of RP10 cells with IPTG. This does not occur in RP10 cells in the absence of IPTG. (B) Fold induction of *MMP-9* mRNA in RP10 cells. *MMP-9* and *GAPDH* mRNA levels were quantified by phosphorimaging. *MMP-9* mRNA levels were normalized to *GAPDH* levels. The fold induction of *MMP-9* mRNA, both in the presence and absence of IPTG, was determined by setting the normalized *MMP-9* level at 0 hours under both conditions to 1.


A
MMP-9
GAPDH
0 Hrs.
2 Hrs.
4 Hrs.
6 Hrs.
8 Hrs.
0 Hrs.
2 Hrs.
4 Hrs.
6 Hrs.
8 Hrs.
+IPTG
-IPTG

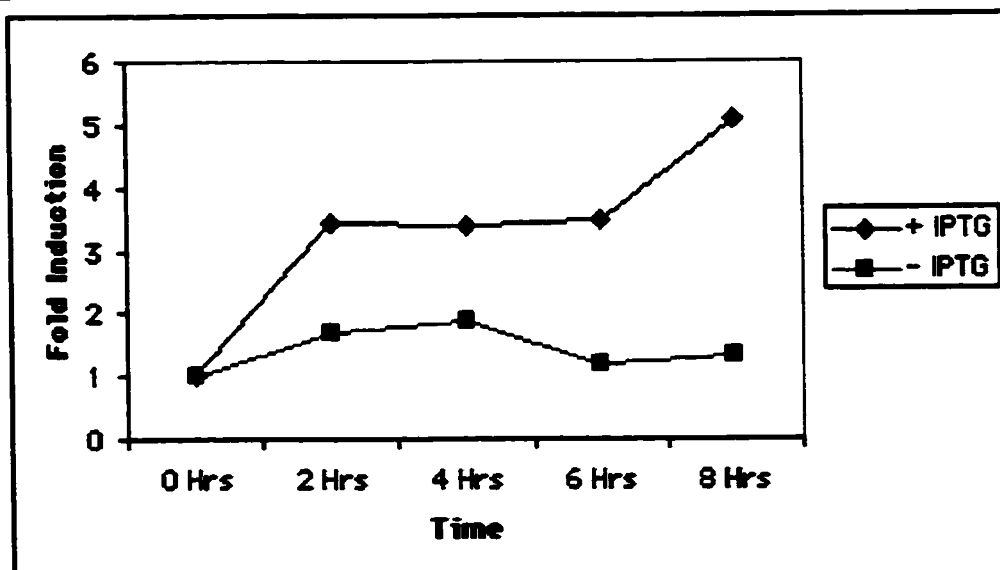
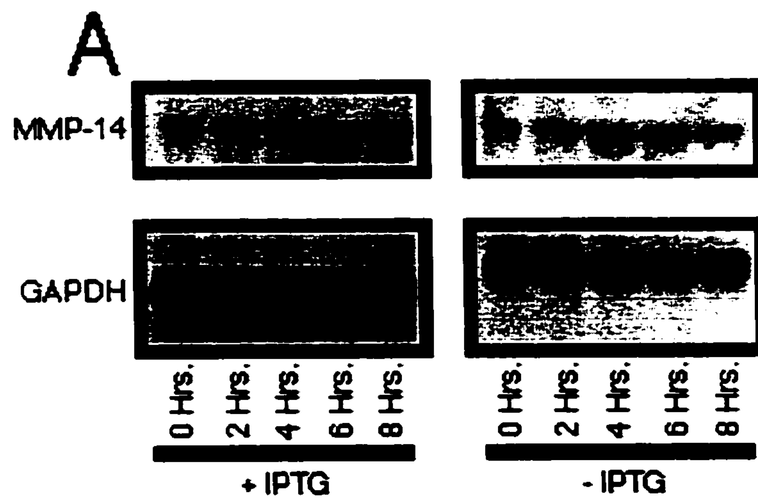
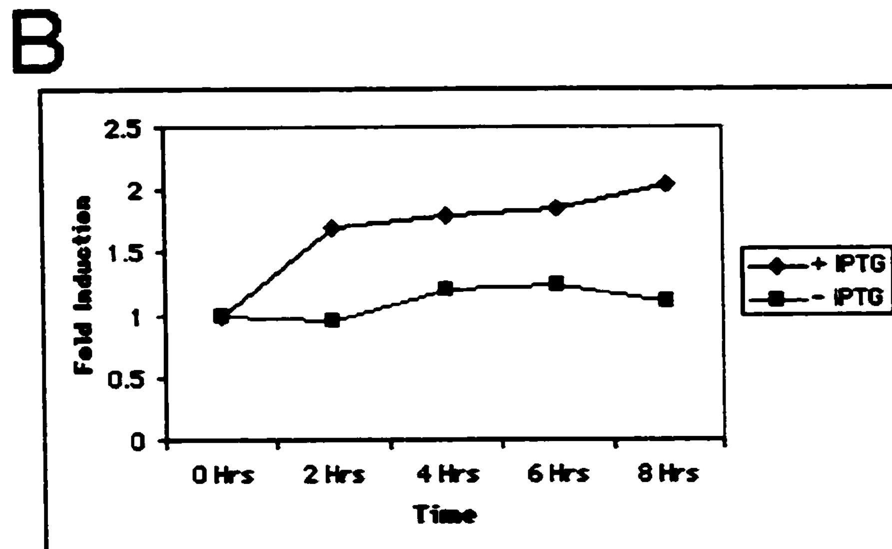

B
Fold Induction
6
5
4
3
2
1
0
0 Hrs
2 Hrs
4 Hrs
6 Hrs
8 Hrs
Time
+ IPTG
- IPTG

Figure 3.8 Induction of *MMP-14* mRNA in RP10 Cells.

(A) Northern blot analysis showing *MMP-14* expression in RP10 cells at various times after induction with IPTG. RP10 cells were grown at 39.5^{0}C for varying lengths of time in the presence or absence of 5 mM IPTG as indicated. Northern blots were carried out using 30 μg of total RNA isolated from these cells. RNA was hybridized with a rat *MMP-14* cDNA probe, which detects a mRNA species of approximately the expected size for *MMP-14*, 4.5 kb (Sato et al., 1994). Blots were stripped and hybridized with a rat *GAPDH* cDNA probe as an internal control. *MMP-14* levels rise relative to *GAPDH* levels upon induction of RP10 cells with IPTG. This does not occur in RP10 cells in the absence of IPTG. (B) Fold induction of *MMP-14* mRNA in RP10 cells. *MMP-14* and *GAPDH* mRNA levels were quantified by phosphorimaging. *MMP-14* mRNA levels were normalized to *GAPDH* levels. The fold induction of *MMP-14* mRNA, both in the presence and absence of IPTG, was determined by setting the normalized *MMP-14* level at 0 hours under both conditions to 1.


A
MMP-14
GAPDH
0 Hrs.
2 Hrs.
4 Hrs.
6 Hrs.
8 Hrs.
+ IPTG
- IPTG


B
Fold Induction
2.5
2
1.5
1
0.5
0
0 Hrs
2 Hrs
4 Hrs
6 Hrs
8 Hrs
Time
+ IPTG
- IPTG

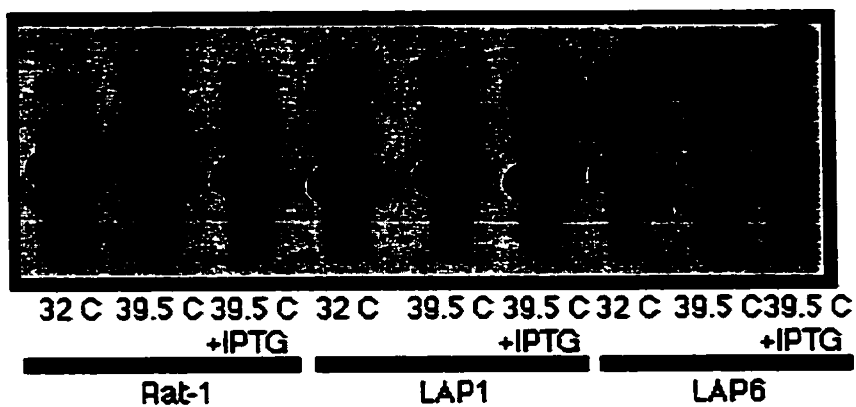
express LAP267, but do not inducibly overexpress *PEA3*. Rat-1 cells were co-transfected with pHβALAP267 and pL1-3neo and selected with G418 at 32^{0}C. G418 resistant cell lines generated in this manner were transfected with pL7CAT to detect LAP267 activity. Transfected cells were grown for 48 hours at 32^{0}C, 39.5^{0}C in the absence of IPTG, or at 39.5^{0}C in the presence of 5 mM IPTG. Extracts were prepared from these cells and CAT assays performed. Two cell lines, designated LAP1 and LAP6, showed elevated CAT activity at 32^{0}C and 39.5^{0}C in the presence of IPTG, but not at 39.5^{0}C in the absence of IPTG (Figure 3.9A). Rat-1 cells transfected with pL7CAT show very low CAT activity that is not elevated in response to lower temperature or the presence of IPTG. CAT activity is not detected in LAP1 or LAP6 cells not transfected with pL7CAT (Figure 3.9B). LAP1 and LAP6 cells show the expected behavior of cells that express LAP267. The fold induction of CAT activity by reduction in temperature or addition of IPTG in transfected LAP1 and LAP6 cells relative to uninduced cells has been determined (Figure 3.9C).

RNase protection experiments have been carried out on LAP1, LAP6 and Rat-1 cells to confirm that *PEA3* mRNA cannot be inducibly overexpressed in these cells in response to IPTG. LAP1 and LAP6 cells were grown at 39.5^{0}C in the presence of IPTG for varying lengths of time. RNA was extracted from these cells and hybridized with the antisense mouse *PEA3* riboprobe, and the antisense rat *GAPDH* riboprobe as an internal control. As expected, mouse *PEA3* mRNA could not be detected in either LAP1 or LAP6 cells. No induction of this mRNA occurred in response to IPTG (Figures 3.10A and 3.10B). In this regard LAP1 and LAP6 cells are indistinguishable from Rat-1 cells (Figure 3.10C).

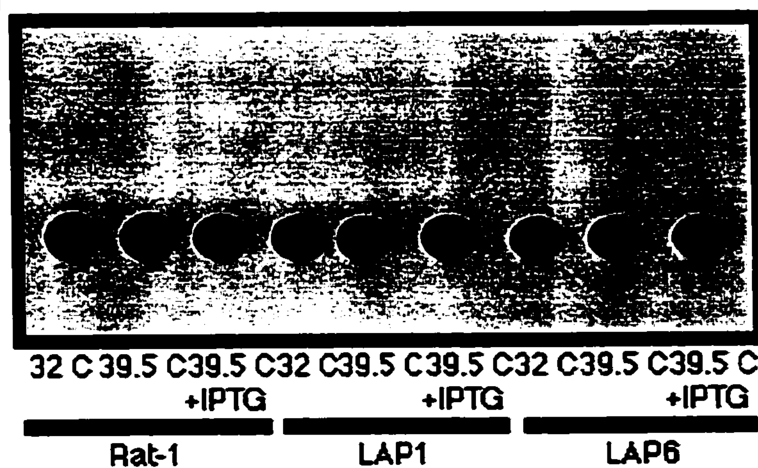
Figure 3.9 Inducible CAT Activity in LAP1 and LAP6 Cell Lines.

(A) CAT assays carried out on extracts of LAP1, LAP6 and Rat-1 cells transfected with pL7CAT. Rat-1, LAP1 and LAP6 cells were transfected with pL7CAT and grown for 48 hours at 32^{0}C or 39.5^{0}C in the absence of IPTG, or at 39.5^{0}C in the presence of 5 mM IPTG. CAT assays were performed on 50 μg of extracts from all cell lines under the various conditions, as indicated. In LAP1 and LAP6 cells, elevated CAT activity is observed in extracts prepared from cells grown at 32^{0}C or at 39.5^{0}C in the presence of IPTG, relative to that observed in extracts prepared from cells grown at 39.5^{0}C in the absence of IPTG. This is not observed in Rat-1 cells. (B) CAT assays carried out on extracts of untransfected Rat-1, LAP1 and LAP6 cells were grown at 32^{0}C or 39.5^{0}C in the absence of IPTG, or at 39.5^{0}C in the presence of 5 mM IPTG as indicated. CAT assays were performed on 50 μg of extracts prepared from these cells. CAT activity was undetectable in all cell lines under all conditions tested. (C) Fold induction of CAT activity in Rat-1, LAP1 and LAP6 cells transfected with pL7CAT. Specific CAT activities (%Acetylation/μg extract) were determined by phosphorimaging. Fold inductions of CAT activity were determined by setting the specific CAT activity of each cell line at 39.5^{0}C to 1. . CAT assays were carried out on three independently isolated samples under each condition tested. Error bars represent the standard error of the mean determined for these samples.

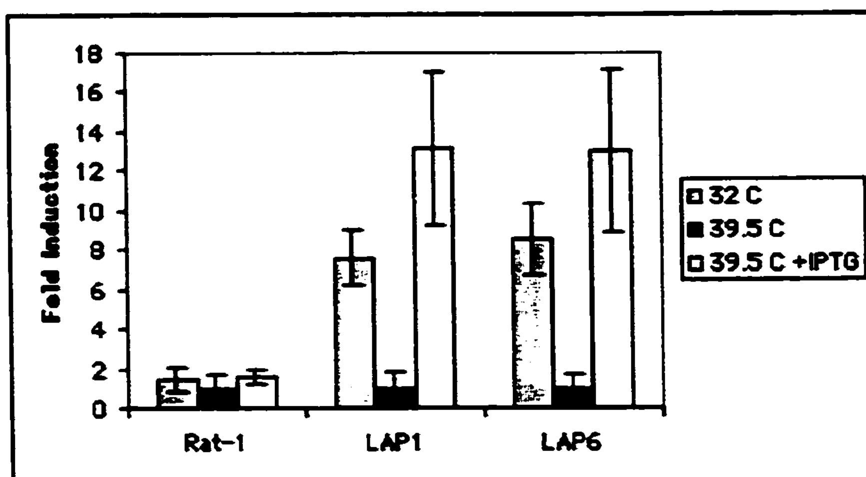
A



B



C



3.2.7 Elevated Expression of *MMP-9* and *MMP-14* Cannot be Detected in LAP6 Cells.

To ensure the elevated expression of *MMP-9* and *MMP-14* observed in RP10 cells (Figures 3.7 and 3.8) are not due to LAP267 activity, northern blots were carried out on RNA isolated from LAP6 cells. LAP6 cells were grown in the presence of IPTG at 39.5^{0}C for varying lengths of time. RNA from these cells was hybridized to a rat *MMP-9* or rat *MMP-14* cDNA probe. A rat *GAPDH* cDNA probe was used as an internal control for RNA loading. *MMP-9* mRNA could not be detected in LAP6 cells at any time after induction with IPTG (Figure 3.11A). *MMP-14* mRNA was detected in LAP6 cells but, unlike in RP10 cells, levels of this mRNA did not change significantly upon addition of IPTG (Figure 3.11B). The fold induction of *MMP-14* mRNA over uninduced LAP6 cells is shown in figure 3.11C. As LAP267 cannot directly activate transcription of *MMP-9* or *MMP-14*, activation of transcription of these genes in RP10 cells most likely carried out by PEA3.

3.3 Discussion

Cell lines that can inducibly overexpress *PEA3* have been generated to be used in the identification of genes whose expression is regulated be PEA3. RP2, RP10, and RP11 cells show inducible expression of both *PEA3* mRNA and a CAT reporter under the control of four PEA3 binding sites. Background levels of *PEA3* expression are lower in RP10 and RP11 cells than in RP2 cells. This is important because the technique chosen to detect changes in gene expression, differential display reverse transcription PCR (DDRT-PCR) detects changes in the expression of any given gene most effectively

Figure 3.10 Mouse *PEA3* mRNA in Not Detectable in Rat-1, LAP1 or LAP6 Cells. RNase protection assays were carried out on 10 µg of RNA isolated from Rat-1, LAP1 and LAP6 cell lines after 0 hours, 2 hours, 4 hours, 6 hours and 8 hours of growth at 39.5°C as indicated. RNA samples were hybridized with antisense mouse *PEA3* and rat *GAPDH* riboprobes. *GAPDH* serves as an internal control for RNA loading. Mouse *PEA3* was not detected in any of the three cell lines under any conditions. (A) RNase protection carried out on 10 µg of Rat-1 RNA preparations. (B) RNase protection carried out on 10 µg of LAP1 RNA preparations. (C) RNase protection carried out on 10 µg of LAP6 RNA preparations.

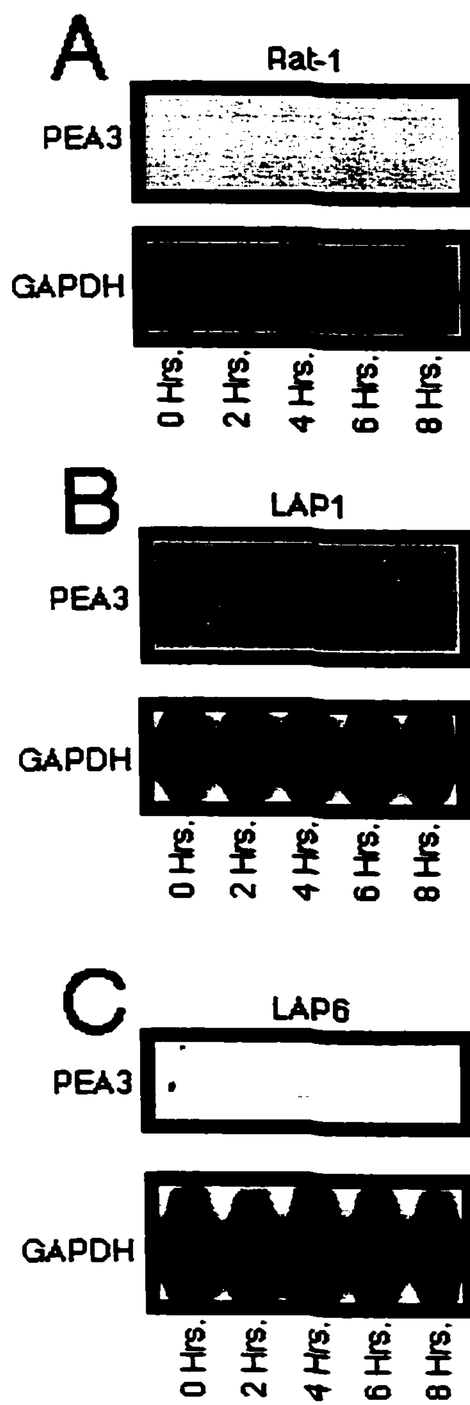
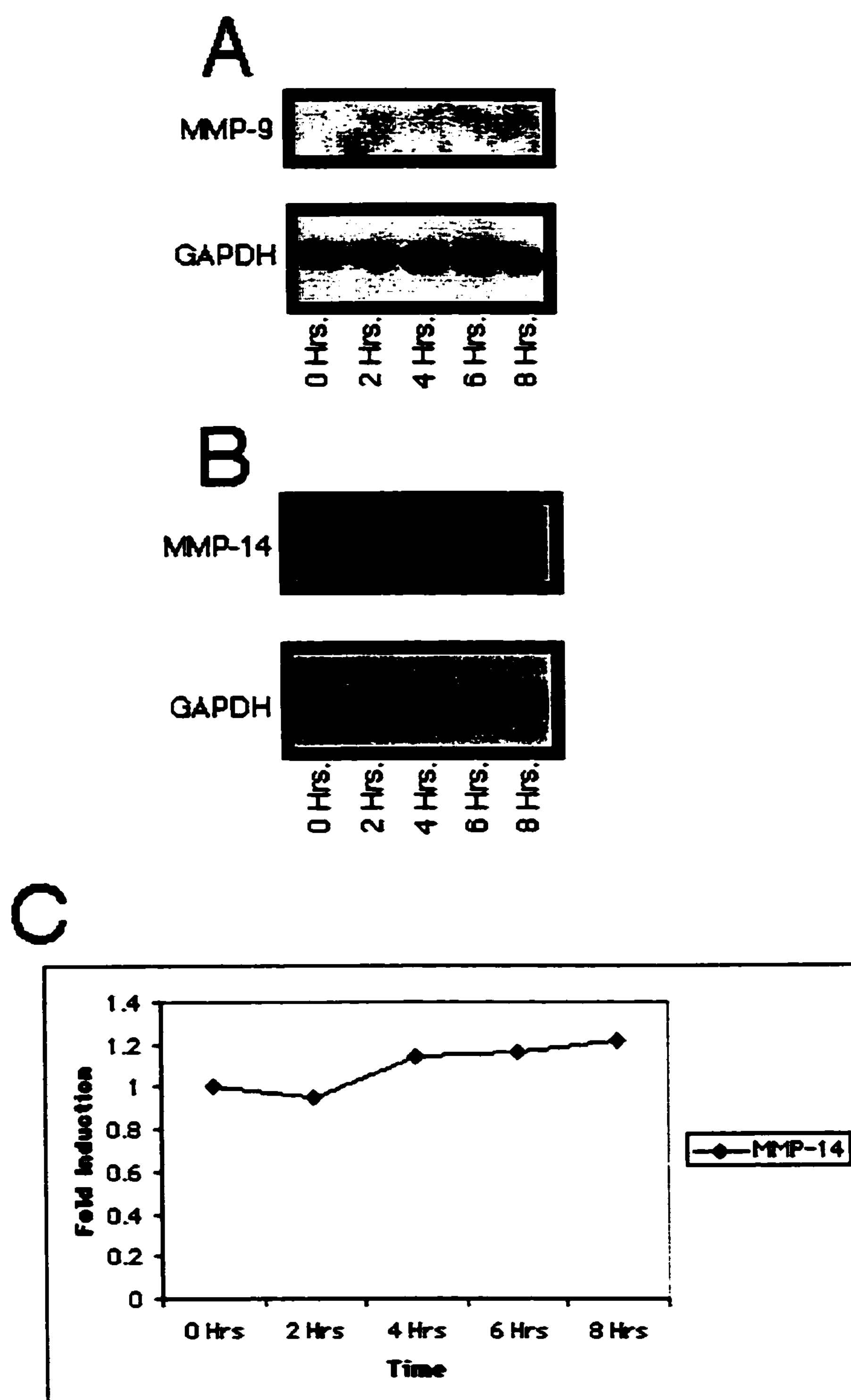

A
Rat-1
PEA3
GAPDH
0 Hrs.
2 Hrs.
4 Hrs.
6 Hrs.
8 Hrs.
B
LAP1
PEA3
GAPDH
0 Hrs.
2 Hrs.
4 Hrs.
6 Hrs.
8 Hrs.
C
LAP6
PEA3
GAPDH
0 Hrs.
2 Hrs.
4 Hrs.
6 Hrs.
8 Hrs.

Figure 3.11 ***MMP-9*** **and** ***MMP-14*** **mRNA Expression is Not Induced in LAP6 Cells.** LAP6 cells were grown at 39.5^{0}C in the presence of 5 mM IPTG for various times as indicated. Northern blot analysis was carried out on 30 μg of total RNA isolated from these cells. (A) Northern blot analysis showing *MMP-9* mRNA expression in LAP6 cells at various times after induction with IPTG. RNA was hybridized with a rat *MMP-9* cDNA probe. Blots were stripped and hybridized with a rat *GAPDH* cDNA probe as an internal control. *MMP-9* mRNA is undetectable in LAP6 cells. (B) Northern blot analysis showing *MMP-14* expression in LAP6 cells at various times after induction with IPTG. RNA was hybridized with a rat *MMP-14* cDNA probe. Blots were stripped and hybridized with a rat *GAPDH* cDNA probe as an internal control. No change in the level of *MMP-14* was observed. (C) Fold induction of *MMP-14* mRNA in LAP6 cells. *MMP-14* and *GAPDH* mRNA levels were quantified by phosphorimaging. *MMP-14* mRNA levels were normalized to *GAPDH* levels. Fold inductions of *MMP-14* mRNA were determined by setting the normalized *MMP-14* level at 0 hours to 1.


A
MMP-9
GAPDH
0 Hrs.
2 Hrs.
4 Hrs.
6 Hrs.
8 Hrs.
B
MMP-14
GAPDH
0 Hrs.
2 Hrs.
4 Hrs.
6 Hrs.
8 Hrs.
C
Fold Induction
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 Hrs
2 Hrs
4 Hrs
6 Hrs
8 Hrs
Time
MMP-14

when the background levels of this gene (in this case, before *PEA3* induction) are low (Guimaraes et al., 1995). Therefore, it is preferable that levels of PEA3 target gene expression be as low as possible before *PEA3* induction. Because of this, PEA3 levels should also be as low as possible before induction. Since RP10 cells also show slightly better inducibility of *PEA3* than RP11 cells, this cell line was chosen as the best for identifying PEA3 target genes.

The expression of *MMP-9* and *MMP-14* increases upon induction of *PEA3* overexpression in RP10 cells. The kinetics of induction of *MMP-9* and *MMP-14* match that of *PEA3* overexpression. These genes have previously been reported to be targets of PEA3 (Higashino et al., 1995; Habelhah et al., 1999). PEA3 is able to drive expression of reporter genes cloned downstream of the *MMP-9* promoter, and transfection of the MCF-7 breast cancer cell line with *PEA3* causes an increase in *MMP-9* expression (Higashino et al., 1995; Kaya et al., 1996). Similarly, transfection of fibrosarcoma-derived cell lines with *PEA3* leads to elevation of *MMP-14* expression (Habelhah et al., 1999). Expression of *MMP-7* is also elevated in response to *PEA3* induction in RP10 cells (Xin and Hassell, unpublished data). Again, this is consistent with the results of others that suggest *MMP-7* is a PEA3 target gene (H. Crawford, personal communication). Like *MMP-9*, PEA3 is able to drive expression of target genes cloned downstream of the *MMP-7* promoter. Since induction of *PEA3* overexpression in RP10 cells causes increased expression of known PEA3 target genes it is likely that these cells can be effective in the detection and identification of novel PEA3 target genes.

The transcription factor LAP267, which induces *PEA3* overexpression in RP10 cells, is a fusion of the *E. coli lac* repressor protein and the HSV VP16 transcriptional

activator. As this protein retains the DNA-binding activity of the *lac* repressor, a protein that has no mammalian homologue, it not expected to activate transcription of endogenous genes in rat cells. However, it is possible that cryptic sites may exist in the promoters of certain genes that LAP267 is able to bind and from which it can activate transcription. The effect of this in RP10 cells would be inducible activation of transcription of these genes, causing them to appear to be PEA3 targets. Therefore, LAP1 and LAP6 cells were created, which express LAP267 but do not inducibly overexpress *PEA3*. These cells were used to ensure that changes in gene expression in RP10 cells are due to *PEA3* overexpression and not the direct activity of LAP267. No change in the expression of *MMP-9* or *MMP-14* is observed in LAP6 cells upon induction of LAP267 activity, demonstrating that this cell line is appropriate for use in this fashion.

Mouse embryo fibroblast (MEF) cell lines have been derived in our lab from both wild type and *PEA3*-null mice (Hastings and Hassell, unpublished data). It is possible that PEA3 target genes could be detected by comparing the pattern of gene expression in wild type and *PEA3*-null cells. However, these cell lines have undergone immortalization, a process during which changes in gene expression occur that may differ from one cell line to the next. No ready means exists to distinguish these differences from differences in gene expression resulting from *PEA3* expression. In RP10 cells, *PEA3* expression can be changed specifically, and resulting changes in gene expression must occur as a result of this change. Furthermore, PEA3 may activate transcription of genes encoding other transcription factors. Changes in gene expression between *PEA3*-null and wild type cell lines may occur as a result of differing expression of these

transcription factors. Again, no ready means of distinguishing between these changes and those that are a direct result of *PEA3* expression exists. If RP10 cells are used, the kinetics with which changes in gene expression occur can be monitored and compared to those of *PEA3* overexpression. Expression of direct targets of PEA3 should be induced with kinetics closely matching those of *PEA3* overexpression as was observed for *MMP-9* and *MMP-14* induction.

In spite of these advantages in the use of RP10 cells, there are potential problems with the use of these cell lines for the identification of PEA3 target genes. Ets proteins generally act synergistically with other transcription factors, such as AP-1, to activate transcription of target genes. Often Ets proteins are incapable of activating transcription of certain targets in the absence of these factors (section 1.6.1; Graves and Pedersen, 1998; Wasylyk et al, 1998). For example, PEA3 synergizes with the transcription factors of the Tcf-Lef family and c-Jun to activate transcription from the *MMP-7* promoter (H. Crawford, personal communication). The activity of PEA3 on this promoter is quite weak in the absence of these proteins. In RP10 cells, the ability of PEA3 to activate transcription of target genes is dependent upon expression of its partner proteins. If these proteins are not expressed in these cells PEA3 may be able to only weakly activate transcription of certain targets, or not be able to activate transcription of some targets at all. Also, expression levels of partner proteins for PEA3 may be limiting for the induction of PEA3 target genes when *PEA3* is overexpressed in RP10 cells. Induction of expression of certain PEA3 target genes in RP10 cells may therefore be weak or not detectable. For example, *MMP-3* has been reported to be a target of PEA3 (Higashino et al., 1995). PEA3 can drive expression of reporter genes cloned downstream of the *MMP-*

3 promoter. Transfection of *PEA3* expressing squamous cell carcinoma-derived cell lines with antisense *PEA3* results in reduced *MMP-3* expression (Hida et al., 1997). Also, work in this lab has shown that *MMP-3* expression can be induced by epidermal growth factor in serum starved wild type MEF cells, but not in *PEA3*-null MEF cell lines (Xin and Hassell, unpublished data). However, no change in *MMP-3* expression is observed in RP10 cells upon induction of *PEA3* overexpression, possibly due to absence of a necessary partner for PEA3 in these cells (Xin and Hassell, unpublished data).

In summary, cell lines have been generated in which *PEA3* overexpression can be induced. Overexpression of *PEA3* in RP10 cells causes increased expression of certain genes whose expression is known to be regulated by PEA3, suggesting that these cells would be useful for the detection of novel PEA3 target genes. RP10 cells have advantages over other systems that may potentially be used to identify PEA3 target genes as *PEA3* expression can be specifically altered in these cells, and the changes in *PEA3* expression are easily monitored. To this end, gene expression in RP10 cells, before and after induction of *PEA3* overexpression has been compared using DDRT-PCR. The results of this analysis will be discussed in the following chapter.

CHAPTER 4

CLONING AND IDENTIFICATION OF PEA3 TARGET GENES.

4.1 Introduction.

In Chapter 3 the generation and characterization of cell lines in which the Ets family transcription factor PEA3 can be inducibly overexpressed was described. These cell lines were generated for use as a tool for the identification of genes whose expression is regulated by PEA3. This may be done by comparing mRNA populations isolated from cells induced to overexpress *PEA3* with mRNA populations isolated from uninduced cells.

The technique chosen to carry out these comparisons was differential display reverse transcription (DDRT)-PCR (Liang and Pardee, 1992) (Figure 4.1). RNA isolated from cells or tissues can be compared, in this case, RP10 cells induced with IPTG and uninduced RP10 cells. mRNA from each cell type is reverse transcribed using an anchored oligo-dT primer capable of annealing to the poly-A tails of mRNA subpopulations. In this case, the degenerate primers T_{12}MA, T_{12}MC, T_{12}MG and T_{12}MT (M represents A, C or G) were used, each of which will lead to reverse transcription of one quarter of the mRNA in each sample (Liang et al., 1993). PCR is carried out on the resulting cDNA populations using the same primer with which reverse transcription was carried out and a decamer oligonucleotide of arbitrary sequence, leading to the amplification of a subpopulation of cDNA fragments of various sizes. These fragments are electrophoresed on a denaturing polyacrylamide gel. Differences in the resulting banding patterns obtained using RNA from different cells with the same primer set

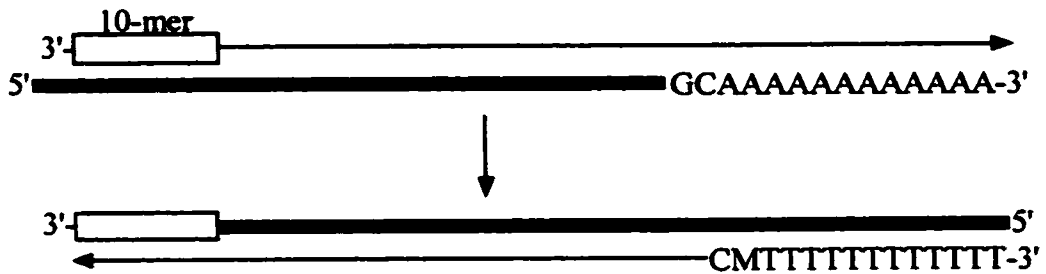
Figure 4.1 The Differential Display Reverse Transcription Polymerase Chain Reaction (DDRT-PCR).

Total RNA is isolated from the cells or tissues to be compared. mRNA subpopulations are reverse transcribed using one of four degenerate primers, $T_{12}MA$, $T_{12}MC$, $T_{12}MG$ or $T_{12}MT$ (M represents A, C, or G). PCR amplification is carried out on the resulting cDNA populations using the same primer used for reverse transcription and a decamer oligonucleotide of arbitrary sequence. PCR is carried out in the presence of a radiolabelled oligonucleotide. The amplified cDNA populations are electrophoresed on a denaturing polyacrylamide gel. Differences in banding patterns arising from the samples being compared represent differences in gene expression between these samples (Liang and Pardee, 1992; Liang et al., 1993). In the experiments to be described, RP10 cells induced to overexpress *PEA3* with IPTG are compared to uninduced RP10 cells.

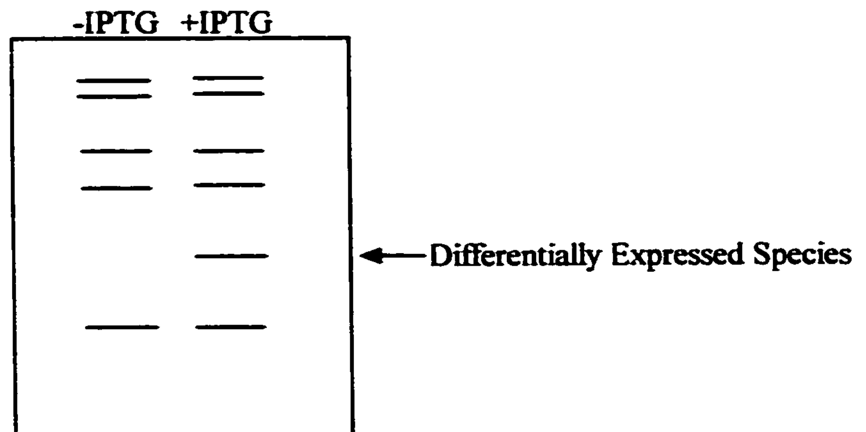
mRNA

Reverse Transcription

PCR



Sequencing Gel



represent differences in the expression levels of various mRNAs between these cells. cDNA bands can be eluted from gels, cloned and sequenced to identify the differentially expressed mRNA species from which they arise.

RNA isolated from uninduced RP10 cells was compared with RNA isolated from RP10 cells induced to overexpress *PEA3* for 24 hours. A number of mRNAs whose expression is altered in response to induction of *PEA3* overexpression were identified and fragments of cDNAs encoding these mRNAs were cloned. Only a small subset of these appear to be direct targets of regulation by PEA3. One of these is the mRNA for the activated leukocyte cell adhesion molecule (ALCAM), a member of the immunoglobulin superfamily of cell adhesion molecules. *ALCAM* mRNA levels increase in RP10 cells in response to *PEA3* overexpression. Furthermore, *ALCAM* expression differs between wild-type MEF cell lines and *PEA3*-null MEF cell lines. Portions of the *ALCAM* promoter have been cloned and sequenced. This promoter contains five potential PEA3-binding sites. In co-transfection experiments, PEA3 can activate transcription of a luciferase reporter gene cloned downstream of this promoter. This evidence suggests that expression of *ALCAM* is activated by PEA3.

4.2. Results.

4.2.1 Comparison of Gene Expression in Induced and Uninduced RP10 Cells by DDRT-PCR.

DDRT-PCR was carried out on RP10 cell RNA to detect mRNA species whose expression level changes in response to *PEA3* overexpression, which can be induced by the addition of IPTG to cells of this type grown at 39.5^{0}C. RNA was isolated from RP10

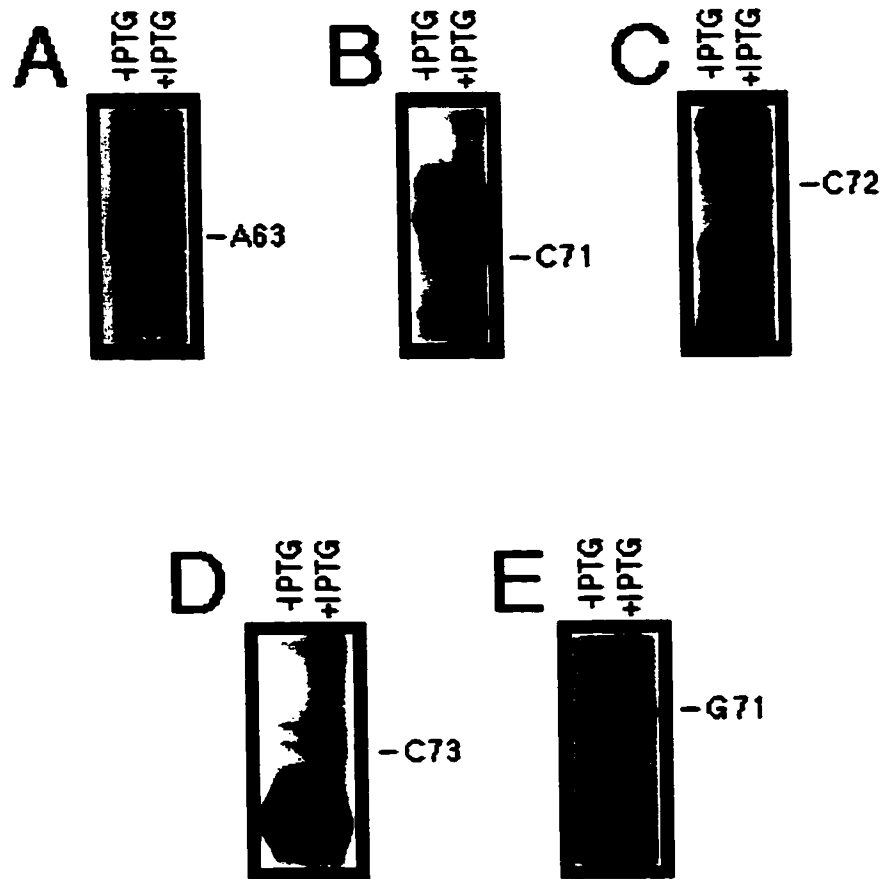
cells that had been grown at 39.5^{0}C for 24 hours, either in the presence or absence of 5mM IPTG. Each RNA sample was reverse transcribed using one of the primers T_{12}MA, T_{12}MC, T_{12}MG or T_{12}MT. PCR amplification was carried out on the resulting cDNA populations using the same primer used for reverse transcription and one of five decamer oligonucleotide primers, AP6, AP7, AP8, AP9 or AP10 (Section 2.16). After electrophoresis of the resulting amplified cDNA populations through a denaturing polyacrylamide gel, a number of bands of differing intensity, representing mRNA species whose expression levels change in response to *PEA3* overexpression, were detected. A total of 33 bands were found for which differences in intensity could be reproducibly observed when DDRT-PCR was carried out on independently isolated RNA preparations. Of these, 21 increase in intensity in response to *PEA3* overexpression, while 11 decrease in intensity. Examples of bands for which changes in expression were observed are shown in Figure 4.2. All 33 of these bands were excised from the gels, and the cDNA fragments eluted and reamplified by PCR using the same primers which gave rise to them by DDRT-PCR. The reamplified cDNA fragments were cloned into the pCR2.1 vector (Invitrogen) and sequenced.

4.2.2 Confirmation of Changes in Gene Expression in RP10 Cells by Reverse Northern Blotting.

As DDRT-PCR is prone to the detection of false positives, changes in gene expression detected in this way must be confirmed by other methods. Also, since inductions of *PEA3* overexpression were carried out for 24 hours in the DDRT-PCR experiments, many of the observed changes in gene expression may not be due directly to

Figure 4.2 Differences in *A63*, *C71*, *C72*, *C73* and *G71* Expression as Observed by DDRT-PCR.

RNA was isolated from RP10 cells grown for 24 hours in the presence or absence of 5 mM IPTG at 39.5^{0}C. Reverse transcription was carried out on 200 ng of each RNA sample using each of the primers T_{12}MA, T_{12}MC, T_{12}MG or T_{12}MT. The resulting subpopulations of cDNAs were PCR amplified using the same primer with which reverse transcription was carried out and one of the decamer oligonucleotides AP6, AP7, AP8, AP9 or AP10. Amplified cDNAs were electrophoresed on a 6% urea/acrylamide gel. A number of bands of differing intensity in the patterns derived from uninduced and induced RP10 cells were observed. (A) Difference in intensity of the band designated *A63*. *A63* intensity is reduced by induction of RP10 cells by IPTG. (B) Difference in intensity of the band designated *C71*. *C71* intensity is increased by induction of RP10 cells with IPTG. (C) Difference in intensity of the band designated *C72*. *C72* intensity is increased by induction of RP10 cells with IPTG. (D) Difference in intensity of the band designated *C73*. *C73* intensity is increased by induction of RP10 cells by IPTG. (E) Difference in intensity of the band designated *G71*. *G71* intensity is increased by induction with IPTG.


A
-IPTG
+IPTG
-A63
B
-IPTG
+IPTG
-C71
C
-IPTG
+IPTG
-C72
D
-IPTG
+IPTG
-C73
E
-IPTG
+IPTG
-G71

PEA3 activity, but may be indirect effects caused by PEA3-regulated expression of other transcription factors. To address these issues reverse northern blot experiments (Zhang et al., 1996) were carried out. RNA was isolated from RP10 cells grown at 39.5^{0}C either in the presence or absence of IPTG for only 8 hours. cDNA probes were generated by reverse transcription of these RNA preparations in the presence of a radiolabelled nucleotide using oligo-dT as a primer. The cDNAs cloned using DDRT-PCR were spotted in duplicate on two filters. Also spotted were the mouse *PEA3* cDNA as a positive control and the rat *GAPDH* cDNA as an internal control. These filters were hybridized with cDNA probes generated from either induced or uninduced RP10 cells. The intensity of hybridization of each cDNA to the two probes was compared by phosphorimaging. Differences in intensity were observed for only five of the 33 cDNAs. These have been designated *A63*, *C71*, *C72*, *C73* and *G71*. Of these *A63* is downregulated in response to *PEA3* overexpression, while the others are upregulated. The results of reverse northern blot experiments showing the changes is expression of *A63*, *C71*, *C72*, *C73* and *G71* are shown in Figure 4.3. The differences in expression as detected by DDRT-PCR are shown in Figure 4.2.

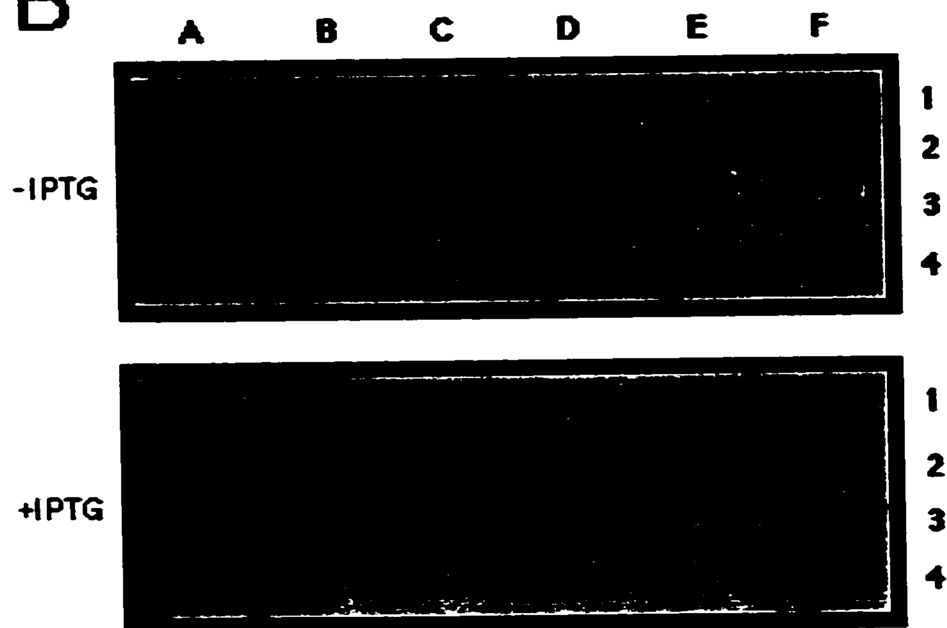
The sequences of these cDNAs were compared with the sequences in the GenBank database. Only *G71* showed homology to anything in this database. *G71* is a 456 base pair cDNA, homologous along its entire length with *MEMD*, a human mRNA, identified by DDRT-PCR, whose expression level has been shown to correlate with metastatic and invasive potential in melanoma-derived cell lines (van Groningen et al., 1995).

Figure 4.3 Reverse Northern Analysis of cDNAs Cloned by DDRT-PCR.

Two nylon membranes were spotted in duplicate with 1 μg of each cDNA to be analyzed. RNA was isolated from RP10 cells grown at 39.5^{0}C, both in the presence and absence of 5 mM IPTG, for 48 hours. cDNA probes were prepared by reverse transcription of these RNA preparations, using oligo-dT as a primer, in the presence of α-[^{32}P]-dCTP. One membrane was hybridized with cDNA probe prepared from uninduced RP10 cells, and the other with cDNA probe prepared from IPTG induced RP10 cells. The intensities of hybridization of each cDNA to both probes was compared by phosphorimaging. The cDNAs spotted in the example shown are listed in panel A. The results of the hybridization are shown in panel B. Hybridizations to *PEA3*, *C71*, *C72*, *C73*, and *G71* are more intense with probe prepared from IPTG induced RP10 cells than with that prepared from uninduced cells. Hybridization with *A63* is less intense with probe prepared from IPTG induced RP10 cells.

A

A	B	C	D	E	F	
A63	C61	C622	C623	C63	C634	1
C644	C66	C71	C72	C73	G72	2
G732	G734	G74	PEA3	A64	G71	3
A61	A62	C66	C67	G74	GAPDH	4



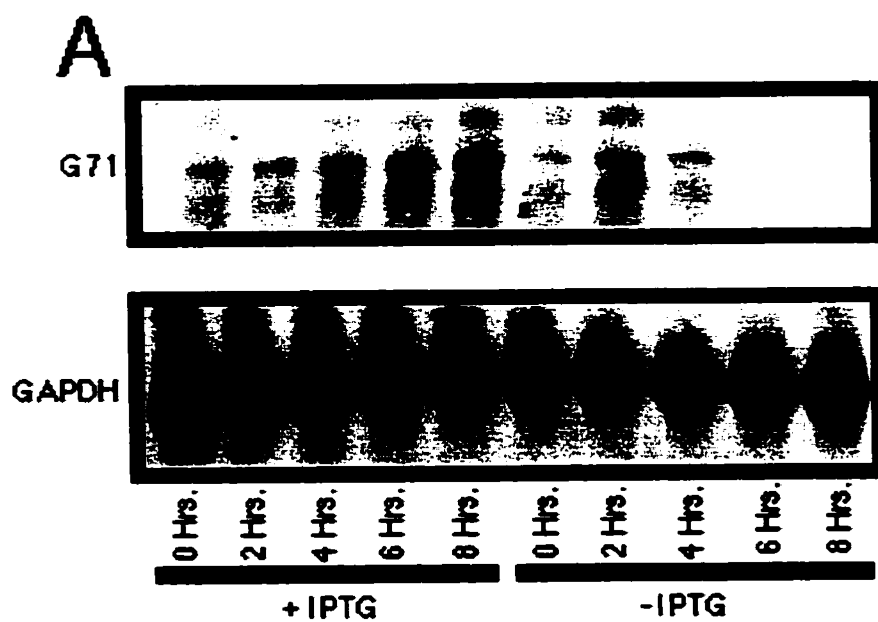
4.2.3 *G71* mRNA Levels are Elevated in RP10 Cells in Response to *PEA3* Overexpression.

RNase protection experiments were carried out to ensure that *G71* levels increase in RP10 cells at times when *PEA3* overexpression occurs. RP10 cells were grown for varying lengths of time at 39.5^{0}C in the presence or absence of 5mM IPTG. RNA was isolated from these cells and analyzed for expression of *G71* mRNA. RNA samples were hybridized with an antisense *G71* riboprobe and an antisense rat *GAPDH* riboprobe as an internal control for RNA loading. Upon induction of RP10 cells with IPTG, *G71* mRNA levels become elevated relative to those of *GAPDH* mRNA (Figure 4.4A). The kinetics of *G71* mRNA induction closely matched those of the *PEA3* mRNA (Figure 3.3B) in these cells, suggesting *G71* expression is directly regulated by PEA3. No change in *G71* mRNA levels is observed in RP10 cells not induced with IPTG. Levels of *G71* and *GAPDH* mRNA were quantified by phosphorimaging. *G71* mRNA levels were normalized to *GAPDH* levels and the fold induction of *G71* mRNA expression over that of uninduced RP10 cells determined (Figure 4.4B). *G71* mRNA levels are elevated 2- to 2.5-fold upon induction of *PEA3* overexpression.

Increased expression of *G71* in RP10 cells was initially detected by DDRT-PCR after 24 hours of induction with IPTG (Figure 4.2E). Therefore, RNase protection experiments were carried out to ensure *G71* levels remain elevated in RP10 cells after at least 24 hours of induction with IPTG. *G71* mRNA levels remain elevated for up to 48 hours after induction with IPTG (Figure 4.5A). A similar 2- to 2.5-fold level of induction of *G71* mRNA is observed over uninduced RP10 cells until this time (Figure 4.5B).

Figure 4.4 Induction of *G71* mRNA Expression in RP10 Cells.

(A) RNase protection analysis of RP10 cell RNA at various times after induction with IPTG. RP10 cells were grown at 39.5^{0}C for varying periods of time in the presence of absence of 5 mM IPTG as indicated. RNase protection assays were carried out on 10 μg of RNA isolated from these cells. The RNA was hybridized with antisense*G71* and rat *GAPDH* riboprobes. *GAPDH* serves as an internal control for RNA loading. Protected species of approximately the expected sizes of 456 base pairs for *G71* and 95 base pairs for *GAPDH* are observed. *G71* mRNA levels become elevated in RP10 cells in the presence of IPTG. No increase in *G71* levels is apparent in RP10 cells in the absence of IPTG. (B) Fold induction of *G71* mRNA in RP10 cells. Levels of *G71* and *GAPDH* mRNA were quantified by phosphorimaging. *G71* mRNA levels were normalized to *GAPDH* levels. The fold induction of *G71* mRNA, both in the presence and absence of IPTG, was determined be setting the normalized *G71* level at 0 hours under both conditions to 1.


A
G71
GAPDH
0 Hrs.
2 Hrs.
4 Hrs.
6 Hrs.
8 Hrs.
0 Hrs.
2 Hrs.
4 Hrs.
6 Hrs.
8 Hrs.
+IPTG
-IPTG

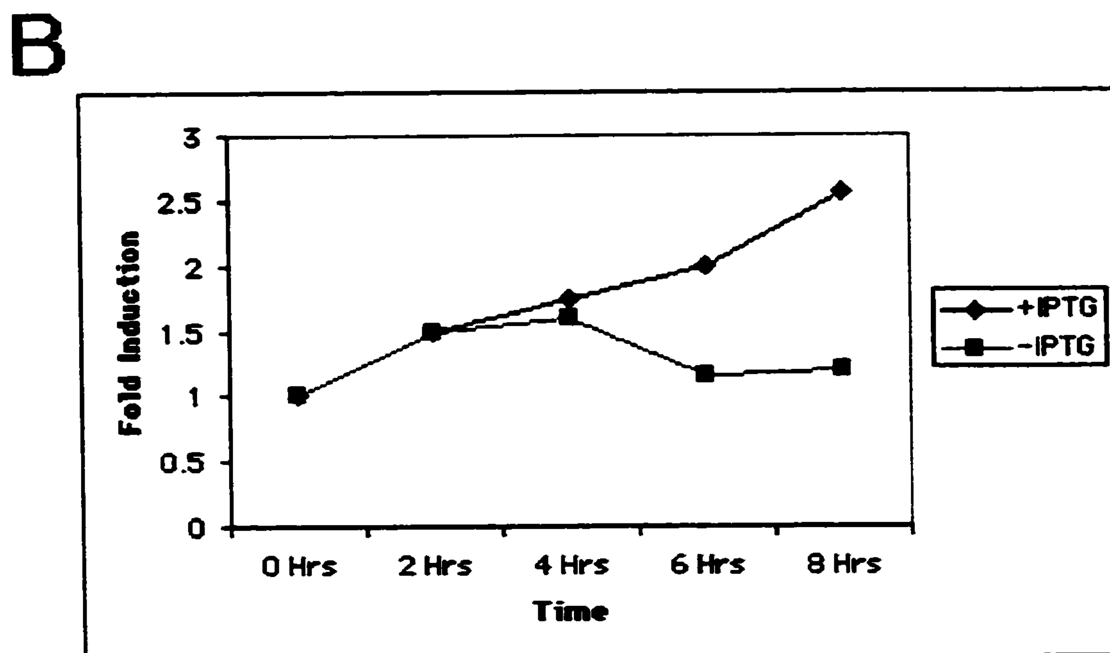
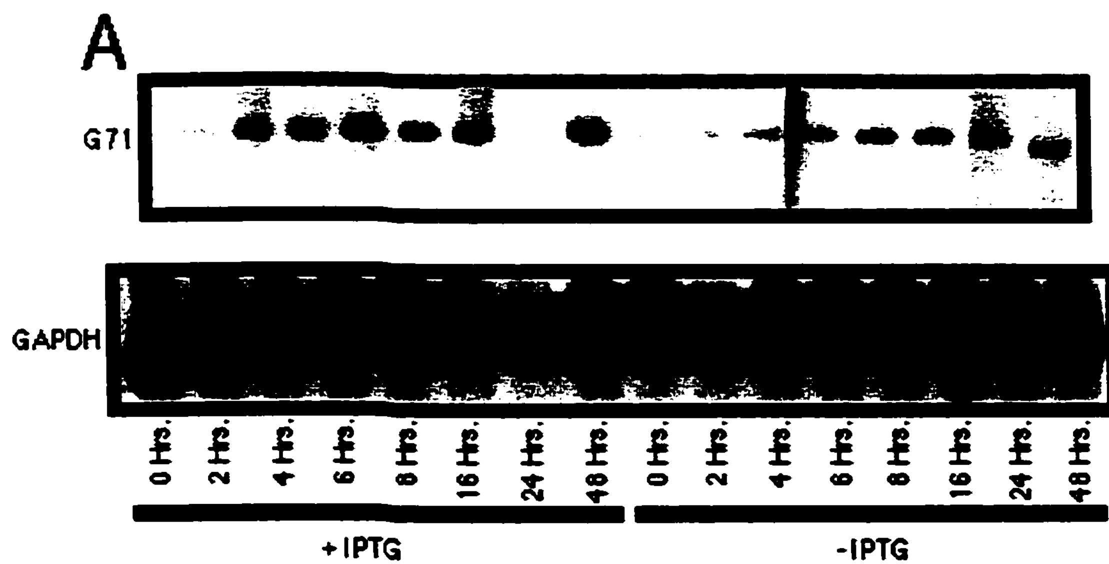
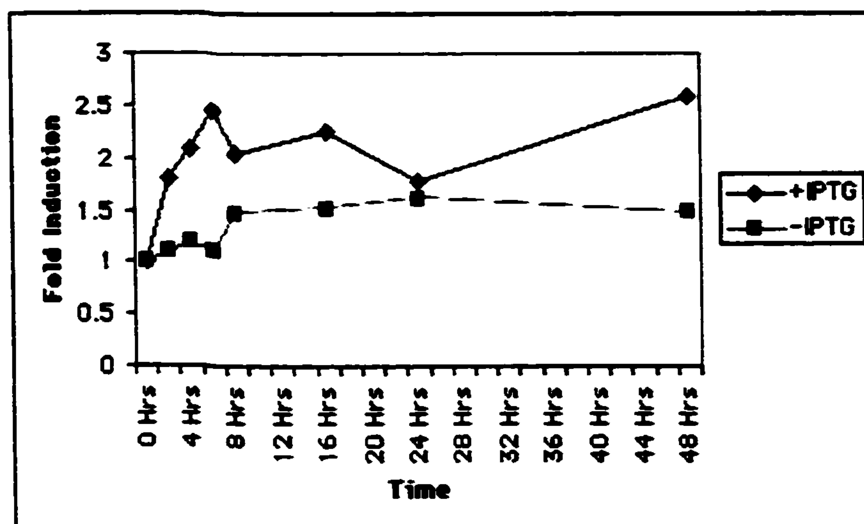

B
Fold Induction
3
2.5
2
1.5
1
0.5
0
0 Hrs
2 Hrs
4 Hrs
6 Hrs
8 Hrs
Time
+IPTG
-IPTG

Figure 4.5 Elevation of *G71* mRNA in RP10 Cells Up to 48 Hours After Induction With IPTG.

(A) RNase protection analysis of RP10 RNA at various times after induction with IPTG. RP10 cells were grown at 39.5^{0}C for varying periods of time in the presence or absence of 5 mM IPTG as indicated. RNase protection assays were carried out on 10 µg of RNA isolated from these cells. The RNA was hybridized to antisense *G71* and *GAPDH* riboprobes. *GAPDH* serves as an internal control for RNA loading. *G71* mRNA levels rapidly become elevated in the presence of IPTG, and remain elevated for up to 24 hours after induction. No significant increase in *G71* mRNA levels is apparent in the absence of IPTG. (B) Fold induction of *G71* mRNA in RP10 cells. Levels of *G71* and *GAPDH* mRNA were quantified by phosphorimaging. *G71* mRNA levels were normalized to *GAPDH* levels. The fold induction of *G71* mRNA, both in the presence and absence of IPTG, was determined by setting the normalized *G71* levels under both conditions to 1.


A
G71
GAPDH
0 Hrs.
2 Hrs.
4 Hrs.
6 Hrs.
8 Hrs.
16 Hrs.
24 Hrs.
48 Hrs.
0 Hrs.
2 Hrs.
4 Hrs.
6 Hrs.
8 Hrs.
16 Hrs.
24 Hrs.
48 Hrs.
+IPTG
-IPTG


B
Fold Induction
3
2.5
2
1.5
1
0.5
0
0 Hrs
4 Hrs
8 Hrs
12 Hrs
16 Hrs
20 Hrs
24 Hrs
28 Hrs
32 Hrs
36 Hrs
40 Hrs
44 Hrs
48 Hrs
Time
+IPTG
-IPTG

Similar RNase protection analysis was carried out using LAP6 cells to ensure that LAP267 is not able to directly activate transcription of *G71* mRNA. LAP6 cells were grown at 39.5^{0}C in the presence of 5 mM IPTG for varying lengths of time. RNA isolated from these cells was hybridized with an antisense *G71* riboprobe as an internal control. *G71* mRNA levels do not change in LAP6 cells upon induction with IPTG (Figure 4.6A). Again, *G71* and *GAPDH* mRNA levels were quantified by phosphorimaging, and *G71* levels normalized to *GAPDH*. This confirmed that *G71* levels do not change significantly relative to *GAPDH* mRNA levels (Figure 4.6B). Therefore, it is most likely that the elevation of *G71* mRNA levels observed in RP10 cells is due to activation of *G71* transcription by PEA3.

4.2.4 Identification of G71 as Activated Leukocyte Cell Adhesion Molecule (ALCAM).

As both the sequences of *G71* and *MEMD*, its human homologue, are derived from the 3'-untranslated regions of the mRNAs, little was known about the identity or function of the protein they encode. Therefore, a cDNA library screen was carried out to obtain a longer *G71* cDNA. The *G71* cDNA obtained from DDRT-PCR was used as a probe to screen a rat brain cDNA library cloned into λGT10 (Clontech). After screening one million phage, a 967 base pair cDNA was isolated that hybridized to the *G71* probe. Upon sequencing of this cDNA, it was discovered that it contained the entire sequence of the *G71* probe at its 3'-end, confirming its identity as a longer *G71* cDNA. Comparison of the sequence of this cDNA with the GenBank database revealed that it contains extensive sequence homology along its entire length with the 3'-untranslated region of the

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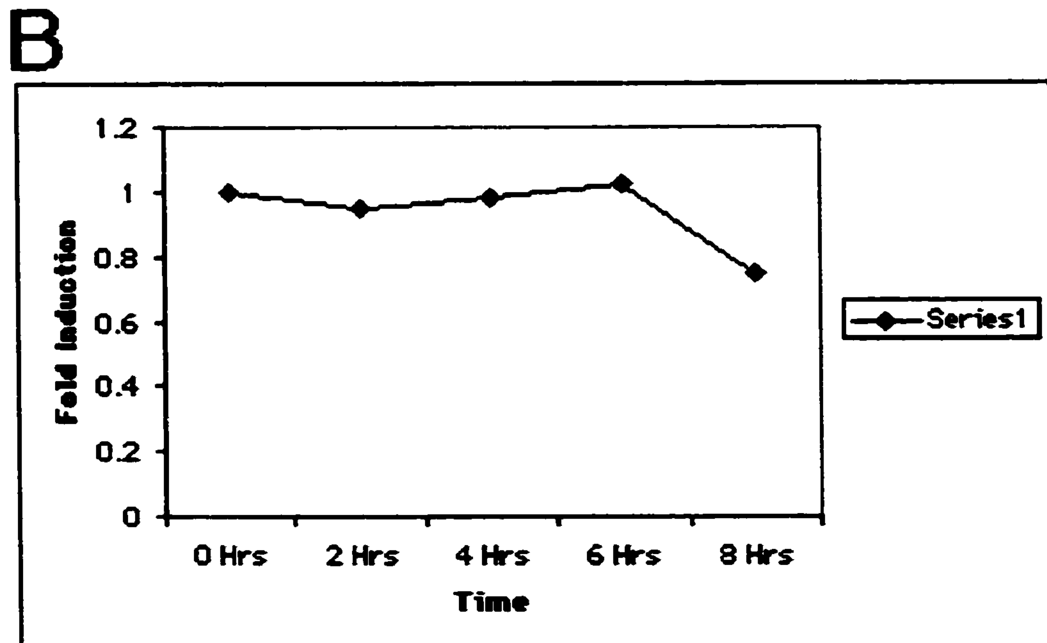
Figure 4.6 Analysis of *G71* mRNA Levels in LAP6 Cells.

(A) RNase protection analysis of LAP6 cell RNA at various times after induction with IPTG. LAP6 cells were grown at 39.5^{0}C for varying periods of time in the presence of 5 mM IPTG as indicated. RNase protection assays were carried out on 10 μg of RNA isolated from these cells. The RNA was hybridized to antisense *G71* and rat *GAPDH* riboprobes. *GAPDH* serves as an internal control for RNA loading. *G71* mRNA levels do not change in LAP6 cells after induction with IPTG. (B) Fold induction of *G71* mRNA in LAP6 cells. Levels of *G71* and *GAPDH* mRNA were quantified by phosphorimaging. *G71* mRNA levels were normalized to *GAPDH* levels. The fold induction of *G71* mRNA was determined by setting the normalized *G71* level at 0 hours of induction with IPTG to 1.

A

B



human mRNA for activated leukocyte cell adhesion molecule (ALCAM) (Bowen et al., 1995) (Figure 4.7). Also, the *MEMD* cDNA has been shown to be identical to the 3'-end of the *ALCAM* mRNA (Degen et al., 1998). The *G71* cDNA is 73% identical to human *ALCAM* at the nucleotide level (Figure 4.7). *G71* is most likely the rat homologue of *ALCAM*.

4.2.5 Induction of *PEA3* Expression by EGF in Mouse Embryo Fibroblast Cell Lines.

Mouse embryo fibroblast (MEF) cell lines have been derived from both wild type and *PEA3*-null mice (Hastings and Hassell, unpublished data) (Section 3.3). It has been demonstrated that stimulation of serum starved wild-type MEF cells with EGF causes increased expression of *MMP-3*, *MMP-7* and *MMP-9*, known PEA3 target genes (Xin and Hassell, unpublished data). This effect of EGF stimulation is not observed in *PEA3*-null MEF cells. RNase protection experiments were carried out to ensure that expression of *PEA3* correlates with increased expression of these *MMPs*.

PEA3-null MEFB10 and MEF1 and wild type MEF4 and MEFD cells were serum starved for 36 hours. These cells were then stimulated with 200 ng/ml EGF for varying lengths of time. RNA was isolated from these cells and hybridized with an antisense mouse *PEA3* riboprobe. This riboprobe is specific for the region of *PEA3* disrupted in the *PEA3*-null cell lines, protecting a 323-nucleotide fragment of the *PEA3* mRNA from RNase digestion in wild-type cells, but not *PEA3*-null cells. RNA samples were also hybridized with an antisense mouse *L32* ribosomal protein riboprobe as an internal control for RNA loading (Figure 4.8A). As expected, *PEA3* mRNA could not be

Figure 4.7 Alignment of Rat *G71* and Human *ALCAM* cDNA Sequences.

The *G71* cDNA sequences were aligned with the 3'-end of the human *ALCAM* cDNA (Degen et al., 1998) using the Lasergene multiple sequence alignment software. Identical nucleotide residues in both sequences are highlighted in red. The *G71* sequence is 73% identical to that of *ALCAM* in this region.

-----CC TACC TG A TA GAAGC TT TA AC G AA - T T Rat
CTCCG TT ATAA GC T CG TGCAT GG GG TT A GC T A A Human

C A C A --- GGGGGGAG A Rat
A C G C TAT AAAAAAGA T Human

TTGATT A A - Rat
TT---- T G A Human

A T GG A CCAAAACAA Rat
G C TA G --------- Human

C TGT A T C A A A A Rat
A G-- G C - T - C G Human

-G T G Rat
TA - T Human

T GC TG C G G GGAGTGAG G C Rat
C AT CA A A A A------- C A Human

G T TC TTTTGTTTGGTTGGTCTTTTTTTTCCTTCCTTCCTTCCTCCCTTC Rat
A A AT --- Human

CTTCCTTCCTCCTTTCTTTCTTTCTTC T T G A Rat
--------------------------- C C T G Human

T C G G G GG G TC Rat
A T A A C AT A AT Human

GAGAGCAGGGAAA TG TA TC A CGGGTAAGGTG Rat
AGAGAGTTATGCT CA AT CT G T---------- Human

- C G C T A C -- CTGG Rat
T T A A C C G AA GAAA Human

ACC T- A AA G GG C TTG- G Rat
GAA AA G CG A AA A CCAC A Human

C T CT CA T C T CG- GA G C C C CG GA C Rat
T C TC AT C T C GAT AG A T T T TA TG G Human

TC G T CGC TCA ACTG G GCT TC G T G Rat
CA A C GAA ATG -TAA A AAG CA A C T Human

GA TTT G GGC G TA T CTAA TG Rat
AG GGC A T-T T AG A T--G AA Human

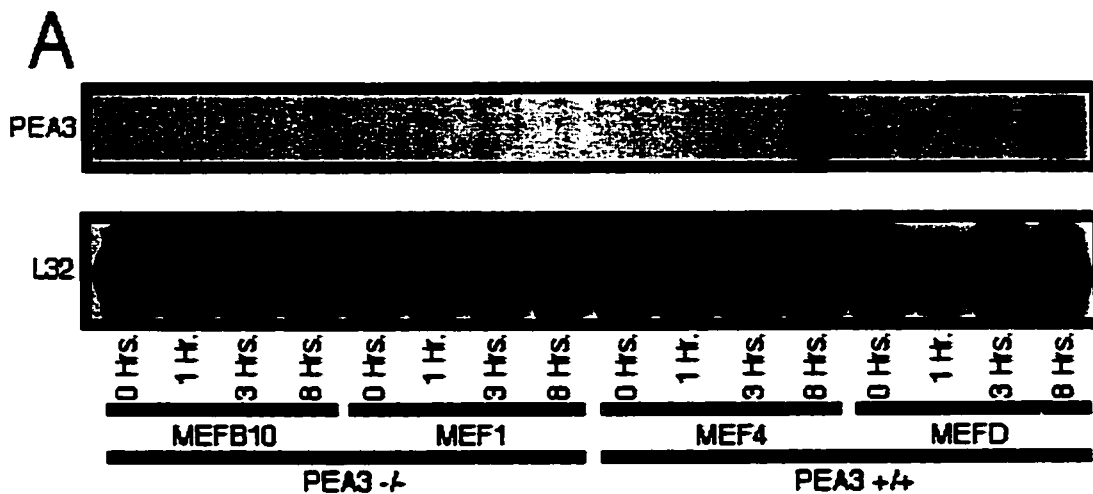
CA C A A C T Rat
AG T G T A - Human

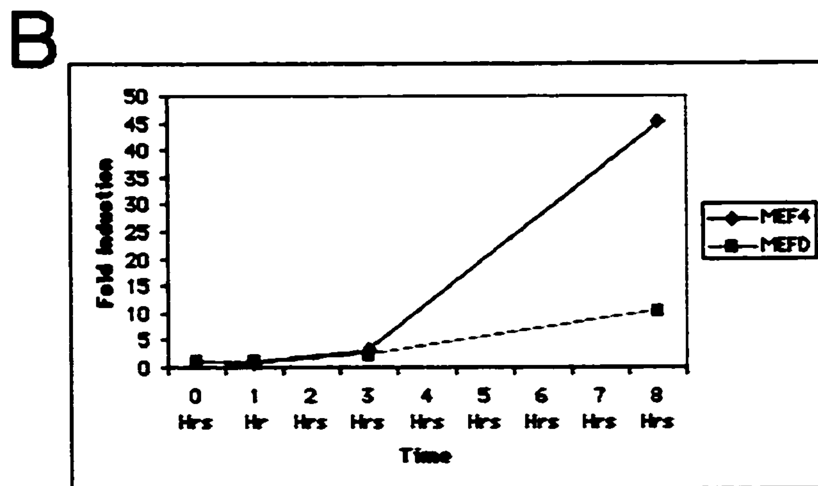
C T - A A A TAAA C Rat
G C T G T G ACTG A Human

T AAA Rat
A Human

Figure 4.8 Induction of *PEA3* Expression in Wild Type by Not *PEA3*-Null MEF Cell Lines.

(A) RNase protection analysis of serum starved MEF cell lines at various times after stimulation with EGF. *PEA3*-null MEFB10 and MEF1, and wild type MEF4 and MEFD cells were serum starved for 36 hours. Each cell line was subsequently stimulated with 200 ng/ml EGF for varying lengths of time as indicated. RNase protection assays were carried out on 10 μg of RNA isolated from these cells. The RNA was hybridized with an antisense mouse *PEA3* riboprobe expected to protect a 323-nucleotide fragment of *PEA3* mRNA from RNase digestion in wild type cells, but not *PEA3*-null cells. RNA was also hybridized to an antisense riboprobe expected to protect a 195-nucleotide fragment of mouse *L32* mRNA. *L32* serves as an internal control for RNA loading. *PEA3* mRNA was not detected in MEFB10 or MEF1 cells. *PEA3* mRNA was also undetectable in with EGF, *PEA3* mRNA became elevated to detectable levels. (B) Fold elevation of *PEA3* mRNA in MEF4 and MEFD cells. Levels of *PEA3* and *L32* mRNA were quantified by phosphorimaging. *PEA3* mRNA levels were normalized to *L32* levels. The fold induction of *PEA3* mRNA was determined by setting the normalized *PEA3* level at 0 hours of EGF stimulation in each cell line to 1.


A
PEA3
L32
0 Hrs.
1 Hr.
3 Hrs.
8 Hrs.
0 Hrs.
1 Hr.
3 Hrs.
8 Hrs.
0 Hrs.
1 Hr.
3 Hrs.
8 Hrs.
0 Hrs.
1 Hr.
3 Hrs.
8 Hrs.
MEFB10
MEF1
MEF4
MEFD
PEA3 -/-
PEA3 +/+


B
Fold Induction
50
45
40
35
30
25
20
15
10
5
0
0 Hrs
1 Hr
2 Hrs
3 Hrs
4 Hrs
5 Hrs
6 Hrs
7 Hrs
8 Hrs
Time
MEF4
MEFD

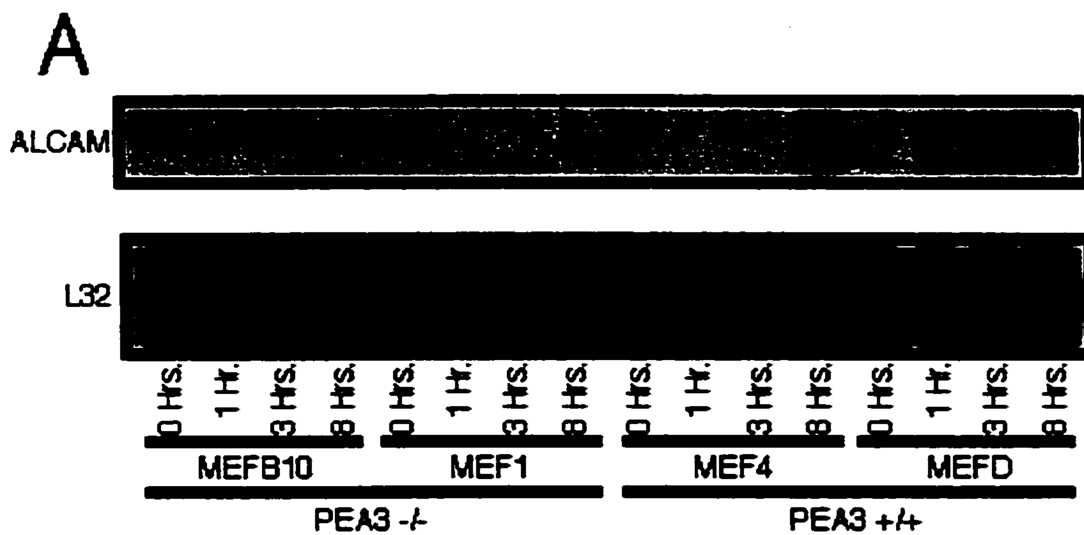
detected in MEFB10 or MEF1 cells with the riboprobe used. *PEA3* mRNA could also not be detected in serum starved MEF4 or MEFD cells prior to EGF stimulation. However, after 8 hours of stimulation with EGF, *PEA3* becomes expressed at detectable levels in both MEF4 and MEFD cells. Levels of *PEA3* and *L32* mRNA were quantified by phosphorimaging. *PEA3* mRNA levels were normalized to *L32*. Fold induction of *PEA3* mRNA levels in EGF stimulated MEF4 and MEFD cells over those of unstimulated, serum starved cells were determined (Figure 4.8B). *PEA3* levels become elevated approximately 45-fold upon EGF stimulation of MEF4 cells, and approximately 10-fold upon EGF stimulation of MEMD cells. This is consistent with the elevation of expression of *MMP* genes observed in these cells upon stimulation with EGF.

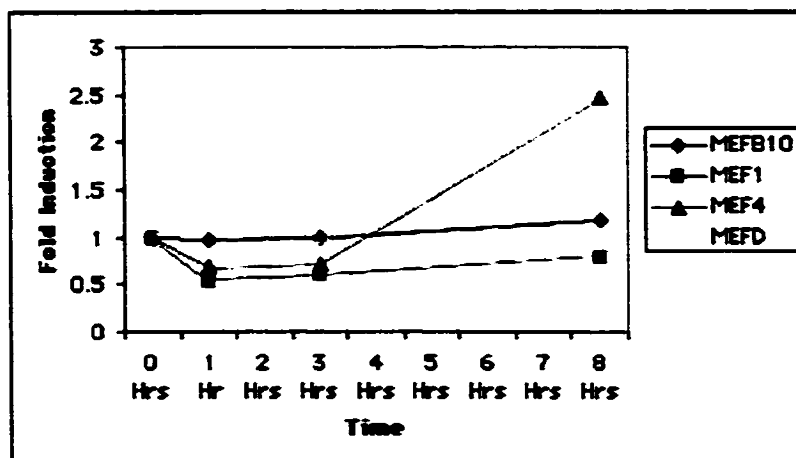
4.2.6 *ALCAM* Expression Levels Increase in Wild Type but Not *PEA3*-null MEF Cells Upon Stimulation with EGF.

RNase protection experiments were carried out to determine whether similar effects on *ALCAM* mRNA expression occur in serum starved MEF cell lines as were observed for *MMP-3*, *MMP-7* and *MMP-9*. A fragment of the 5'-end of the mouse *ALCAM* cDNA (Bowen et al., 1997) was obtained by RT-PCR carried out on mouse brain RNA. This cDNA was used to produce an antisense mouse *ALCAM* riboprobe. RNA was isolated from serum starved MEFB10, MEF1, MEF4 and MEFD cells that had been stimulated with 200 ng/ml EGF for varying lengths of time. This RNA was hybridized with the antisense *ALCAM* and mouse *L32* riboprobes and RNase protection analysis carried out (Figure 4.9A). Wild-type MEF4 and MEFD cells express higher levels of *ALCAM* mRNA than *PEA3*- null MEFB10 and MEF1 cells under all conditions

Figure 4.9 Induction of *ALCAM* Expression in Wild Type but Not *PEA3*-Null MEF Cell Lines.

RNase protection analysis of serum starved MEF cell lines at various times after stimulation with EGF. *PEA3*-null MEFB10 and MEF1, and wild type MEF4 and MEFD cells were serum starved for 36 hours. Each cell line was subsequently stimulated with 200 ng/ml EGF for varying lengths of time as indicated. RNase protection assays were carried out on 10 μg of RNA isolated from these cells. The RNA was hybridized to an antisense riboprobe expected to protect a 499-nucleotide fragment of mouse *ALCAM* mRNA from RNase digestion. RNA was also hybridized with an antisense mouse *L32* riboprobe as an internal control for RNA loading. *ALCAM* mRNA levels do not change in MEFB10 or MEF1 cells upon stimulation with EGF. However, *ALCAM* mRNA levels become elevated in MEF4 and MEFD cells after 8 hours of EGF stimulation. (B) Fold elevation of *ALCAM* mRNA in MEF cell lines. Levels of *ALCAM* and *L32* mRNA were quantified by phosphorimaging. *ALCAM* mRNA levels were normalized to *L32* levels. The fold induction of *ALCAM* mRNA was determined by setting the normalized *ALCAM* level at 0 hours of EGF stimulation in each cell line to 1.


A
ALCAM
L32
0 Hrs.
1 Hr.
3 Hrs.
8 Hrs.
0 Hrs.
1 Hr.
3 Hrs.
8 Hrs.
0 Hrs.
1 Hr.
3 Hrs.
8 Hrs.
0 Hrs.
1 Hr.
3 Hrs.
8 Hrs.
MEFB10
MEF1
MEF4
MEFD
PEA3 -/-
PEA3 +/+


B
Fold Induction
3
2.5
2
1.5
1
0.5
0
0 Hrs
1 Hr
2 Hrs
3 Hrs
4 Hrs
5 Hrs
6 Hrs
7 Hrs
8 Hrs
Time
MEFB10
MEF1
MEF4
MEFD

tested. *ALCAM* mRNA levels do not change in MEFB10 or MEF1 cells upon stimulation with EGF. However, in both MEF4 and MEFD cells, *ALCAM* mRNA levels rise after 8 hours of EGF stimulation in a manner similar to *PEA3* mRNA. Levels of *ALCAM* and *L32* mRNA were quantified by phosphorimaging. *ALCAM* levels were normalized to *L32* and the fold elevation of *ALCAM* mRNA levels over unstimulated cells of the same type determined (Figure 4.9B). In MEF4 cells, *ALCAM* mRNA levels become elevated approximately 2.5-fold after 8 hours of EGF stimulation. In MEFD cells, *ALCAM* mRNA levels become elevated approximately 2-fold under these conditions. This data is consistent with the elevation of *G71* (*ALCAM*) mRNA levels in RP10 cells observed upon induction of *PEA3* overexpression.

4.2.7 Cloning and Characterization of the Mouse ALCAM Promoter.

RT-PCR experiments were carried out on a mouse brain RNA sample to obtain the 5'-end of the mouse *ALCAM* cDNA. This cDNA was used as a probe to screen a SV129 mouse genomic library. This led to the isolation of a 1135 base pair genomic DNA fragment that hybridized to this probe and upon sequencing was found to contain the 5'-end of the *ALCAM* cDNA, as well as 862 base pairs of upstream sequences. Figure 4.10A shows the upstream sequence from nucleotide positions -862 to +4 relative to the reported 5'-end of the *ALCAM* cDNA (Bowen et al., 1997; Degen et al., 1998). Transcription factor binding sites in these sequences were identified using the Matinspector software (Quandt et al., 1995). These upstream sequences do not contain a consensus TATA element. However, a close match to the consensus initiator (Inr) element 5'-YYAN(A/T)YY-3' is found at nucleotide position -27 (Jahaverty et al., 1994).

Figure 4.10 ***ALCAM*** **Promoter Sequence and Promoter Constructs.**

(A) Sequence of the mouse *ALCAM* promoter. The sequence from nucleotide positions -862 to +4 relative to the reported 5'-end of the *ALCAM* cDNA are shown. Transcription factor binding sites were found using the Matinspector software. The *ALCAM* promoter contains five sites that match the consensus for the *PEA3* DNA-binding site. These are located at -143, -265, -574, -613 and -770. Binding sites for AP-1, C/EBPβ and Tcf transcription factors are also shown. These factors can cooperate with PEA3 to activate transcription. The sequence contains a consensus initiator element and a binding site for USF, a factor that synergizes with initiator-binding proteins to initiate transcription from these elements. (B) Structure of reporter constructs containing *ALCAM* promoter regions. The *ALCAM* promoter sequences from nucleotides -862 to +4 (ALCAM -862), -508 to +4 (ALCAM -508) and -263 to +4 (ALCAM -263) were cloned upstream of a luciferase reporter in the pGL3basic vector (Promega). Black boxes represent the positions of *PEA3*-binding consensus sites in these constructs. The 5'-ends of the ALCAM -263 and ALCAM -508 constructs are indicated by square brackets in panel A.

A

-862 GATCTCAGATCCTATGTCTTTGGGGCGCTGAGAATCCCCTGGTAAGAGCCAGACACTGGCGAATCC[illegible]CCCCCTACTC[illegible]CT

-762 TCATTGGCCAGCGTGTCCTGTGGGAGGCGGAG[illegible]CCCGCCCACCAGAGCGCGGGGGCTGGGGCATCAGGCTCTTCTCCCACCAGGGGCCGC

-662 CAGCTCTGCTCAGTCTCCGACTTGGTCCTGCAGCGCCTCTAGG[illegible]CGCTACCAACCACCTGCTCCGCGCTGCCG[illegible]GGGCGG

-562 GAGAGAGGAGCGGGAGGAGCAGGAGGAGCGGGAGGAGCGGGAGGAGGAGACAGGCAGCTTACACACGCCTTCCCGTCCTCCACTCAGAGCAGCCGGAGCC

-462 CGCGGCCGCCGCCACTGCTCCAGCTGCCGCCGCTACAGCCTCTGCCTCGGCTGCCACCTGAGGAGACCCACCGTCGGGACCCCGCGGCGCTGCCCCGCCG

-362 ACCCTTCTCTGCTCCCGGTGATCCGTGCACGGTGCAACTCTCATCACTCTTCTTATCCTCT[illegible] CEBPB AGAAGAGAAACTACTAGAAGAC[illegible]

-262 [illegible]GAAACCGCGTCTAC[illegible]ACTGAAGCGTCTCCTGGAATAAGAGGGTCGCCCGTCCTGGGAGTAGCTGCCAGAGA CEBPB CACTGAAGAAAGGGTG

-162 TGGAGGGGGGGAGGGAGGAGTTGC[illegible] CEBPB GGAGGAAAGTTGCGTGTGGCACAGACCCGACGGCGCAGCGCCACAGTCCCCGGGGGACCGT USF [illegible]

-62 CEBPB GTTGGAAAAAAGTCGCTGTCCCCCTAAGGCGAGACCCACAAGCGAGCGGGCCCCGTGGCCGGGG +4 [illegible] USF

B

ALCAM -862: -860 [construct] Luciferase

ALCAM -508: -508 [construct] Luciferase

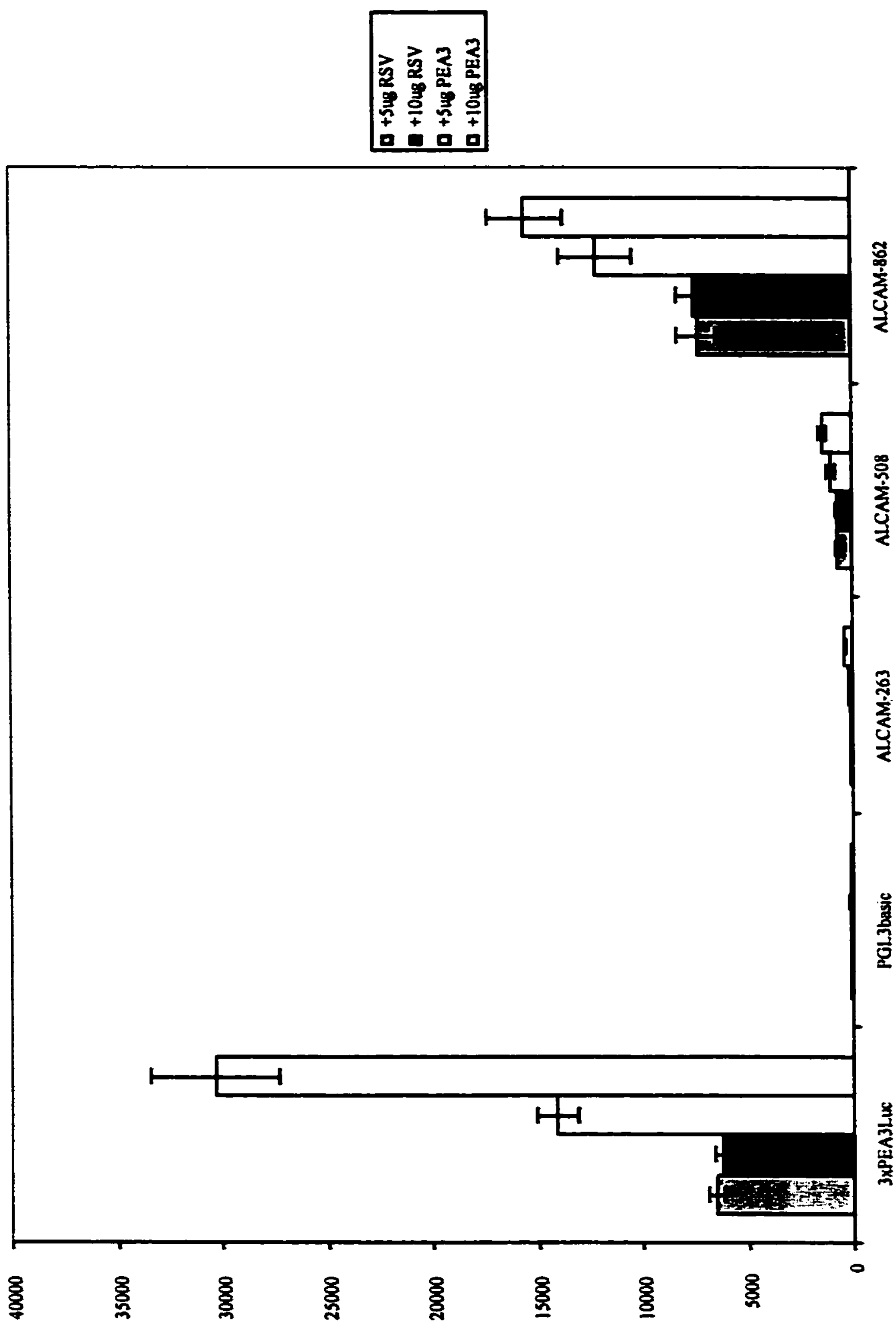
ALCAM -263: -263 [construct] Luciferase

Binding sites for upstream stimulatory factor 1 (USF1), are found near this element, at positions -13, -71 and -135. USF1 interacts synergistically with Inr binding proteins to initiate transcription (Roy et al., 1997). It is likely that transcription of *ALCAM* is initiated at this Inr element. The *ALCAM* promoter contains five matches to the PEA3 DNA-binding element 5'-(T/C)(T/G)CCGGA(A/T)(G/C)CG-3' (Bowman and Hassell, unpublished data). These are located at nucleotide positions -127, -198, -568, -608 and -764. Also shown in Figure 4.10A are binding sites for the AP1, Tcf-Lef and C/EBPβ transcription factors, which are known to cooperate with PEA3 to activate transcription (Wasylyk et al., 1990; H. Crawford, personal communication; L. Howe, personal communication). The presence of these sites in the *ALCAM* promoter is consistent with the ability of PEA3 to activate transcription of this gene. The *ALCAM* promoter also contains many transcription factor binding sites that are not shown, including sites for AP2, AP4, ATF, c-Myb, CREB, MyoD, NFκβ, N-Myc and Sp1.

Experiments were carried out to test the ability of PEA3 to activate transcription from *ALCAM* promoter sequences. Reporter plasmids were constructed in which fragments of the *ALCAM* promoter, containing the sequences from -862 to +4 (ALCAM -862), -508 to +4 (ALCAM -508), or -263 to +4 (ALCAM -263) were placed upstream of the luciferase reporter gene in the pGL3 basic vector (Promega) (Figure 4.10B). The positions of putative PEA3-binding sites in these constructs are shown as black boxes in Figure 4.10B. The 5'-ends of the ALCAM -508 and -263 constructs are shown as square brackets in Figure 4.10A. MCF-7 cells were co-transfected with 5 μg or 10 μg of pRSV-PEA3, encoding *PEA3* under the control of the Rous Sarcoma Virus promoter, or 5 μg or 10 μg of pRSV empty vector (Invitrogen) (Figure 4.11). As a positive control, MCF-7

Figure 4.11 Specific Luciferase Activities of MCF-7 Cells Transfected with *ALCAM* Promoter Constructs and *PEA3*.

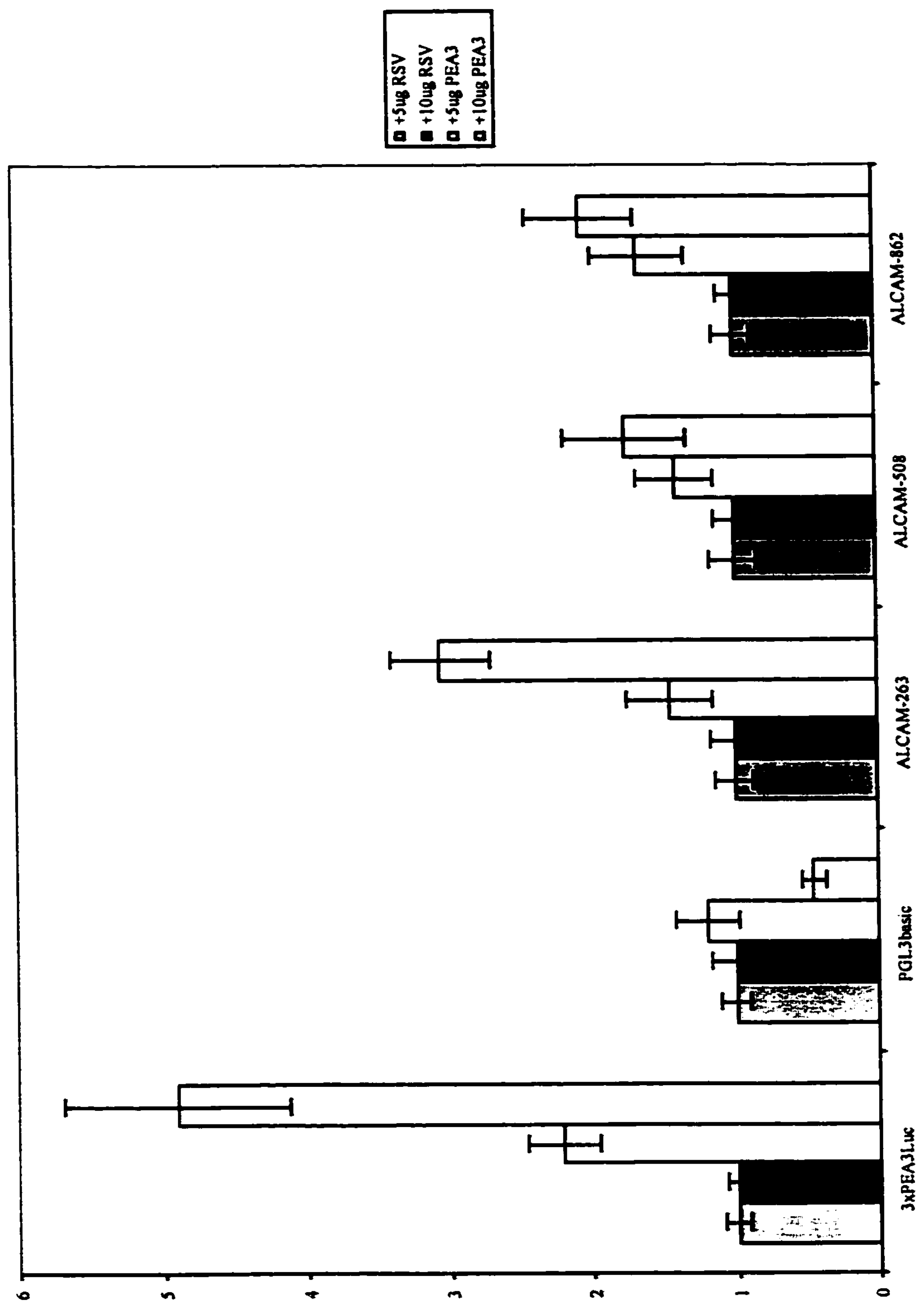
MCF-7 cells were cotransfected with 5 μg of each *ALCAM* promoter construct and either 5 μg or 10 μg of RSV-PEA3 or the RSV empty vector. Cells were cotransfected with the promoterless pGL3basic vector (Promega) and RSV-PEA3 or RSV empty vector as a negative control. Cells were cotransfected with 3xPEA3Luc, encoding luciferase under the control of three PEA3-binding sites, and RSV-PEA3 or RSV empty vector as a positive control. Luciferase assays were carried out on 50 μg of cell extracts prepared 48 hours after transfection. The ALCAM -263 construct shows very low basal promoter activity in MCF-7 cells. This activity is increased by the additional sequences present in ALCAM -508 and ALCAM -862. The activity of all three *ALCAM* promoter constructs is increased by PEA3. The data shown are the mean values obtained from three independent experiments carried out in triplicate (n=9), with error bars representing standard deviation.



+5ug RSV
+10ug RSV
+5ug PEA3
+10ug PEA3
40000
35000
30000
25000
20000
15000
10000
5000
0
3xPEA3Luc
PGL3basic
ALCAM-263
ALCAM-508
ALCAM-862

cells were co-transfected with pRSV of pRSV-PEA3 and the 3xPEA3Luc reporter, encoding luciferase under the control of three PEA3-binding elements derived from the polyomavirus enhancer (Sheppard and Hassell, unpublished data). As a negative control, MCF-7 cells were co-transfected with pRSV of pRSV-PEA3 and the promoterless pGL3 basic vector. The activity of the ALCAM -263 promoter construct, when co-transfected with pRSV empty vector is quite low. However, the presence of additional promoter sequences in the ALCAM -508 and ALCAM -862 constructs confer higher basal activities upon these constructs in MCF-7 cells. When co-transfections with pRSV-PEA3 are carried out, the activities of all three promoter constructs are increased. The fold elevation of luciferase expression driven from the *ALCAM* promoter constructs in the presence of pRSV-PEA3 over that observed in the presence of an equal amount of pRSV empty vector has been determined (Figure 4.12). The activity of the ALCAM -263 promoter construct is elevated approximately 3-fold upon co-transfection with 10 μg of pRSV-PEA3, while the activities of the ALCAM -508 and ALCAM -862 constructs are elevated approximately 2-fold under the same conditions. No similar increase in luciferase expression is observed when the promoterless pGL3 basic vector is co-transfected with pRSV-PEA3. Expression of luciferase from the 3xPEA3Luc vector increases approximately 5-fold upon co-transfection with 10 μg of pRSV-PEA3. The magnitude of the increase in activity of the *ALCAM* promoter constructs in the presence of pRSV-PEA3 is consistent with the elevation of *ALCAM* (*G71*) expression in RP10 cells upon induction of *PEA3* overexpression (Section 4.2.3). For each combination of vectors, luciferase activity was measured from three samples from three independent experiments (n = 9).

Figure 4.12 Fold Activation of *ALCAM* Promoter Construct Activities by PEA3. Specific luciferase activities shown in Figure 4.11 were normalized by setting the luciferase activity of extracts prepared from cells cotransfected with each promoter construct and RSV empty vector to 1. ALCAM -263 promoter activity is activated approximately 3-fold upon cotransfection with RSV-PEA3. ALCAM -508 and ALCAM -862 are activated approximately 2-fold upon cotransfection with RSV-PEA3. The data shown are the mean values obtained from three independent experiments carried out in triplicate (n = 9), with error bars representing standard deviation.


+5ug RSV
+10ug RSV
+5ug PEA3
+10ug PEA3
0
1
2
3
4
5
6
3xPEA3Luc
PGL3basic
ALCAM-263
ALCAM-508
ALCAM-862

A statistical analysis of the data shown in Figures 4.11 and 4.12 is shown in table 4.1. The specific luciferase activities (in relative light units/µg extract) are shown for each sample for all three cotransfections. The mean, variance and standard deviation for each combination of vectors in each cotransfection experiment was determined. As well, the overall mean, variance and standard deviation for all three cotransfection experiments were determined, and are shown at the bottom of Table 4.1. These overall values are shown in Figure 4.11. The variance in specific luciferase activities measured in individual cotransfection experiments was always less than that seen when the data from all three transfections was pooled. Fold inductions in mean specific luciferase activity upon cotransfection with 5 or 10 µg of *PEA3* are also shown for each cotransfection experiment as well as the overall fold induction for all three cotransfections. The overall fold inductions are shown in Figure 4.12. The statistical significance of the increases in mean luciferase activity upon cotransfection with 5 or 10 µg of *PEA3* were tested using a two-tailed t-test, using both the data from each individual cotransfection and the overall data form all three cotransfection experiments. These increases were found to be statistically significant ($P<0.05$) in all cases except when the pGL3basic reporter vector was used.

A greater activation of luciferase expression by PEA3 occurs from the ALCAM -263 promoter construct than the ALCAM -508 and ALCAM -862 promoter constructs. This implies that PEA3 activates transcription from the PEA3-binding site at nucleotide position -127 in these experiments, as this is the only consensus PEA3-binding site present in the ALCAM -263 construct.

Table 4.1. Statistical Analysis of Data Shown in Figures 4.11 and 4.12

	3xPEA3luc				pGL3basic				ALCAM-263				ALCAM-508				ALCAM-862			
	5ug RSV	10ug RSV	5ug PEA3	10ug PEA3	5ug RSV	10ug RSV	5 ug PEA3	10 ug PEA3	5ug RSV	10ug RSV	5 ug PEA3	10 ug PEA3	5ug RSV	10ug RSV	5 ug PEA3	10 ug PEA3	5ug RSV	10ug RSV	5 ug PEA3	10 ug PEA3
Tran1:Sam1	6003	5704	13027	28563	96	103	126	56	150	136	199	364	812	849	1052	1321	7215	7524	11896	15374
Tran1:Sam2	5962	5842	13015	26208	104	112	132	72	132	122	175	362	774	701	1293	1496	7996	7214	13542	17592
Tran1:Sam3	6345	6004	13224	27542	115	108	121	43	139	154	162	392	703	762	964	1551	6854	7812	12659	13541
Mean	6103	5850	13089	27438	105	108	126	57	140	137	179	373	763	771	1103	1456	7355	7517	12699	15502
Variance	44222	22548	13772	1394670	91	20	30	211	82	257	352	281	3061	5532	29011	14425	340741	89441	678529	4115002
St. Dev.	210	150	117	1181	10	5	6	15	9	16	19	17	55	74	170	120	584	299	824	2029
Fold Induction			2.14	4.69			1.20	0.53			1.27	2.71			1.45	1.89			1.73	2.06
P(T-test)			1.16E-05	8.45E-04			0.040	0.019			0.05	6.26E-05			0.06	2.34E-03			1.26E-03	0.019
Tran2:Sam1	6542	6432	14445	30016	67	74	78	21	75	102	109	311	652	726	1096	1467	8765	8782	14396	18502
Tran2:Sam2	6754	6212	13954	29542	71	82	85	52	65	96	126	327	694	698	854	1227	7762	8142	12996	16431
Tran2:Sam3	6326	6141	14151	31568	75	96	96	37	84	78	132	331	643	765	910	1396	8042	7998	13451	16202
Mean	6541	6262	14183	30375	71	84	86	37	75	92	122	323	663	730	953	1363	8196	8307	13614	17045
Variance	45797	23020	61054	1123009	16	124	82	240	90	156	142	112	741	1132	16049	15200	279498	174765	510008	1605247
St. Dev.	214	152	247	1060	4	11	9	16	10	12	12	11	27	34	127	123	529	418	714	1267
Fold Induction			2.17	4.65			1.22	0.44			1.64	3.51			1.44	1.87			1.66	2.05
P(T-test)			2.74E-06	5.13E-04			0.17	0.016			6.48E-03	2.08E-05			0.052	8.58E-03			6.90E-04	0.0037
Tran3:Sam1	6983	6674	15590	34321	93	136	162	72	115	117	152	351	491	599	751	901	5615	6954	9371	14621
Tran3:Sam2	7014	6524	15458	34562	126	145	154	54	92	123	175	374	573	653	699	1146	7002	6221	10542	12304
Tran3:Sam3	6827	6653	14968	33541	135	131	132	79	126	104	136	332	602	679	773	1292	6546	7002	9654	14999
Mean	6941	6617	15339	34141	118	137	149	68	111	115	154	352	555	644	741	1113	6388	6726	9856	13975
Variance	6696	4398	71601	189680	326	34	161	111	201	63	256	295	2210	1110	963	26025	333163	127728	248875	1419378
St. Dev.	100	81	328	534	22	7	16	13	17	10	20	21	58	41	38	198	707	438	611	1459
Fold Induction			2.21	5.16			1.27	0.50			1.39	3.07			1.33	1.73			1.54	2.08
P(T-test)			1.80E-04	9.01E-05			0.12	3.42E-03			0.046	5.61E-04			0.013	0.049			3.23E-03	0.0087
Mean	6486	6204	14092	30330	99	109	121	54	112	117	154	352	671	720	960	1325	7317	7516	12121	15507
Variance	157424	125253	1008747	9183591	530	520	759	310	927	507	800	656	9824	4965	35302	38356	795156	517687	2949486	3315465
St. Dev.	397	354	1004	3030	23	23	28	18	30	23	28	26	99	70	188	198	892	720	1717	1821
Fold Inuction			2.17	4.89			1.23	0.50			1.38	3.01			1.42	1.84			1.66	2.06
P(T-test)			5.72E-11	8.32E-10			0.063	1.31E-05			0.0045	3.14E-14			0.0010	1.40E-06			5.06E-06	2.71E-08

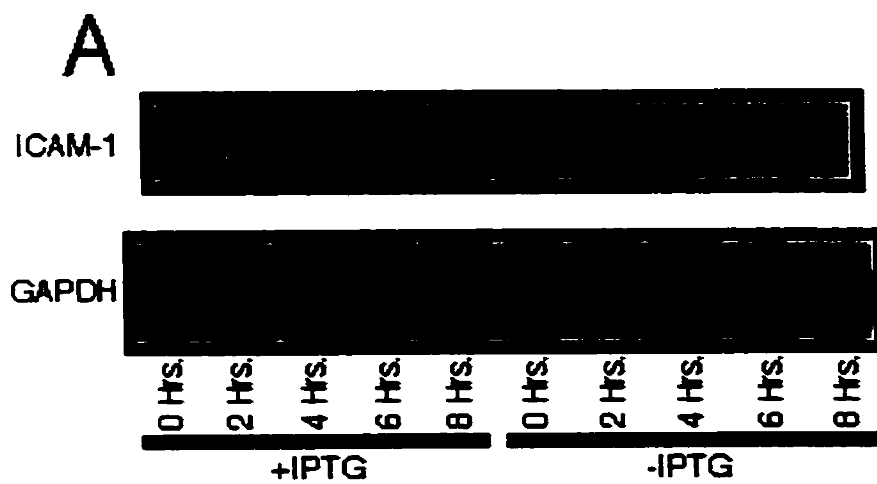
Experiments have been conducted to determine if PEA3 can act synergistically with Tcf-Lef transcription factors on the *ALCAM* promoter, as has been observed for the *MMP7* promoter. MCF-7 cells were co-transfected with the *ALCAM* promoter constructs in combination with pRSV-PEA3 and pCANβcateninΔ90, which encodes a constitutively active form of β-catenin. No difference in luciferase activity was observed between extracts prepared from these cells and cells co-transfected with only pRSV-PEA3 and the reporter constructs, indicating that Tcf-Lef factors cannot cooperate with PEA3 on this promoter (data not shown).

4.2.8 *PEA3* Overexpression Does Not Influence Expression of *ICAM-1* or *N-CAM* in RP10 Cells.

The ability of PEA3 to regulate the expression of certain other immunoglobulin-like cell adhesion molecules has been tested. RT-PCR experiments were carried out on RP10 RNA using primers specific for the mRNAs of a number of these CAMs. Expression of *ICAM-1* and *N-CAM* mRNA could be detected in RP10 cells. cDNAs obtained from these experiments were used to generate antisense riboprobes for rat *ICAM-1* and *N-CAM*. RNase protection experiments using these riboprobes were carried out to determine whether expression of *ICAM-1* or *N-CAM* was altered upon induction of PEA3 overexpression. RP10 cells were grown at 39.5^{0}C for varying lengths of time in the presence or absence of 5 mM IPTG. RNase protection assays were performed on RNA isolated from these cells. Neither the levels of *ICAM-1* mRNA (Figure 4.13A) or *N-CAM* mRNA (Figure 4.14A) change significantly upon induction of *PEA3* overexpression. Quantification of *ICAM-1* and *N-CAM* mRNA levels by phosphorimaging confirmed that

Figure 4.13 Analysis of *ICAM-1* mRNA Expression in RP10 Cells.

(A) RNase protection analysis of RP10 cell RNA at various times after induction with IPTG. RP10 cells were grown at 39.5^{0}C for varying periods of time in the presence or absence of 5 mM IPTG as indicated. RNase protection assays were carried out on 10 µg of RNA isolated from these cells. The RNA was hybridized with an antisense riboprobe expected to protect a 442-nucleotide fragment of the rat *ICAM-1* mRNA from RNase digestion. RNA was also hybridized with an antisense rat *GAPDH* riboprobe as an internal control for RNA loading. *ICAM-1* mRNA levels do not change significantly in RP10 cells in either the presence or absence of IPTG. (B) Fold induction of *ICAM-1* mRNA in RP10 cells. Levels of *ICAM-1* and *GAPDH* were quantified by phosphorimaging. *ICAM-1* mRNA levels were normalized to *GAPDH* levels. The fold induction of *ICAM-1* mRNA, both in the presence and absence of IPTG, was determined by setting the normalized *ICAM-1* level at 0 hours under both conditions to 1.


A
ICAM-1
GAPDH
0 Hrs.
2 Hrs.
4 Hrs.
6 Hrs.
8 Hrs.
0 Hrs.
2 Hrs.
4 Hrs.
6 Hrs.
8 Hrs.
+IPTG
-IPTG

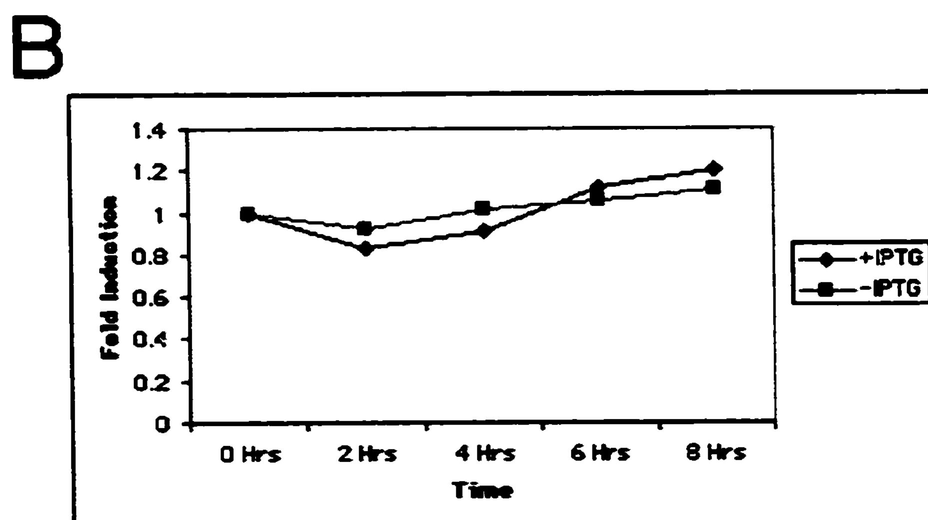
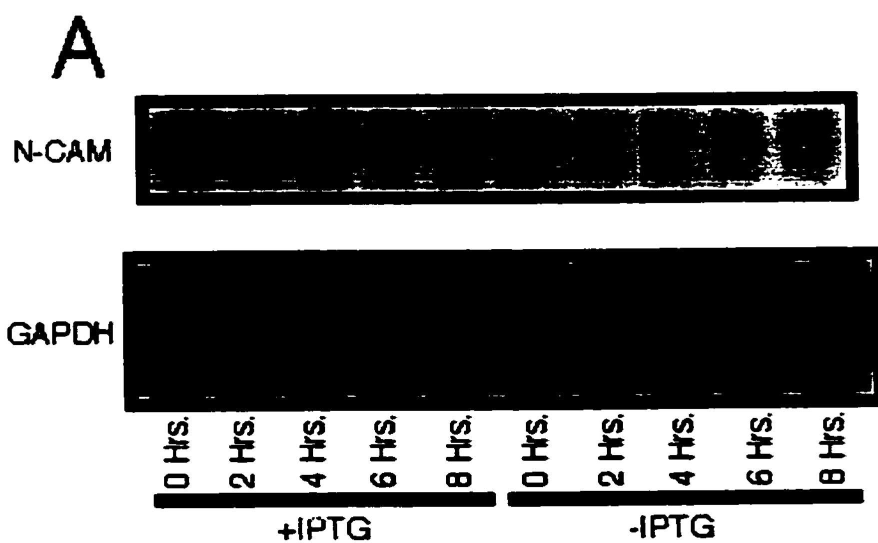
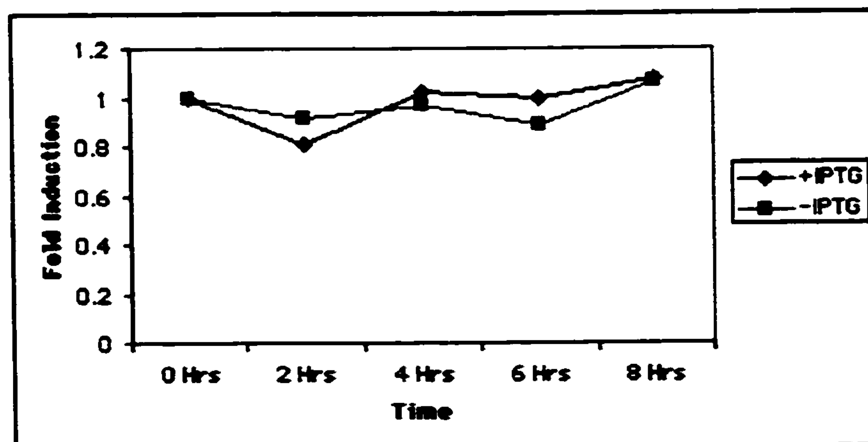

B
Fold Induction
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 Hrs
2 Hrs
4 Hrs
6 Hrs
8 Hrs
Time
+IPTG
-IPTG

Figure 4.14 Analysis of *ICAM-1* mRNA Expression in RP10 Cells.

(A) RNase protection analysis of RP10 cell RNA at various times after induction with IPTG. RP10 cells were grown at 39.5^{0}C for varying periods of time in the presence or absence of 5 mM IPTG as indicated. RNase protection assays were carried out on 10 µg of RNA isolated from these cells. The RNA was hybridized with an antisense riboprobe expected to protect a 420-nucleotide fragment of the rat *N-CAM* mRNA from RNase digestion. RNA was also hybridized with an antisense rat *GAPDH* riboprobe as an internal control for RNA loading. *N-CAM* mRNA levels do not change significantly in RP10 cells in either the presence or absence of IPTG. (B) Fold induction of *N-CAM* mRNA in RP10 cells. Levels of *N-CAM* and *GAPDH* were quantified by phosphorimaging. *N-CAM* mRNA levels were normalized to *GAPDH* levels. The fold induction of *N-CAM* mRNA, both in the presence and absence of IPTG, was determined by setting the normalized *N-CAM* levels at 0 hours under both conditions to 1.


A
N-CAM
GAPDH
0 Hrs.
2 Hrs.
4 Hrs.
6 Hrs.
8 Hrs.
0 Hrs.
2 Hrs.
4 Hrs.
6 Hrs.
8 Hrs.
+IPTG
-IPTG


B
Fold Induction
1.2
1
0.8
0.6
0.4
0.2
0
0 Hrs
2 Hrs
4 Hrs
6 Hrs
8 Hrs
Time
+IPTG
-IPTG

levels of these mRNAs are not elevated in IPTG induced RP10 cells relative to uninduced cells (Figures 4.13B and 4.14B).

4.3 Discussion.

A modest but consistent activation of *ALCAM* (*G71*) expression is observed upon induction of *PEA3* overexpression in RP10 cells. Elevated levels of *ALCAM* mRNA have been observed in induced RP10 cells by DDRT-PCR, reverse northern blotting and RNase protection analysis carried out on independently isolated RNA preparations. The kinetics of the elevation of *ALCAM* mRNA levels closely match those of *PEA3* overexpression upon IPTG induction of RP10 cells, suggesting that this is a direct effect of PEA3. Furthermore, *ALCAM* mRNA levels remain elevated for up to 48 hours after induction of RP10 cells with IPTG. This is consistent with the detection of a difference in *ALCAM* mRNA levels by DDRT-PCR between uninduced RP10 cells and RP10 cells induced with IPTG for 24 hours. No change in the level of *ALCAM* expression occurs in LAP6 cells in the presence of IPTG, demonstrating LAP267 is incapable of directly effecting this change in RP10 cells.

ALCAM mRNA levels increase only 2- to 2.5-fold in RP10 cells upon induction of *PEA3* overexpression. This is a modest degree of elevation, but is consistent with that observed for *MMP-14* (Figure 3.8), a known PEA3 target (Habelhah et al., 1999). The modest activation of *ALCAM* and *MMP-14* expression may be due to the nature of RP10 cells (Section 3.3). Ets proteins, including PEA3, generally must interact with other transcription factors to activate transcription (Graves and Petersen, 1998). Such transcription factors, able to cooperate with PEA3 to activate *ALCAM* expression may be

present in limiting amounts or absent in RP10 cells, preventing large increases in *ALCAM* expression when *PEA3* is overexpressed.

Comparison of *ALCAM* expression in wild type and *PEA3*-null MEF cells has also demonstrated a coordinate upregulation of *PEA3* and *ALCAM* expression, consistent with the activation of *ALCAM* expression by *PEA3*. As has been discussed (Section 3.3), there are potential problems with the use of these cells for the identification of PEA3 target genes due to the inability to control for PEA3-independent differences in gene expression between these cell lines. For example, *ALCAM* is expressed at higher levels under all conditions tested in MEFB10 cells than in MEF1 cells (both *PEA3*-null) and in MEFD cells than in MEF4 cells (both wild-type) (Figure 4.9). However, it has been observed that expression of the known PEA3 target genes *MMP-3*, *MMP-7*, and *MMP-9* become elevated in serum starved wild-type MEF cells upon addition of EGF (Xin and Hassell, unpublished data). Consistent with this observation, it has been demonstrated that *PEA3* expression becomes elevated in wild-type MEF cells after 8 hours of stimulation with EGF. *ALCAM* expression levels rise in these cells after a similar period of stimulation with EGF. Since a similar increase in *ALCAM* expression is not observed in *PEA3*-null MEF cells under the same conditions, this suggests regulation of *ALCAM* expression by PEA3. Again, the activation of *ALCAM* expression in MEF4 and MEFD cells is modest, but is consistent with that observed in RP10 cells.

Co-transfection of MCF-7 cells with RSV-PEA3 and constructs in which the luciferase reporter gene has been cloned downstream from fragments of the *ALCAM* promoter demonstrate the ability of PEA3 to activate transcription from this promoter. Expression of luciferase from all three promoter constructs used increases in the presence

of PEA3, with luciferase expression increasing as higher concentrations of RSV-PEA3 are used in these experiments. The ability of PEA3 to activate reporter gene expression from the *ALCAM* promoter is consistent with the coordinate upregulation of *PEA3* and *ALCAM* expression observed in both RP10 and MEF cells. Taken together these data strongly suggest that PEA3 is able to activate the expression of *ALCAM*.

CHAPTER 5

GENERAL DISCUSSION

5.1 The *ALCAM* Promoter.

A mouse genomic DNA fragment containing the 5'-end of the mouse *ALCAM* cDNA, as well as 862 base pairs upstream of these sequences, has been obtained (Section 4.2.7). It is clear that these upstream sequences have promoter activity, as the level of expression of luciferase is considerably higher in MCF-7 cells when it is cloned downstream of these sequences than in a promoterless vector (Figure 4.11). While these upstream sequences do not contain a TATA box, there is a sequence closely matching the consensus initiator element 5'-YYAN(A/T)YY-3' (Jahaverty et al., 1994) located between positions -33 and -27 relative to the reported 5'-end of the *ALCAM* cDNA (Bowen et al., 1997; Degen et al., 1998) (Figure 4.10A). Also present near this element, at positions -13, -71 and -135, are binding sites for the transcription factor USF, which is known to interact synergistically with Inr binding proteins, such as TFII-I, to activate Inr-dependent transcription (Roy et al., 1997). As no consensus Inr elements are found at the sequences corresponding to the reported 5-end of the *ALCAM* cDNA, it is likely that the reported cDNA sequences are incomplete and *ALCAM* transcription is initiated from the Inr element at position -33.

Consistent with the ability of PEA3 to activate *ALCAM* transcription, the *ALCAM* promoter sequences contain five matches to the consensus PEA3-binding element 5'-(T/C)(T/G)CCGGA(A/T)(G/C)CG-3' (Bowman and Hassell, unpublished data), located at positions -127, -198, -568, -608 and -764. However, it is likely that only the site at

position -127 is functional, as PEA3 can activate luciferase expression from all three *ALCAM* promoter conustructs used in this study (Figures 4.11 and 4.12). In fact, the construct ALCAM -263, which contains only the PEA3-binding site at position -127, shows the highest activation of luciferase expression by PEA3 of all the *ALCAM* promoter constructs.

The *ALCAM* promoter sequences isolated in this study also contain consensus binding sites for a number of other transcription factors, some of which are known to act synergistically with PEA3. For example, the *ALCAM* promoter sequences contain three consensus AP-1 binding sites (Angel and Karin, 1991) and a consensus binding site for members of the Tcf-Lef family of transcription factors (Geise et al., 1991), both of which synergize with PEA3 to activate transcription from the *MMP-7* promoter (H. Crawford, personal communication). Similarly, the *ALCAM* promoter contains four consensus binding sites for C/EBPβ, which has been shown to act synergistically with PEA3 to activate transcription from the cyclooxegenase-2 (*COX-2*) promoter (L. Howe, personal communication). The transcription of both *MMP-7* and *COX-2* is activated by growth factors of the wingless (Wnt) family (Crawford et al., 1999; Howe et al., 1999). This suggests that PEA3 may be important in the response to signals generated by these factors, with Wnts either directly leading to activation of PEA3 or to activation of transcription factors that synergize with PEA3.

Wnt signalling is initiated by the binding of Wnts to members of the frizzled family of transmembrane growth factor receptors (Bhanot et al., 1996). This results in a number of intracellular responses, the best characterized of which is the stabilization of β-catenin. In the absence of a Wnt signal, β-catenin exists in a complex with either

cadherins and the actin cytoskeleton, or with the proteins axin, adenomatous polyposis coli (APC) and glycogen synthase kinase 3β (GSK3β) (Hart et al., 1998; Ikeda et al., 1998; Eastman and Grosscheldt, 1999). When in complex with GSK3β, β-catenin becomes phosphorylated, leading to its ubiquitination and proteosome mediated degradation (Orford et al., 1997). Wnt signals, transmitted via members of the dishevelled (Dsh) family of proteins, lead to the inhibition of β-catenin phosphorylation by GSK3β, stabilization of β-catenin and elevated levels of free β-catenin (Paphkoff et al., 1996; Eastmann and Grosscheldt, 1999). Free β-catenin is able to translocate to the nucleus and interact with and activate members of the Tcf-Lef family of transcription factors (Behrens et al., 1996). When not interacting with β-catenin, Tcf-Lef factors may actively repress transcription, possibly due to interactions with transcriptional repressor proteins (Brannon et al., 1997; Nusse, 1999). These interactions are disrupted by the binding of β-catenin to Tcf-Lef factors, which causes the conversion of these factors from transcriptional repressors to activators.

While stabilization of β-catenin and activation of Tcf-Lef factors is the best characterized method through which Wnt signals are transduced, this process can occur through a number of different pathways (Arias et al., 1999). Wnt proteins are able to interact with many extracellular and cell surface proteins, and impinge upon many intercellular signaling pathways. For example, Wnts can signal through Dsh proteins to activate kinases of the JNK family (Li et al., 1999). These kinases can, in turn, activate the transcription factors c-Jun and PEA3, which, as has been discussed, are able to synergize with Tcf-Lef factors to activate transcription from certain promoters, such as

that of *MMP-7*. In the case of *MMP-7*, Wnts are able to generate multiple signals that impinge upon the same promoter (H. Crawford, personal communication).

Inappropriate expression of *wnts*, or other genes involved in Wnt-mediated signaling pathways, have been associated with the development of certain cancers, including breast and colorectal cancer. For example, transgenic mice that overexpress *wnt-1* in their mammary epithelium, show mammary gland hyperplasia and develop adenocarcinomas (Tsukamoto et al., 1988). Also, *wnt* overexpression has been detected in a proportion of naturally occurring human breast tumors (Huguet et al., 1994; Iozzo et al., 1995). Interestingly, PEA3, which is thought to be involved in Wnt-mediated signaling, has also been associated with the development and progression of breast cancers (Benz et al., 1997).

As has been discussed, *COX-2* expression is activated in response to Wnt signaling (Howe et al, 1999). Consistent with this, the *COX-2* promoter contains two potential binding sites for Tcf-Lef factors. However, overexpression of β-catenin is by itself insufficient to cause activation of transcription from this promoter. This suggests that transcription factors other than Tcf-Lef family members, activated by Wnt-mediated signaling pathways, are required for efficient transcriptional activation of the *COX-2* promoter. Perhaps multiple transcription factors must be present for an efficient response to Wnts, such as is the case for the *MMP-7* promoter, where Tcf-Lef factors, c-Jun and PEA3 must all be present for the most efficient response (H. Crawford, personal communication).

A similar scenario may be true for the *ALCAM* promoter. This promoter contains sites for a number of factors known to act synergistically with PEA3. It is possible that a

number of these factors, as well as PEA3, must be present in an active state for large increases in *ALCAM* transcription to occur. Cotransfection of MCF-7 cells with both *PEA3* and activated β-catenin does not result in higher levels of transcription from the *ALCAM* promoter than is observed upon transcription with *PEA3* alone, indicating that PEA3 does not synergize with Tcf-Lef factors on this promoter. However, it is possible that PEA3 may be able to act synergistically with C/EBPβ, c-Jun or other transcription factors to produce stronger effects of the *ALCAM* promoter than the modest transcriptional activation caused by PEA3 alone.

5.2 The Biology of ALCAM.

ALCAM (also known as BEN, SC-1, DM-GRASP and CD166) is a member of the immunoglobulin-like class of cell adhesion molecules (Section 1.4.2.3). It is comprised of an extracellular domain containing two V-type immunoglobulin domains and three C-type immunoglobulin domains in the order VVCCC, a transmembrane domain and a short cytoplasmic domain (Burns et al., 1991; Tanaka et al., 1991; Porquie et al., 1992a; Bowen et al., 1995). *ALCAM* is expressed in a variety of different cell types during various stages of development, including neurons, thymic epithelial cells, activated T cells and monocytes, fibroblasts, endothelial cells and keratinocytes (Bowen and Afuffo, 1999). Also, ALCAM has been shown to have a variety of diverse biological functions.

One of the better understood functions of ALCAM is its role in the development of the nervous system of a number of organisms. *ALCAM* is transiently expressed in dorsal root ganglia sensory neurons, sympathetic neurons, motor neurons of the spinal

cord and brain, cerebellar climbing fibers, retinotectal projections and the dorsal funiculus and floorplate cells of the spinal cord (Burns et al., 1991; Pourquie et al., 1992b; Pollerberg and Mack, 1994). *ALCAM* expression in these cells generally occurs during axonogenesis with ALCAM protein being localized to the axon as it extends towards its target cells. A number of experiments have suggested that the interaction of ALCAM with cell surface molecules of neighboring cells is important for this process. For example, anti-ALCAM antibodies are able to inhibit the extension of neurites from sympathetic ganglia (Burns et al., 1991). Conversely, many neuronal cell types that express *ALCAM* will extend neurites on substrates to which purified ALCAM has been adsorbed (Burns et al., 1991; DeBernardo and Chang, 1995). The interaction of ALCAM with other neuronal cell proteins, such as Ng-CAM, has also been shown to stimulate neurite extension (DeBernardo and Chang, 1996).

ALCAM is expressed in motor neurons during times of axonogenesis and synaptogenesis, after which its expression is downregulated (Pourquie et al., 1992a). Similarly, during muscle development, myoblasts express *ALCAM* until synapses with motor neurons are formed, after which *ALCAM* expression is rapidly downregulated and ALCAM protein becomes restricted to the sites of neuromuscular contact (Fournier-Thibault et al., 1999). It appears that a homotypic interaction of ALCAM molecules of the motor neuron with those of the myoblast is important in the formation of neuromuscular contacts. Also, denervation of mature muscle fibers leads to a restoration of *ALCAM* expression that is maintained until regeneration of the motor nerve and reinnervation of the muscle occurs. This suggests that all myogenic cells express *ALCAM* in the absence of innervation (Fournier-Thibault et al., 1999).

ALCAM is also expressed in a number of cells of the immune system, including activated B cells, T cells and monocytes, and thymic epithelial (TE) cells (Wee et al., 1994; Patel et al., 1995; Bowen et al., 1995). *ALCAM* is expressed in mitogen-activated T cells, with expression levels peaking 48 hours after stimulation and returning to undetectable levels by 8 days after activation (Bowen et al., 1995). *ALCAM* is also expressed in monocytes under conditions in which differentiation into macrophages occurs. *ALCAM* expression in these cells is augmented by the cytokines interleukin-3 (IL-3), macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GMCSF) (Levesque et al., 1998).

ALCAM is a ligand for another cell surface protein of the immune system, CD6, which it binds with a 1:1 stoichiometry (Bowen et al., 1995; Bowen et al., 1996). CD6 is a member of the scavenger receptor cysteine-rich (SRCR) family of cell surface proteins, which are characterized by domains homologous to the type I macrophage scavenger receptor (Aruffo et al., 1991; Resnick et al., 1994). *CD6* is expressed in thymocytes and mature T cells (Aruffo et al., 1991). The interaction of CD6 molecules on the surface of thymocytes with ALCAM molecules on the surface of TE cells has been shown to be important for the binding of thymocytes to the TE and their differentiation into mature T cells. For example, the binding of thymocytes to the TE is inhibited by both anti-CD6 and anti-ALCAM antibodies (Vollger et al., 1987; Bowen et al., 1995). Conversely, COS cells transfected with CD6 gain the ability to interact with TE cells, which is inhibited by both anti-CD6 and anti-ALCAM antibodies (Bowen et al., 1995).

The interaction of ALCAM with CD6 may also contribute to T cell activation. Anti-CD6 antibodies can induce or enhance T cell activation under certain conditions,

suggesting that signaling through CD6 may be important for this process (Horimoto et al., 1988; Gangeni et al., 1989). For example, ALCAM molecules on the surface of macrophages may interact with CD6 molecules on the surface of T cells during antigen presentation at sites of inflammation, contributing to T cell activation (Bowen et al., 1995; Levesque et al., 1998).

ALCAM has also been implicated in osteogenesis. *ALCAM* is expressed in mesenchymal stem cells of the bone marrow, which are capable of differentiating into a number of tissues, including bone, cartilage, tendon, muscle or fat (Bruder et al., 1998). Upon differentiation of these cells, *ALCAM* expression is lost. Furthermore, osteogenic differentiation of these cells is accelerated in the presence of anti-ALCAM antibodies. However, the precise role ALCAM plays in osteogenic differentiation is currently not understood.

ALCAM is able to bind high-density lipoprotein (HDL) and may play a physiological role as an HDL receptor (Matsumoto et al., 1997). HepG2 or CHO cells transfected with *ALCAM* show elevated HDL-binding activity. Also, the differentiation of monocytes into macrophages results in increased ability of these cells to bind HDL. Again, the role of ALCAM in lipid metabolism is not well understood at this time.

Finally, ALCAM has been implicated in tumor formation and progression, particularly in breast cancer and melanoma. While *ALCAM* is not normally expressed in the mammary epithelium, it is expressed at high levels in certain breast cancer derived cell lines (Bowen et al., 1995). Similarly, *ALCAM* is expressed in a number of melanoma derived cell lines, with expression level correlating with the invasive and metastatic potential of these cells (van Groningen et al., 1995; Degen et al., 1998).

5.3 Possible Biological Significance of Activation of *ALCAM* Transcription by PEA3.

There is significant reason to believe that the activation of *ALCAM* transcription by PEA3 observed in this study is biologically significant. While there is significant overlap in the expression patterns of *PEA3* and *ALCAM*, this overlap is not perfect. For example, *PEA3* is not expressed in any of the cells of the hematopoetic system or leukocytes, while *ALCAM* is expressed by the thymic epithelium and activated T cells and monocytes (Laing, 1998; Chotteau-Lelivre et al., 1997; Wee et al, 1994; Patel et al., 1995; Bowen et al., 1995). Therefore, PEA3 is not likely to play a role in *ALCAM* expression in these tissues. Since the *ALCAM* promoter contains binding for many transcription factors (Section 4.2.7), one would not expect PEA3 to be absolutely necessary for *ALCAM* expression. Moreover, a requirement for other transcription factors to act synergistically with PEA3 could explain a lack of *ALCAM* expression in tissues that express *PEA3*.

5.3.1 Possible Roles for PEA3 and ALCAM in Nervous System and Muscle Development.

The co-expression of *ALCAM* and *PEA3* in myoblasts and neurons during neuromuscular development suggests important functions for these molecules in this process. *PEA3* is expressed in myoblasts as these cells are proliferating and migrating to their appropriate positions (Laing, 1998). When these cells cease dividing and differentiate into myocytes, *PEA3* expression in downregulated. Similarly, during muscle repair, *PEA3* expression becomes activated during satellite cell proliferation and

is downregulated as they differentiate into myocytes (Taylor et al., 1997; Section 1.7.3). *ALCAM* is also expressed in myogenic cells during periods of proliferation and migration, with downregulation of *ALCAM* expression occurring as these cells differentiate and contacts with motor neurons are formed (Fournier-Thibault et al., 1999). The similar expression patterns of *PEA3* and *ALCAM* suggest that PEA3 may be important for *ALCAM* expression in these cells. As the interaction of ALCAM molecules on myogenic cells and motor neurons is thought to be important in the formation of neuromuscular contacts, this implicates PEA3 in this process as well.

During the development of the nervous system, *PEA3* is expressed in subpopulations of spinal cord motor neurons that are destined to innervate specific muscles, and dorsal root ganglia sensory neurons that will provide sensory input from the same muscles (Lin et al., 1998). *PEA3* expression occurs in motor neurons as axons are being extended into the limb and is thought to be important for the targeting of these neurons to their appropriate muscles. Activation of *ALCAM* expression by PEA3 may be important in this process. *ALCAM* is expressed in a number of neuronal cell types during nervous system development, including spinal cord motor neurons and dorsal root ganglia sensory neurons, which also express *PEA3* (Burns et al., 1991). The interactions of cell surface molecules, such as ALCAM, on extending axons with cell surface molecules on neighboring neurons or ECM molecules is one factor that helps to guide these axons to their proper targets (Hynes and Lander, 1992). Changes in the strength of these interactions may cause an axon to alter the direction of its extension as this targeting process occurs. Therefore, as *PEA3* becomes expressed in motor and sensory

neurons, increased *ALCAM* expression, leading to changes in the path of axon extension towards the proper target cells, may occur.

While the pattern of *PEA3* expression suggests a role in neuromuscular development, no gross abnormalities in this process have been observed in *PEA3*-null mice (Laing, 1998). It has been suggested that this is due to redundancy of *PEA3* function, with the expression of another *ets* gene or genes able to compensate for the loss of its expression. Studies on *ER81*-null mice suggest that *ets* gene expression is important for axon targeting. During development of the chick nervous system, *ER81*, like *PEA3*, is expressed in spinal cord motor neuron pools destined to innervate specific muscles, and the dorsal root ganglia sensory afferent neurons that will provide feedback from these muscles (Lin et al., 1998). During mouse development, *ER81* is expressed in the corresponding motor neuron pools, but is expressed in all sensory afferent neurons instead of specific pools of these neurons (S. Arber, personal communication). The sensory afferents of *ER81*-null mice fail to form connections with spinal cord motor neurons, suggesting that *ER81* expression is required for proper axon guidance in these cells. If *PEA3* does, in fact, play a role in the proper targeting of the neuronal cell populations in which it is expressed, it is quite likely that elevation of *ALCAM* expression levels contributes to this process.

Male *PEA3*-null mice are sterile, possibly due to an ejaculatory of erectile dysfunction (Laing, 1998; Section 1.7.3). As these processes are controlled by the autonomic nervous system, this phenotype may be the result of defects in the autonomic innervation of the corpus cavernosum smooth muscle. An erectile defect may be the result of insufficient parasympathetic innervation of the cavernous smooth muscle,

inhibiting smooth muscle relaxation and preventing blood flow into the cavernous spaces. Also, an over-active sympathetic nervous system could have the same result (Giuliano et al., 1995). *ALCAM* is expressed in autonomic neurons and has been shown to support nurite extension from sympathetic ganglia (Burns et al., 1991). Therefore, ALCAM may be involved in the proper pathfinding of autonomic neurons during development. While the expression of *PEA3* in sympathetic or parasympathetic neurons has not been studied, it is possible that its loss in these cells may result in the male sterility observer in *PEA3*-null males. It may be hypothesized that *PEA3* expression in sympathetic or parasympathetic neurons destined to innervate the cavernous smooth muscle is required for proper pathfinding of these neurons, and that activation of *ALCAM* expression by PEA3 is important for this process. Loss of *PEA3* expression in these cells would then result in reduced *ALCAM* expression, which would contribute to inappropriate axon pathfinding and improper autonomic innervation of the cavernous smooth muscle.

5.3.2 Possible Roles for PEA3 and ALCAM in Tumor Progression.

Both *PEA3* and *ALCAM* expression have been associated with increased invasive and metastatic potentials of tumor cells. For example, transfection with *PEA3* can confer an invasive phenotype upon the non-invasive MCF-7 breast cancer cell line (Kaya et al., 1996). Similarly, *ALCAM* expression levels in melanoma derived cell lines correlate with the invasive and metastatic potentials of these cells (van Groningen et al., 1995). Therefore, the ability of PEA3 to activate *ALCAM* expression may contribute to the increased invasive and metastatic abilities associated with *PEA3* overexpression in tumor cells.

One might expect that tumor cells that overexpress *ALCAM* would adhere to one another more tightly, and thus would be less likely to metastasize, exactly the opposite of what has been observed. However, a number of features of ALCAM biology suggest how *ALCAM* overexpression may contribute to increased metastatic ability. Vascular endothelial cells have been shown to express *ALCAM* (Bowen and Aruffo, 1999). *ALCAM* expressing tumor cells that have escaped into the vasculature could interact with and adhere to *ALCAM* expressing endothelial cells. This would facilitate the escape of the tumor cell from the vasculature, and thus the formation of secondary tumors. Since ALCAM can engage in a number of heterophilic interactions, endothelial cells may also express other ALCAM ligands as well, similarly contributing to the ability of *ALCAM* expressing tumor cells to escape the vasculature.

ALCAM overexpression may also contribute to tumor progression by the disruption of anti-tumor immunological responses. ALCAM molecules on the surface of tumor cells may interact with ALCAM on the surface of activated T cells or antigen presenting macrophages infiltrating the tumor. Similarly, tumor cell ALCAM may interact with CD6 molecules on the surface of T cells. These interactions may interfere with the normal interactions of T cells with antigen presenting cells and thereby disrupt the normal immune response to the tumor.

5.4 Summary and Future Perspectives.

In this study, PEA3 has been shown to activate transcription of the gene encoding the cell adhesion molecule ALCAM. While the magnitude of this activation is modest, it has been consistently observed using a number of systems. The presence of other

transcription factors that can act synergistically with PEA3 may be required for a greater induction of *ALCAM* activity. This can be tested by co-transfection experiments, using the *ALCAM* promoter/reporter constructs (Section 4.2.7), *PEA3* and various combinations of activated β–catenin, *c-jun*, *C/EBPβ* and other transcription factors that might interact with PEA3.

While modest, the activation of *ALCAM* expression by PEA3 is very likely biologically significant. This finding has shed light on the possible roles PEA3 may play in nervous system development. Transcriptional activation of *ALCAM* by PEA3 may be important for the axonal pathfinding of certain motor, sensory and autonomic neurons. To confirm this, experiments must be carried out to determine if the expression levels of *ALCAM* become elevated in *PEA3*-expressing motor and sensory neurons at times when *PEA3* is expressed. Furthermore, the expression of *PEA3* in sympathetic and parasympathetic ganglia during development must be confirmed, particularly for those that innervate the male reproductive system. If *PEA3* is in fact expressed in these tissues, *ALCAM* expression levels must again be tested to determine if they increase when *PEA3* is expressed.

Finally, the activation of *ALCAM* expression by PEA3 may contribute to the ability of PEA3 to confer an invasive phenotype upon certain tumor cells. The ability of PEA3 to activate the expression of various metalloproteinases, and the ability of these molecules to contribute to the metastatic process has been well documented (Sections 1.4.1.2 and 1.7.2). However, elevated levels of ALCAM in *PEA3*-expressing tumor cells may also contribute to this process. To confirm this, experiments must be conducted to compare *ALCAM* expression levels in *PEA3*-expressing tumors with normal tissue

adjacent to these tumors, or with non-*PEA3*-expressing tumors of the same type to determine it a correlation between *PEA3* and *ALCAM* expression in these tissues indeed exists.

CHAPTER 6

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