

**PEA3 SUBFAMILY TRANSCRIPTIONAL ACTIVATION OF OSTEOPONTIN,
A TRANSFORMATION-ASSOCIATED PROTEIN**

By

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ABSTRACT

PEA3, ERM, and ER81 comprise a subfamily of ETS transcription factors that upregulate genes correlated with an increased metastatic potential of tumors. In mouse embryo fibroblast (MEF) cells, PEA3 is required for transformation by activated Ras or Neu, but the means by which PEA3 mediates Ras-transformation is not clear. Osteopontin (OPN) expression is induced upon H-ras-transformation and purified PEA3 can bind the OPN promoter by gel-shift analysis. In this study, OPN expressed higher transcript levels in the wildtype MEF 4 cell line than the PEA3 null MEF 1 cell line and was further characterized as a potential PEA3 target gene by Northern blot analyses and transient transfection studies. Northern blot analyses of 4 wildtype MEF (4, 100, 101, 104), 5 PEA3 null MEF (1, 115, B5, B10, B12), and 5 MEF 1 retransformant cell lines that stably reexpress PEA3 showed a good correlation between OPN and ERM transcript levels in 9/11 cell lines although at least 2 PEA3 subfamily members were coexpressed in 8/11 cell lines that expressed high OPN transcript levels. This suggested that the PEA3 subfamily additively regulated OPN and that ERM protein was more abundant than PEA3 and ER81 protein levels in the MEF cell lines. The relative PEA3 subfamily protein levels remain to be clarified. Transient transfection assays in the HEK 293-1C cell line indicated that the OPN promoter was responsive to PEA3 and that the promoter region between -258 to -88 was required for maximal OPN promoter activity. There are 16 candidate core ETS binding sites in the -777/+79 OPN promoter which could be responsible for PEA3 subfamily transactivation. The OPN promoter was more active in the MEF 4 cell line than the MEF 1 cell line, corresponding to their relative number of

expressed PEA3 subfamily members. Ectopic expression of dominant negative PEA3 suppressed OPN promoter activity in the MEF 4 cell line. Furthermore, ectopic expression of PEA3, ERM, or ER81 increased OPN promoter activity in the MEF 1 or COS-1 cell line. Thus OPN is transcriptionally regulated by the PEA3 subfamily and represents a target gene that can mediate the progression of tumor cells.

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ABBREVIATIONS

GAPDH	glyceraldehyde 3-phosphate dehydrogenase
MEF	mouse embryo fibroblast
MMTV	mouse mammary tumor virus
OPN	osteopontin
PEA3	polyomavirus enhancer activator 3
RDA	representational difference analyses

INTRODUCTION

1. PEA3 is a member of the ETS family of transcription factors

PEA3 (polyomavirus enhancer activator 3) belongs to the Ets transcription factor family (Xin et al., 1992). There are over 30 Ets members that are represented in metazoans that function in development, growth and oncogenesis (Dittmer and Nordheim, 1998). *ETS* genes are segregated into 13 subfamilies depending on their sequence similarity (Laudet et al., 1999). Multiple Ets proteins are expressed with varying degrees of tissue specificity within a given species. All Ets proteins have a conserved 84 to 90 amino acid winged helix-turn-helix ETS domain that is required and sufficient for DNA binding (Karim et al., 1990). The ETS domain recognizes a 9 to 15 base pair sequence with a 5'-GGA^A/T-3' core and the specificity of binding is determined by the flanking nucleotides (Wasylyk et al., 1993).

The activity of Ets proteins is mediated and modulated by domains that are well conserved within subfamilies (Dittmer and Nordheim, 1998). For instance, the PEA3 subfamily has a highly conserved amino terminal acidic domain responsible for transcriptional activation (de Launoit et al., 1997). Auto-inhibitory domains that negatively regulate DNA binding flanking the ETS domain are found in Elk-1 (Janknecht et al., 1994), Ets-1 (Donaldson et al., 1996; Skalicky et al., 1996) and the PEA3 subfamily (Bojovic and Hassell, 2001; Brown et al., 1998; Laget et al., 1996). The pointed domain present in about half of the *ETS* genes plays a role in protein-protein interactions, which can affect Ets activity (Carroll et al., 1996; Golub et al., 1996).

Most Ets proteins except GABP α bind to DNA as monomers but cooperation between Ets proteins and other transcription factors can facilitate DNA binding (Bassuk and Leiden, 1997). For example, binding of a AP-1 components c-Jun and c-Fos to an AP-1 binding site can transcriptionally activate the tissue inhibitor of metalloproteinases-1 (TIMP-1) whereas binding of an adjacent Ets-1 protein alone can not transactivate TIMP-1 expression (Logan et al., 1996). However, Ets-1 can physically complex with c-Jun and c-Fos to synergistically activate TIMP-1 expression (Logan et al., 1996).

Signal transduction pathways can also modulate DNA binding, protein-protein interaction and transactivation activity of Ets proteins (Graves and Petersen, 1998). Upstream regulation from a MAP kinase has been implicated for various *ETS* genes. ERK 1 or ERK2 and SAPK phosphorylate ELK-1 at the same sites *in vivo* as *in vitro* and a mutation in these sites disrupts transactivation (Treisman, 1996). Similarly, ERK2 phosphorylates Ets-1 (Rabault et al., 1996) and a transactivation domain mutation abolishes Ras activation (Yang et al., 1996). Both MAPK and PKA can induce ERM activity (Janknecht et al., 1996). ER81 is also downstream of the Ras/Raf/MEK/ERK pathway (Janknecht, 1996).

2. Ets proteins are implicated in oncogenesis

Overexpression or chromosomal translocation of *ETS* genes can lead to a variety of mouse and human cancers. For example, ectopic overexpression of PU.1 in mice causes erythroleukemia (Moreau-Gachelin et al., 1996) and Ets-1 is overexpressed in 64% of gastric adenocarcinomas in humans (Nakayama et al., 1996). Translocation of the amino

terminal end of the *ETS* gene TEL to other transcription factor such as AML-1 (Shurtleff et al., 1995), or to tyrosine kinases such as the PDGF beta receptor (Carroll et al., 1996) or ABL (Golub et al., 1994) can cause human myelomonocytic leukemia. Ewing's sarcoma in humans is predominantly caused by a chromosomal translocation of the amino-terminal region of EWS to the DNA-binding domain of the *ETS* gene FLI-1 (Delattre et al., 1992). Fusion of EWS to four other *ETS* genes ERG (Sorensen et al., 1994), ETV1 (Jeon et al., 1995), E1AF (Kaneko et al., 1996; Urano et al., 1996), or FEV (Peter et al., 1997) can also cause Ewing's sarcoma.

3. The PEA3 subfamily is associated with oncogenesis

The PEA3 subfamily of Ets proteins consists of three members conserved between zebrafish, mice and humans: PEA3/E1AF/ETV4 (Higashino et al., 1995; Roehl and Nusslein-Volhard, 2001; Xin et al., 1992), ER81/ETV1 (Brown and McKnight, 1992; Jeon et al., 1995) and ERM/ETV5 (Laget et al., 1996; Monte et al., 1994; Roehl and Nusslein-Volhard, 2001). The PEA3 subfamily share a 95% sequence similarity within the ETS domain, 85% sequence similarity in their acidic domain and are 50% homologous in sequence overall (Monte et al., 1995). There are negatively regulatory elements that flank the ETS domain and transactivation domain in PEA3 that respectively inhibit DNA binding and activation (Bojovic and Hassell, 2001). The autoregulatory regions flanking the ETS domain can complex with USF-1 to relieve PEA3 autoinhibition (Greenall et al., 2001).

Several growth signals can induce PEA3 activity, including the tumor promoter TPA (Gutman and Wasylyk, 1990), EGF (Rorth et al., 1990), FGF-2 (D'Razio et al., 1997), FGF-3 and FGF-8 (Raible and Brand, 2001). The transactivation of the PEA3 subfamily is mediated by the Ras/MAP kinase and protein kinase A signal transduction pathways (Janknecht, 1996; Janknecht and Hunter, 1996). The expression of the PEA3 subfamily overlaps temporally and spatially throughout mouse development (Chotteau-Lelievre et al., 1997). In adult mice ERM expression is more widespread (Monte et al., 1994; Monte et al., 1995) than PEA3 (Xin et al., 1992) and ER81 (Monte et al., 1995). All three PEA3 subfamily members have the highest expression in the brain (Monte et al., 1995) and to a lesser extent in the mammary gland (Shepherd et al., 2001).

PEA3 is overexpressed in 76% of human breast tumors (n=74) (Benz et al., 1997). About 20% to 30% of all breast cancers are HER2/Neu positive breast cancers, of which 93% overexpress PEA3 (Benz et al., 1997). In transgenic MMTV-Neu and MMTV-polyomavirus middle T antigen mice, PEA3 mRNA is overexpressed in mammary adenocarcinomas and in most of the tumors metastasized to the lung (Trimble et al., 1993). Further studies show that all 3 PEA3 subfamily members are coordinately upregulated in MMTV-Neu mammary tumors in comparison to age-matched control or adjacent normal mammary tissue (Trevor Shepherd, personal communication). The relationship between the PEA3 subfamily and HER2/Neu mammary adenocarcinomas has been further investigated *in vivo*.

Several lines of evidence show that PEA3 is required for Neu induced transformation by the Ras signal transduction pathway. Activated Neu can transform the NIH 3T3 cell

line and cotransfection of dominant negative PEA3 decreases the number of transformed cells, suggesting that Ets proteins are involved in Neu-induced tumorigenesis (Trevor Shepherd, personal communication). To define the role of PEA3 in activated Ras or Neu transformed cell, mouse embryonic fibroblast (MEF) immortalized cell lines derived from mice with a targeted knockout of PEA3 as well as syngeneic wildtype mice were infected with constitutively active forms of Ras or Neu (Gina Fidalgo, personal communication). Activated Ras or Neu can transform wildtype MEF cell lines whereas PEA3 null cell lines are refractory to transformation. Furthermore, cell lines derived from the PEA3 null MEF 1 cell line that have PEA3 stably transfected in them have a restored capacity to be transformed by Ras or Neu (Gina Fidalgo, personal communication). Since PEA3, ERM, and ER81 have increased transcript levels in MMTV-Neu mammary adenocarcinomas, the entire PEA3 subfamily likely mediates Ras or Neu mediated transformation by regulating downstream target genes.

PEA3 activates multiple proteins involved in degrading major components of the extracellular matrix that can lead to extracellular matrix remodeling, tumor invasion, and metastasis. PEA3 regulates representatives from 4 different major classes of matrix metalloproteinases (MMP), including MMP-1/interstitial collagenase (Higashino et al., 1995), MMP-3/stromelysin 1 (Kaya et al., 1996) and MMP-7/matrilysin (Crawford et al., 2001), MMP-9/gelatinase B (Kaya et al., 1996), and MMP-14/MT1-MMP (Habelhah et al., 1999; Nerlov et al., 1992). PEA3 also regulates other proteins that regulate MMP activity including urokinase plasminogen activator (uPA) enhancer (Rorth et al., 1990), and TIMP-1 (Edwards et al., 1992). In addition, PEA3 cooperates with AP-1 for optimal

transactivation of uPA (Nerlov et al., 1992) and MT1-MMP (Habelhah et al., 1999; Nerlov et al., 1992). The increased expression and activity of many of these extracellular matrix proteinases leads to increased cell invasiveness (Westermarck and Kahari, 1999). Overexpression of PEA3 has been shown to induce MMP-9 in a human mammary cell line and results in increased metastasis (Kaya et al., 1996), which can be reversed with transient transfection of antisense PEA3 (Hida et al., 1997). Hence PEA3 can mediate cell-migration and metastasis of tumor cells by upregulation of target gene expression. The identity of more PEA3 target genes would help elucidate the mechanism by which the PEA3 subfamily function in tumorigenesis.

4. Osteopontin and oncogenesis

Osteopontin (OPN) is a secreted glycosylated phosphoprotein ligand that can bind to integrins, increase cell adhesion to the extracellular matrix, and increase cell migration (Denhardt and Guo, 1993). OPN can act as a macrophage chemoattractant and stimulate immune responses (Denhardt and Noda, 1998). OPN is highly expressed in bone, cartilage, kidney, lung, and brain. OPN is secreted through many body fluids and OPN protein is associated with luminal surfaces of epithelial cells by immunohistochemistry (Brown et al., 1992). Many stimuli can induce OPN expression including growth factors such as EGF, hormones including estrogen and calcitriol (vitamin D₃), and chemical carcinogens such as TPA (Craig and Denhardt, 1991).

The glycine-arginine-glycine-aspartic acid-serine (GRGDS) motif in OPN protein is required for binding to integrins (Xuan et al., 1995), thereby increasing cell adhesion and

facilitating cellular rearrangements required in development and other processes. OPN can bind integrins $\alpha_5\beta_3$ (Oldberg et al., 1986), $\alpha_5\beta_5$ (Hu et al., 1995), $\alpha_5\beta_1$ (Liaw et al., 1995), $\alpha_6\beta_1$ (Smith et al., 1996), $\alpha_8\beta_1$ (Denda et al., 1998), and $\alpha_4\beta_1$ (Bayless et al., 1998; Bayless and Davis, 2001). Exposure to the GRGDS motif in OPN is modified by proteolytic cleavage of OPN by thrombin (Senger et al., 1994) to facilitate recognition by integrins $\alpha_9\beta_1$ and $\alpha_4\beta_1$ (Bayless and Davis, 2001). MMP-3 and MMP-7 proteolysis of OPN increases cell adhesion and motility in tumor cells by an unknown mechanism (Agnihotri et al., 2001). OPN is also recognized by glycoprotein receptor CD44 to affect immune responses (Weber et al., 1996).

OPN is a subject of particular interest because the expression of OPN has been correlated with activated ras and src mediated transformed cell lines. V-src transformed cells have higher OPN expression (Tezuka et al., 1996) and cells from c-src null mice have decreased OPN expression levels in comparison to wildtype cells (Chackalaparampil et al., 1996), suggesting that OPN contributes to v-src mediated transformation. OPN transcript levels are also correlated with ras transcript levels and increased malignancy in a series of activated H-ras-transformed 3T3 cell lines (Chambers et al., 1992; Guo et al., 1995). Transfection of ectopic OPN in benign tumors cells causes progression to malignant cells (Oates et al., 1996; Chen et al., 1997). Furthermore, transient transfection of antisense OPN in a H-ras-transformed Rat-1 cell line reduces the tumor number when transplanted in nude mice (Gardner et al., 1994) and reduces the number of soft agar colonies formed in TPA-transformed JB6 epidermal cells (Su et al., 1995). H-ras transformed 3T3 cells from OPN null mice form fewer colonies in soft agar

assays than cells from wild-type mice, consistent with a role of OPN in tumor progression (Wu et al., 2000). Furthermore, H-ras transformed 3T3 cell lines derived from OPN null mice form tumors more slowly when injected in syngeneic wildtype or nude mice than transformed cells from wildtype mice (Wu et al., 2000). Thus functional studies with activated ras and src transformed cell lines indicate that OPN expression is not required for tumor development but increases tumor progression.

OPN expression is increased in many types of primary human cancers, including breast cancer (Brown et al., 1992). OPN protein is localized to tumor cells (Gillespie et al., 1997; Fisher et al., 2000) and OPN expression correlates with increased breast tumor metastasis (Tuck et al., 1997; Tuck et al., 1998). OPN mRNA and protein is upregulated in the mammary tumors of MMTV-v-H-ras or MMTV-c-myc mice, in comparison to normal mammary glands (Rittling and Novick, 1997). This is consistent with previous observations that OPN is a transformation-associated protein. However, surprisingly there is no difference in mammary tumor incidence or growth rate in MMTV-v-H-ras or MMTV-c-myc crossed with OPN null mice and wildtype mice (Feng and Rittling, 2000). Chemical carcinogen MNNG induced skin tumors in OPN null mice similarly have no difference in tumor incidence or growth rate in comparison to wildtype mice (Crawford et al., 1998). These results from OPN null mice suggest that OPN is not required for tumor development, in contrast to previous observations. However, H-ras transformed 3T3 cell lines derived from OPN null mice injected in wildtype mice form tumors more slowly than injected transformed wildtype cell lines (Wu et al., 2000). Therefore, OPN null mice may overexpress another integrin ligand with a redundant function as OPN,

thereby compensating for OPN in tumorigenesis. Thus tumor studies utilizing OPN null mice indicate that the role of OPN in tumorigenesis is more complex when subject to endogenous factors in mice and the mechanism by which OPN null mice compensate for OPN in H-ras or c-myc induced transformation remains unclear.

Upregulated OPN expression in tumors prompted investigations into the mechanism of OPN regulation, which is thought to be at the level of transcription (Denhardt and Noda, 1998). The OPN promoter has been cloned for the mouse (Craig and Denhardt, 1991), pig (Zhang et al., 1992), and human (Hijiya et al., 1994) and is well conserved up to -204 nucleotides (Hijiya et al., 1994). Oncogenes promote OPN promoter activity through several regions. A v-src transformed 3T3 cell line has increased OPN transcriptional activity relative to a parental NIH 3T3 cell line which is attributed to a CCAAT box-binding factor (Tezuka et al., 1996). A H-ras transformed 3T3 cell line also has higher activity than the parental NIH 3T3 cell line in the promoter region with an inverted CCAAT box, as well as in an OPN promoter region termed the Ras-Activated Enhancer (RAE) (Guo et al., 1995). The H-ras and v-src oncogenes mediate their effects on OPN transcriptional activity through transcription factors which have not yet been identified.

Transcriptional activators include Tcf-4 and β -catenin which upregulate OPN and induce metastasis of Rat mammary 37 cells (El-Tanani et al., 2001). Either c-myc or the upstream stimulatory factor (USF) binds E-box motifs, and mutational studies show that factors binding the E-boxes can synergistically upregulate OPN transcription with octamer motif-binding proteins (Oct 1/Oct 2) in human malignant astrocytoma cell lines

(Wang et al., 2000). Few OPN promoter studies have been made to identify the transcription factors that may mediate the transformation of tumor cells.

Other transcription factors have been identified that affect OPN expression in bone morphogenesis. Transcriptional repression of OPN by two homeobox transcription factors, Hox-c-8 and Hox-c-9 are counteracted by the transforming growth factor (TGF) and bone morphogenic protein (BMP) signaling pathways (Shi et al., 1999; Shi et al., 2001; Hullinger et al., 2001). The binding of Hox-c-8 with the OPN promoter is relieved by BMP2 stimulated Smad 1 binding and interaction with Hox-c-8 (Shi et al., 1999; Hullinger et al., 2001). Similarly TGF β stimulated Smad 4 interacts with and relieves repression due to a Hox-c-9 and Smad 3 complex that independently bind the OPN promoter (Shi et al., 2001). The intricate balance between the homeobox factors and TGF/BMP signaling modulate the spatial and temporal regulation of bone morphogenesis. SF-1 response elements in the OPN promoter are recognized by the nuclear receptors, estrogen receptor alpha and estrogen receptor related alpha receptor, and are also thought to play a role in bone development (Vanacker et al., 1998). OPN is a major non-collagenous bone protein and therefore many transcription factors thus identified have been related to bone morphogenesis.

Diverse members of the Ets family of transcription factors transactivate the OPN promoter to serve different roles. Ets-1 and Ets-2 can both bind and transactivate OPN in an osteoblast-like cell line (Vary et al., 2000). Ets-1 and PEBP2 α , a runt family protein, can each bind independently to adjacent sites on the OPN promoter, to synergistically upregulate OPN transcription in NIH 3T3 cells (Sato et al., 1998). Ets-1 is expressed in

proliferating pre-osteoblastic cells and Ets-2 is expressed in differentiating and mature osteoblasts suggesting that both Ets-1 and Ets-2 regulate OPN transcription in different phases of bone development (Vary et al., 2000). In contrast, PEA3 transcripts are not expressed in the osteoblast-like MC3T3-E1 cell line (Vary et al., 2000), suggesting that PEA3 is unlikely to play a role in bone development. Induction of another Ets protein, PU.1, increases the expression of OPN transcripts in murine erythroleukemia cells and may play a role in monocyte maturation (Yamada et al., 2001). Finally, a gel-shift assay shows that purified GST-PEA3 protein can bind to a -740/-713 DNA fragment of the OPN promoter that is more active in a H-ras-transformed 3T3 cell line relative to a parental NIH 3T3 cell line, suggesting that PEA3 may upregulate OPN transcriptional activity in response to ras-induced transformation (Guo et al., 1995). Multiple Ets family members can regulate OPN, although the ability and function of the PEA3 subfamily to regulate OPN expression remains to be elucidated.

OBJECTIVES

1. To identify potential PEA3 target genes from a difference product library whereby cDNA from the PEA3 null MEF 1 cell line was subtracted from the wildtype MEF 4 cell line cDNA. Subsequently OPN was identified as a candidate PEA3 target gene.
2. To determine whether PEA3 was required for OPN expression by assessing the relative transcript levels of PEA3, ERM, ER81 and OPN in 4 wildtype and 5 PEA3 null MEF cell lines. Relative OPN and the PEA3 subfamily transcript levels in 5 independent MEF 1 retransformant cell lines that stably reexpressed PEA3 were also assessed to determine whether an abundance of PEA3 subfamily members was sufficient to induce OPN expression.
3. To evaluate PEA3 responsive regions in the OPN promoter in the HEK 293-1C cell line, a cell line with low PEA3 expression. The relative OPN transcriptional activities in the MEF 4 cell line and the MEF 1 cell line were also compared and related to differences in OPN transcript levels.
4. To assess the ability of ectopic dominant-negative PEA3 to suppress OPN promoter activity in the MEF 4 cell line. Conversely, the ability of the PEA3 subfamily to upregulate OPN promoter activity was assessed in the PEA3 null MEF 1 cell line and COS-1 cell line.

MATERIALS

Life Technologies in Burlington, Ontario was the supplier of DNA size markers, BenchMARK prestained protein size markers, *Taq* DNA polymerase 1, 10X PCR buffer, TRIzol, LipofectAMINE, and restriction endonucleases. Life Technologies also supplied GeneScreen Nylon membrane and Kodak products.

Qiagen in Mississauga, Ontario was the supplier of the SuperFect Transfection Reagent and DNA purification kits.

The Strip-EZ kit and ULTRAhyb solution were supplied by Ambion (Austin, Texas, USA).

The Reporter Lysis System was purchased from Promega Corporation, Madison, Wisconsin, USA.

METHODS

1. Identification of putative PEA3 target genes

MEF 4 cell line cDNA that did not correspond to the MEF 1 cell line cDNA were selectively amplified by representation difference analyses and were cloned into a pCR2.1 library (Jihou Xin, personal communication). Bacterial colonies were streaked out and grown (37°C, overnight) on Luria-Bertolli agar plates containing 0.1 mg/ml ampicillin (LB-amp plates). Single colonies were picked and grown shaking overnight at 37°C in LB containing 0.1 mg/ml ampicillin (LB-amp). Bacteria was stored at -80 °C in 25% glycerol.

Colonies were diluted in 20 µl sterile double distilled Millipore filtered water (ddH₂O) and heated (99 °C, 5 minutes) in the Perkin Elmer GeneAmp PCR system 9600. A master mix (27.75 µl) was added to a volume of 47.75 µl consisting of 5 µl 10X PCR buffer (Gibco BRL) 0.62 mM MgCl₂, 125 uM deoxyribonucleotides (Amersham), and 1.3 µM of each primer AB 17075 (5'-GAGCTCGGATCCACTAGTAACG-3') and AB 17076 (5'-GCCGCCAGTGTGATGGATATCT-3') that flank the multiple cloning site of pCR2.1. Samples were denatured (95 °C, 5 minutes) and kept at 80 °C for 30 seconds while 1.25 U Taq DNA polymerase I (Gibco BRL) was added. PCR amplification was made by 25 cycles of denaturing (94 °C, 30 seconds), annealing (60 °C, 30 seconds), and extension (72 °C, 50 seconds) and followed by an extension (72 °C, 7 minutes) and hold cycle (4 °C).

PCR products (10 µl/50 µl) were resolved on a 1.5% agarose gel containing ethidium bromide (1 µg/ml) and visualized with ultraviolet light. Purified plasmids (PCR Purification Kit, Qiagen) were sequenced (Central Facility at the Institute for Molecular Biology and Biotechnology, McMaster University) using M13 Forward and Reverse primers that correspond to the pCR2.1 vector. Sequences were imported into Lasergene Navigator and identified by comparison to known genes in GenBank.

2. Plasmid preparation

a. Plasmids

The 5' OPN promoter deletion plasmids described in Guo et al., 1995, were generously donated by David T. Denhardt (Department of Biological Sciences, Rutgers University, Piscataway, New Jersey). They include the empty reporter vector pXP2-luciferase as well as the mouse 5' OPN promoter deletion mutants -777/+79, -670/+79, -472/+79, -258/+79, and -88/+79 fused to a firefly luciferase reporter. The longest OPN promoter extends from -777 to +79, the 3' end of exon 1 (Craig and Denhardt, 1991). Another reporter plasmid pGL3-44-5XPEA3-luciferase (Bojovic and Hassell, 2001) has five optimal PEA3 binding sites and was used as a positive control for PEA3 subfamily expression plasmid activity and a negative control for empty expression plasmid activity.

Expression plasmids included pCANmyc, pCANmycPEA3, pCANmycERM, pRc/RSV and pRSVmycER81. PCANmycΔNPEA3En is a myc epitope-tagged ETS domain of PEA3 fused to the *Drosophila melanogaster* engrailed repression domain (Trevor Shepherd, private communication).

b. Transformation

DH5 α *E. coli* cells were made competent by the calcium chloride method as described in Current Protocols of Molecular Biology. Plasmids (50 ng to 1 μ g) were transformed into *E. coli* DH5 α competent cells, incubated on ice 30 minutes, heat shocked (37°C, 45 seconds), incubated on ice 2 minutes, mixed with 0.9 ml SOC broth (Gibco BRL), incubated (37°C, 1 hour), and plated out on LB-amp plates. Individual colonies were picked and grown in LB-amp (37°C, overnight) and stored with 25% glycerol at -80°C. Plasmid DNA was purified as described in manufacturer's directions using Miniprep or Maxiprep Kits (Qiagen).

c. Restriction endonuclease digestions for probe templates

Restriction endonuclease digestions were carried out as directed by the manufacturer (Gibco BRL). Purified plasmids from the difference product library were digested with EcoRI to be used as cDNA probe templates. The OPN probe template was a 0.5 kb EcoRI cDNA fragment. The ETS domain is highly homologous between the PEA3 subfamily members, therefore in order to avoid cross-hybridization between ETS domains the PEA3, ERM, and ER81 cDNA probe templates were isolated from the 5' region of the genes. The mouse PEA3 subfamily probe templates were derived from a 0.3 kb Kpn1 fragment from pGEM-7-PEA3, a 0.5 kb EcoRI fragment from PCR11-5'ERM, and a 0.2 kb ER81 HdIII/Xba1 fragment from pBS-ER81. As an internal control, mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcripts were assessed with a

0.6 kb XhoI cDNA fragment from pBS-GAPDH. Products were mixed with 1 µg/ml ethidium bromide, fractionated in a 0.9% low melting point agarose gel, and visualized under UV light for comparison to a 100 bp DNA size marker (Gibco BRL). Probe templates were excised from the gel and purified by QiaQuick Gel Purification (Qiagen).

3. Northern blot analyses of the PEA3 subfamily, OPN, and GAPDH

a. RNA extraction

Total RNA was isolated from cells with 2 ml TRIzol Reagent (Gibco BRL) as described by the manufacturer's directions. Two ml of Trizol was used per 100 mm² plate of cells. RNA was stored in diethyl pyrocarbonate (DEPC)-treated ddH₂O at -80°C. Trevor Shepherd generously donated total RNA from the FM3A mammary cell line.

b. Northern blot analysis

Total RNA was quantified by spectrophotometer analyses (Beckman DU 640) at an optical density of λ260. For each sample 20 µg of total RNA was denatured with 1X MOPS running buffer [20 mM 3-(N-morpholino)-propanesulfonic acid (pH 7), 5 mM sodium acetate, 1 mM EDTA (pH 8)], 16.5 % formaldehyde, and 50% formamide, to a total volume of 20 µl, and heated in a 65°C waterbath for 10 minutes (as described in Current Protocols in Molecular Biology). The denatured RNA was chilled ice for 5 minutes and then mixed with both 2 µg ethidium bromide and 2 µl formaldehyde loading buffer (1 mM EDTA (pH 8), 0.25% Bromphenol Blue, 0.25% Xylene Cyanol, 50% glycerol).

A 1% denaturing agarose gel was prepared by melting 1% (w/v) agarose in 1X MOPS in a microwave and adding 9% (v/v) formaldehyde after the agarose solution had cooled. Samples were run with 1X MOPS running buffer and 9% formaldehyde at 70 V for 3 hours. Equal RNA loading and size were checked by UV visualization of the 18S (1.8 kb) and 28S (4.7 kb) ribosomal bands (Current Protocols in Molecular Biology). Gels were rinsed and washed in 10X SSC at room temperature for 30 minutes. RNA was transferred by capillary action with 10X SSC overnight onto a positively charged nylon membrane (GeneScreen Plus, NEN Life Science Products). The next day the membranes were UV cross-linked (UV Crosslinker, Stratagene), heat-sealed in a plastic bag, and prehybridized or stored at -20°C.

Ultrasensitive hybridization buffer (ULTRAhyb, Ambion) was used to prehybridize the Northern blots (42 °C, 30 minutes). Probes were incubated overnight at 42°C, rinsed, and washed as the protocol suggested.

Northern blots were visualized by autoradiography using BioMax MS Film (Kodak) and a BioMax MS Intensifying Screen (Kodak).

c. Quantification of transcripts by phosphorimager analyses

The Northern blots were exposed to a Phosphor Screen (Molecular Dynamics) and the screen scanned into the program ImageQuant 3.3 with a Phosphorimager (Molecular Dynamics). Each area where a transcript was expected or observed and an equal area above it were quantified and defined as the background. The background area was subtracted from the area of the expected fragment. Equally sized areas were analyzed

within a Northern blot. The quantification data was exported into Microsoft Excel to normalize the transcript levels of each gene to that of GAPDH.

d. Probe synthesis

DNA probes were made using random primers or as described in the Strip-EZ DNA probe synthesis kit (Ambion). The advantage of the kit was that it utilized a modified dCTP that facilitated subsequent stripping of the blots. In a volume of 9 μ l, 25 ng DNA and DEPC-ddH₂O were boiled for 5 minutes and cooled on ice. To the denatured template was added 1X Decamer Solution, 1X Reaction Buffer -dATP, 1X dCTP, 50 uCi [α -P³²]-ATP, and 1 U Exonuclease-free Klenow DNA Polymerase 1 to a final volume of 25 μ l, as suggested by the kit protocol. DNA probes were synthesized in a waterbath at 37 °C for 30 minutes and purified by ProbeQuant G-50 Micro Columns (Pharmacia Biotech) as directed by the manufacturer. The purified probes were boiled for 5 minutes and put on ice for 1 minute before the denatured probes were hybridized to the membrane.

e. Stripping of Northern Blots

Northern blots were stripped of the radiolabelled DNA probes for subsequent hybridization with other DNA probes. The Northern blots incubated with Strip-EZ probes (Ambion) were stripped by washing the blots with 1X Degredation Buffer at 65 °C for 10 minutes, and then washing with 1X Blot Reconstitution Buffer with 0.1% SDS at 65 °C for 10 minutes, as suggested by the Strip-EZ kit (Ambion).

4. Identifying regulatory elements in the OPN promoter

The sequence of the mouse OPN promoter and some putative transcription factor binding sites were obtained from Genbank (Accession D14816). Known OPN regulatory elements were identified from functional studies (Hullinger et al., 2001; Shi et al., 2001; Shi et al., 1999; Wang et al., 2000; Vary et al., 2000; Sato et al., 1998; Guo et al., 1995). Binding sites of Ets proteins were defined as the sequences that matched 5'-GGA^A/T-3' that is recognized by most Ets proteins (Wasylyk et al., 1993). Matches to the optimal PEA3 binding site 5'-TCGCCGGAACCG-3' present in pGL3-44-5XPEA3-luciferase were searched for but were not found. Thus matches to 5'-AGGAAG-3' were defined as candidate PEA3 binding sites, as it is functionally recognized by PEA3 in the polyomavirus enhancer (Martin et al., 1988, Xin et al., 1992).

Potential transcription factor binding sites were identified by searches of the Transfac database using MatInspector (Genomatix). The Transfac database is based on probability matrices of multiple transcription factor binding sequences (Quandt et al., 1996). When multiple recognition sequences of a transcription factor are compared the most invariable nucleotides are defined as the core whereas the overall sequence is defined as the matrix. The parameters for each regulatory element were defined to have a 100% sequence similarity to the core recognition sequence to maintain specificity for each transcription factor. The overall sequence similarity of each regulatory element was defined as 80%, to allow for flexibility in nucleotide sequences. The OPN promoter from -777 to +79 and the sequences matching transcription factor binding sites were graphically represented in Canvas 5.0.

5. Luciferase reporter assays to determine osteopontin promoter activity

a. Cell maintenance

Cells were incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide. Independent mouse embryo fibroblast (MEF) cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (CS), 1% penicillin, 1% streptomycin, and 1% fungizone (Laing et al., 2000). Independent MEF 1 retransformant cell lines were grown as above but were also supplemented with 1.5 µg/ml puromycin (MEF C5-1) or 1 µg/ml blastocidin (MEF 1C, 1J, 1H, 1M) to retain the PEA3 expressing plasmids (Gina Fidalgo, personal communication). Human embryonic kidney (HEK) 293-1C cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin, 1% streptomycin, 1% fungizone, and geneticin (300 µg/ml) (Jason Peters, personal communication). The monkey kidney COS-1 cell line was grown in DMEM media supplemented with 10% FBS, 1% penicillin, 1% streptomycin, and 1% fungizone (Bojovic and Hassell, 2001).

b. Transient transfection assays

For each condition assayed by transient transfection, luciferase assay results were averaged from two to three independent wells. Duplicate experiments were assayed using different preparations of DNA. The total amount of DNA used in each transfection was balanced with calf thymus DNA (Boehringer Mannheim) to 1 µg per well for a 6-well plate, or 2 µg per well for a 12-well plate. In each experiment, an amount of calf thymus

DNA equal to the amount of total DNA was transfected to serve as a negative control (data not shown). For the experiments with expression vectors, the total amount of expression vector backbones between samples were balanced with empty expression vectors (e.g. pCANmyc) to an equal amount of DNA. The PEA3 subfamily expression vectors and the corresponding empty expression vectors, equal to the maximal amount tested in an experiment, were also transiently cotransfected with 0.25 μ g of pGL3-44-5XPEA3-luciferase as positive or negative control for PEA3 subfamily activity (data not shown).

The HEK 293-1C cell line was seeded at a density of 1.2×10^5 cells per well of a 12 well plate and then incubated for 24 hours. The OPN reporter (0.25 μ g) was transiently cotransfected with 0 to 0.75 μ g pCANmycPEA3 in triplicate according to the manufacturer's directions for SuperFect Transfection Reagent (Qiagen). Each DNA sample (1 μ g) was mixed with 0.1 ml DMEM containing 1% nonessential amino acids. SuperFect Transfection Reagent (Qiagen) was added (15 μ l) to the DNA mixture, vortexed 10 seconds, and incubated at room temperature for 15 minutes. HEK 293-1C media (0.3 ml), as defined above, was added to the DNA/Superfect lipid mixture. Media was aspirated from the cells and subsequently each well was incubated 2 hours with 0.4 ml of the DNA/Superfect/media mixture. Cells were aspirated and 1 ml fresh DMEM supplemented with 10% FBS, 1% penicillin, 1% streptomycin, 1% fungizone, and geneticin (300 μ g/ml) was added per well. The transfected cells were incubated 24 hours at 37°C in a humidified incubator with 5% carbon dioxide before whole cell lysate was harvested to assay for luciferase activity.

The MEF cell lines were seeded at a density of 0.7×10^5 cells/well in a 12-well plate and incubated for 24 hours. Each DNA sample was transfected in triplicate. They were transfected with 1 μ g DNA and 2 μ l LipofectAMINE (GibcoBRL) as suggested by the manufacturer's instructions. For each sample this mixture was pooled with 0.3 ml DMEM and then incubated for 30 minutes at room temperature. The MEF cells were washed with 1 ml DMEM and incubated for 1 hour with 0.1 ml of the DNA/LipofectAMINE mixture and 0.4 ml DMEM. Cells were then washed twice and incubated with 1 ml DMEM supplemented with 10% CS, 1% penicillin, 1% streptomycin, and 1% fungizone. Cells were incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide. After 24 hours whole cell lysate was harvested to assay for luciferase activity.

The transient cotransfections of the COS-1 cell line were described as above except scaled up in volume to increase the cell lysate yield for Western blot analysis. Cells were seeded on 6-well plates at 1.25×10^5 cells/well, total DNA was increased to 2 μ g, and the amount of LipofectAMINE used increased to 6 μ l (adapted from Bojovic and Hassell, 2001). The COS-1 cell line was grown in DMEM media supplemented with 10% FBS, 1% penicillin, 1% streptomycin, and 1% fungizone and incubated at 37°C in a humidified atmosphere containing 5% carbon dioxide. The COS-1 cells were transfected in quadruplicate and harvested 24 hours later: 2 wells were harvested for luciferase activity and 2 wells were harvested for Western blot analysis.

c. Luciferase assays

Whole cell lysate from transiently transfected cell lines were harvested with the Reporter Lysis system (Promega) as directed by the manufacturer, so that firefly (*Photinus pyralis*) luciferase activity could be quantified. The cells were kept on ice to inhibit protease activity and were washed twice with cold 1X PBS. Cells on 12-well and 6-well plates respectively were covered with 0.10 ml or 0.15 ml 1X Reporter Lysis Buffer. The dishes were rocked at room temperature for 15 minutes and the whole cell lysate scraped into a tube. Following a 15 second centrifugation of the sample at room temperature, the supernatant was transferred to a new tube. Samples were immediately assayed or stored at -80°C. Cell lysate (20 µl) was mixed with 0.1 ml of the Luciferase Assay Reagent (Promega) substrate and the relative luciferase units measured for 10 seconds in a luminometer (Lumat LB905 or Lumat LB9507).

d. Protein quantification

Total protein concentration was determined by dilution of 20 µl cell lysate with 1 ml 1X Bradford Reagent (BioRad). A dilution series (0 to 20 µg) in duplicate of bovine serum albumin was also analyzed for comparison. The absorbance at 595 nm was measured with a spectrophotometer (Beckman DU 640).

e. Luciferase activity analysis

Relative light units (RLU) measuring luciferase activity was divided by total protein (µg) to determine the normalized luciferase activity (RLU/µg). The average of duplicate

or triplicate samples was reported for each experiment. Each figure is a representative experiment. The error bars in the transient transfection figures represent the average deviation of each RLU/ μ g and were calculated using Microsoft Excel. To take into account the activity of the empty reporter vector, the fold-activation for a given expression vector concentration was determined by dividing the transcriptional activity of the OPN promoter reporter by the empty reporter backbone. The extent that the OPN transcriptional activity was higher in the MEF 4 cell line than in the MEF 1 cell line was measured at each OPN promoter reporter concentration: the transcriptional activity of the empty reporter vector pXP2-luc was subtracted from the transcriptional activity of the OPN reporter in the MEF 4 cell line, and then the difference was divided by the OPN reporter transcriptional activity in the MEF 1 cell line.

6. Western blot analyses of PEA3 subfamily expression vectors

a. Cell lysates

Whole cell lysate was harvested from the COS-1 cell line similar to Bojovic and Hassell (2001). COS-1 cells were washed twice with cold 1X PBS, scraped in 0.8 ml 1X PBS, and transferred to a 1.5 ml tube. The cells were centrifuged for 3 minutes at 7000 rpm and then the supernatant was aspirated. The concentrated COS-1 cells were resuspended in 30 μ l lysis buffer (0.5% Nonidet P-40, 25 mM Tris-HCL, pH 7.4, 2.5 mM EDTA, pH 8, 200 mM NaCl, 10 μ g/ml aprotinin, 2 μ g/ml PMSF, 10 μ g/ml leupeptin, and 10 μ g/ml pepstatin) and lysed for 25 minutes on ice. The cell lysate was centrifuged for 5

minutes at 13000 rpm, the supernatant was pooled and then stored immediately at -80°C or used for Western blot analyses.

b. Western blot analyses

Proteins were resolved on Western blots as described in Current Protocols of Protein Science. Proteins were mixed with 1X loading dye (250 mM Tris-HCl, pH 6.8, 500 mM DTT, 10% SDS, 0.5% bromophenol blue, 50% glycerol), boiled for 3 minutes and cooled on ice. Protein samples and 15 µl BenchMARK prestained protein marker (GibcoBRL) were resolved in a 10% SDS-PAGE gel for 5 hours with 200 V, or at 4°C overnight with 50 V. The protein was transferred overnight by electroblotting with 20 V at 4°C onto an Immobilon-Plus nylon membrane (Millipore). Western blots were stored at 4°C in heat-sealed plastic bags.

Western blots were blocked overnight at 4°C in blocking buffer (BB) [5% skim milk powder in TBS-T (10mM Tris-HCl, pH7.3, 150 mM NaCl, 0.05% Tween 20)]. Primary antibodies were diluted in fresh BB and incubated for 3 hours at room temperature. Mouse anti-myc antibody (9.1 mg/ml) was diluted 1:100. The rabbit anti-grb2 antibody was diluted 1:200 and used as a loading control for COS-1 whole cell lysates. The Western blots were washed three times in 1X TBS-T, blocked 1 hour at room temperature with BB, and incubated with 1:5000 horseradish peroxidase-conjugated goat anti-mouse antibodies (1 mg/ml, KPL) in BB for 1 hour at room temperature. The blots were washed 3 times in 1X TBS-T. Renaissance Western blot chemiluminescence Reagent Plus (NEN Life Science Products) was prepared as directed by the manufacturer

and incubated with Western blots for 1 minute. Protein was visualized by exposure to film (X-Omat Blue XB-1, Kodak).

RESULTS

1. Identification of OPN as a potential PEA3 regulated gene

Immortalized mouse embryonic fibroblast (MEF) cell lines had previously been isolated from mice with a targeted knockout of PEA3 as well as from syngeneic PEA3 wildtype mice (Laura Hastings, personal communication). In contrast to wildtype MEF cell lines, PEA3 null MEF cell lines were refractory to transformation by activated Ras or Neu (Gina Fidalgo, personal communication). Furthermore, stable reexpression of PEA3 in the PEA3 null MEF 1 cell line restored the ability to be transformed by activated Ras or Neu, indicating that PEA3 was required for transformation (Gina Fidalgo, personal communication).

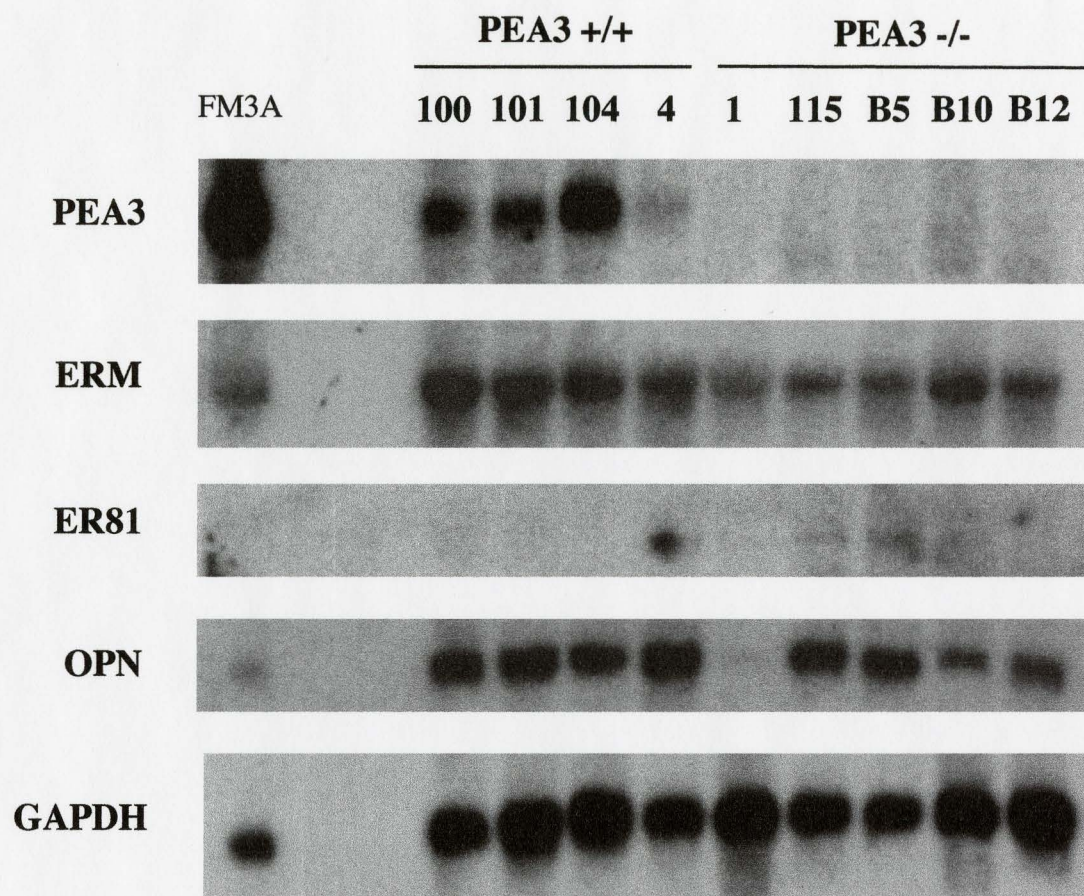
To isolate transformation-associated PEA3 target genes, cDNA from the PEA3 null MEF 1 cell line was subtracted from the wildtype MEF 4 cell line cDNA by representational difference analysis (RDA) (Jihou Xin, personal communication). RDA uses subtractive hybridization to subtract out genes with common expression levels in two cell lines and amplifies genes that have higher expression levels in one cell line (Lisitsyn, 1995). The differentially expressed cDNA were utilized to construct a difference product library and represented genes that were potentially regulated by PEA3.

PCR amplification and sequencing of the difference product library identified osteopontin (OPN) in 8 out of 74 (11%) independent colonies. OPN expression has been correlated with H-ras-transformed cells (Chambers et al., 1992; Guo et al., 1995; Gardner et al., 1994; Wu et al., 2000), consistent with the profile of a transformation-associated PEA3 target gene. Furthermore, purified PEA3 has been shown to bind the OPN promoter in a region of the promoter that is more active in H-ras transformed cell line than the parental NIH 3T3 cell line (Guo et al., 1995). To confirm that OPN was differentially expressed in the wildtype MEF 4 and PEA3 null MEF 1 cell lines, OPN expression was assayed by Northern blot analysis. The wildtype MEF 4 cell line was greater than 10-fold higher than in the PEA3 null MEF 1 cell line (see Figure 1), confirming the possibility that OPN could be a PEA3 target gene.

a. Relative PEA3 subfamily and OPN mRNA in wildtype and PEA3 null MEF cell lines

To determine whether OPN was differentially regulated in other wildtype versus PEA3 null MEF cell lines, Northern blot analyses using PEA3, OPN, and GAPDH DNA probes were assayed in 4 wildtype (4, 100, 101, 104) and 5 PEA3 null (1, 115, B5, B10, B12) MEF cell lines (Figure 1). PEA3 transcript levels did not correlate with OPN transcript levels (Figure 1). Therefore ERM and ER81 transcript levels were also investigated by Northern blot analyses to determine whether there was a correlation between PEA3 subfamily member and OPN transcript levels. GAPDH transcript levels served as an internal loading control within a cell line. The PEA3 null MEF 1 cell line and wildtype MEF 4 cell line were used in the RDA that identified OPN as a potential

Figure 1. Relative transcript levels of OPN and the PEA3 subfamily members in 4 wildtype MEF cell lines and 5 PEA3 null MEF cell lines by Northern blot analysis. RNA from the FM3A mammary cell line served as a positive control for PEA3 and ERM transcripts, and it also expressed low OPN transcript levels. Each cell line was extracted for total RNA and 20 µg was fractionated in each lane. The Northern blot was probed, stripped, and reprobed successively with 25 ng PEA3, ERM, ER81, OPN, and GAPDH cDNA. The GAPDH transcript levels served as control for equal loading. The Northern blot was repeated with independently derived RNA and similar results were observed. PEA3 transcripts were only detected in the wildtype MEF cell lines. ERM transcripts were found in all the MEF cell lines assayed whereas ER81 transcripts were only observed in two PEA3 null MEF cell lines (115, B5). OPN was expressed in all the MEF cell lines surveyed, although OPN transcript levels in the MEF 1 cell line was substantially lower than in the other MEF cell lines. Thus the expression profile of the PEA3 subfamily and OPN showed a good correlation between ERM and OPN transcripts but high OPN transcript levels were correlated with at least 2 PEA3 subfamily members in 6/8 MEF cell lines.



PEA3 regulated gene and previous Northern blot analyses had confirmed that OPN expression was higher in the MEF 4 cell line than in the MEF 1 cell line. Therefore the MEF 1 and MEF 4 cell lines were included as controls for OPN expression and for comparison to the other MEF cell lines. The mouse mammary carcinoma cell line FM3A expresses high levels of PEA3 transcripts and was used as a positive control for PEA3 expression (Xin et al., 1992).

The FM3A cell line expressed PEA3, ERM, and OPN transcript (Figure 1). PEA3 was expressed in all the wildtype PEA3 MEF cell lines but not in the PEA3 null MEF cell lines, as expected. ERM was expressed in all the MEF cell lines and ER81 transcripts were detected in two of the PEA3 null MEF cell lines (115, B5) and perhaps also the wildtype MEF 4 cell line. OPN was expressed in all the MEF cell lines surveyed, although OPN transcript levels in the MEF 1 cell line was substantially lower than in the other MEF cell lines.

Note that with the exception of the PEA3 null MEF 1 cell line, OPN transcript levels were similar between the wildtype MEF cells and the PEA3 null MEF cell lines. Therefore OPN expression did not require PEA3 expression. The FM3A cell line also expressed high levels of PEA3 transcripts but low levels of OPN transcripts in comparison to the MEF cell lines. Thus PEA3 transcript levels did not correlated to OPN transcript levels in either the FM3A cell line or MEF cell lines. Also note that although ERM and OPN expression correlated in most MEF cell lines, the PEA3 null MEF 1 cell line expressed ERM at a level comparable to the other MEF cell lines, but expressed a substantially lower level of OPN expression than the other MEF cell lines. Therefore

there was no absolute correlation between ERM expression levels and OPN transcripts in the MEF cell lines. The MEF cell lines that expressed ER81 also expressed OPN, but OPN expression was not dependent on ER81 since many OPN expressing MEF cell lines did not express ER81.

Thus all the MEF cell lines that expressed OPN coexpressed at least one PEA3 subfamily member. There was a better correlation between ERM and OPN transcripts than PEA3 or ER81 and OPN transcripts, suggesting that ERM was more likely to regulate OPN transcription than PEA3 or ER81. Interestingly, the MEF cell lines that expressed high levels of OPN transcripts, with the exception of B10 and B12, also expressed more than 1 PEA3 subfamily member. The PEA3 subfamily may additively upregulate OPN transcriptional activity. Transcriptional regulation of OPN in the MEF cell lines could be due to a minimal protein level of one PEA3 subfamily member or the total contribution of multiple PEA3 subfamily members.

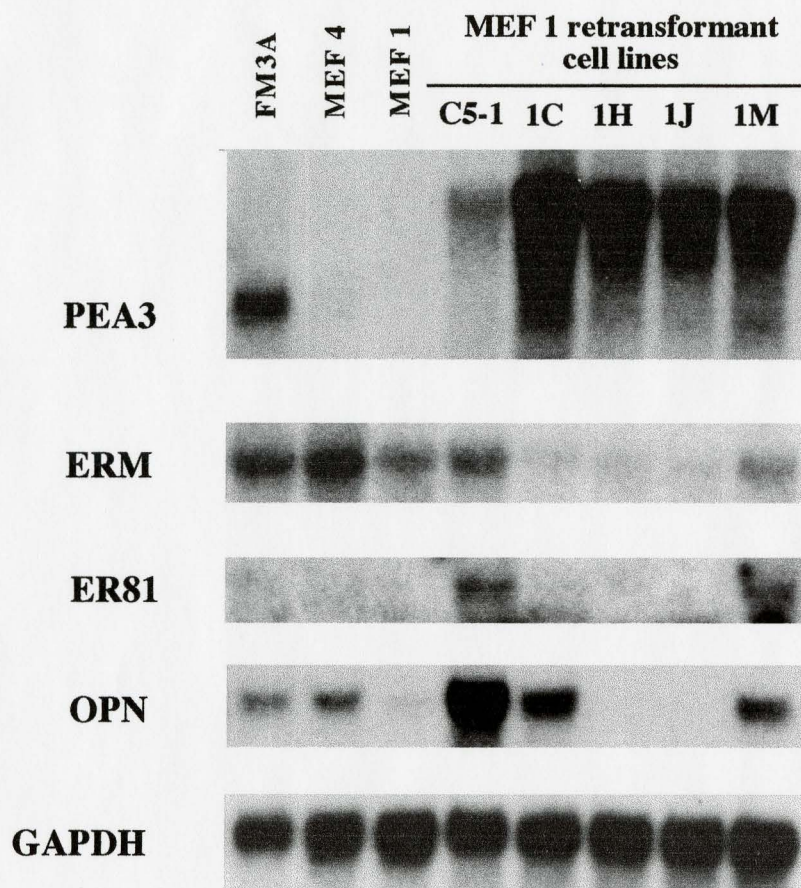
b. Relative PEA3 subfamily and OPN mRNA levels in MEF 1 retransformant cell lines

The PEA3 null MEF 1 cell line expressed ERM transcript levels comparable to the other MEF cell lines, yet the MEF 1 cell line expressed considerably lower OPN transcript levels (Figure 1). Independent MEF 1 retransformant cell lines stably reexpress higher levels of ectopic PEA3 transcripts and protein in comparison to the parental MEF 1 cell line (Gina Fidalgo, personal communication). To determine whether increased PEA3 subfamily transcript levels would correspond to increased OPN transcript levels, 5 independent MEF 1 retransformant cell lines (C5-1, 1C, 1H, 1J, 1M) were assayed by

Northern blot analyses for relative PEA3 subfamily, OPN, and GAPDH transcript levels. Upregulated OPN transcript levels in response to maintained ERM and increased PEA3 transcript levels in the MEF 1 retransformant cell lines would suggest that abundance of multiple PEA3 subfamily members contributed to OPN transcriptional regulation. RNA from the FM3A cell line served as a positive control for endogenous PEA3 and ERM (Figure 2). OPN transcripts were also observed in the FM3A cell line, consistent with Figure 1. The MEF 4 cell line and the parental MEF 1 cell line were included as controls for OPN expression and for comparison to the MEF 1 retransformant cell lines. Note that a prolonged autoradiograph exposure detected higher PEA3 transcripts in the MEF 4 cell line than the MEF 1 cell line, consistent with Figure 1. Compare the relative PEA3 transcript levels in the FM3A and MEF 4 cell lines between Figures 1 and 2. The autoradiograph shown in Figure 2 was selected such that distinct PEA3 transcripts in the MEF 1 retransformant cell lines were visualized.

All of the MEF 1 retransformant cell lines had higher PEA3 expression in comparison to the parental MEF 1 cell line and the MEF 4 cell line (Figure 2). The MEF 1 retransformant cell lines also expressed larger PEA3 transcripts than the endogenous PEA3 transcript observed in the FM3A cell line, due to the bicistronic messages in the vectors used to derive the MEF 1 retransformant cell lines (Gina Fidalgo, personal communication).

Figure 2. Northern blot analyses of the PEA3 subfamily and OPN in 5 MEF 1 retransformant cell lines. Relative expression levels of OPN and the PEA3 subfamily members were assayed by Northern blot analyses in 5 independent MEF 1 cell lines that stably expressed ectopic PEA3 to determine whether increased PEA3 subfamily transcript levels would correlate with increased OPN expression. Each cell line was extracted for total RNA and 20 μ g was fractionated in each lane. The Northern blot was probed, stripped, and reprobed successively with 25 ng PEA3, ERM, ER81, OPN, and GAPDH cDNA. GAPDH transcript levels served as an internal loading control. FM3A RNA served as a positive control for PEA3 and ERM transcripts. RNA from the wildtype MEF 4 cell line and PEA3 null MEF 1 cell line served as controls for OPN transcripts and the parental MEF 1 cell line served as a reference for the 5 MEF 1 retransformant cell lines. A Northern blot with independent RNA preparations yielded similar results. PEA3 expression was substantially higher in the 5 MEF 1 retransformant cell lines in comparison to the parental MEF 1 cell line. ERM was expressed in the C5-1 and 1M cell lines at similar levels to the MEF 1 cell line. ER81 transcripts were not detected in the MEF 1 cell line, but were observed in the C5-1 and 1M cell lines. OPN transcript levels were also higher in 3 MEF 1 retransformant cell lines (1C, C5-1, 1M) than the parental MEF 1 cell line. Thus, at least 2 PEA3 subfamily members were coexpressed in 2/3 cell lines that express high OPN mRNA levels. In addition, high PEA3 mRNA levels and low ERM mRNA levels did not correlate with high OPN transcript levels.



In the MEF 1 Retransformant cell lines, ERM and ER81 transcripts were only detected in the MEF C5-1 and 1M cell lines (Figure 2). The loss of ERM expression in MEF 1 retransformants 1C, 1H, and 1J cell lines in comparison to the parental MEF 1 cell line was attributed to clonal variation in the cell lines. Variations in expression levels of other transcription factors might have contributed to changes in OPN transcript levels. However, the effect of the PEA3 subfamily was addressed later by transfection assays.

OPN transcripts were detected at higher levels in the MEF C5-1, 1C, and 1M cell lines than the parental MEF 1 cell line. Note that in comparison to the parental MEF 1 cell line, the MEF C5-1 and 1M cell lines maintained ERM expression levels but also had upregulated PEA3 and ER81 expression coincident with upregulated OPN expression, suggesting that all PEA3 subfamily members contributed to OPN expression. Note also that although the MEF 1H and 1J cell lines expressed substantially higher PEA3 levels than the parental MEF 1 cell lines, there was a decrease in ERM expression levels and there was no upregulation of OPN expression. This indicated that high expression levels of one PEA3 member were not sufficient to induce OPN expression. Therefore with the exception of the MEF 1C cell line, OPN expression in the MEF 1 retransformant cell lines correlated with the expression of at least 2 PEA3 subfamily members.

Northern blot analyses of the 14 MEF cell lines showed that 11/11 of the MEF cell lines that expressed OPN also coexpressed a PEA3 subfamily member, suggesting that the PEA3 subfamily could upregulate OPN expression (Figure 1 and 2). In 8/11 MEF cell lines that expressed OPN, more than 1 PEA3 subfamily member was coexpressed, suggesting that high OPN transcript levels correlated with the combined expression of at

least 2 PEA3 subfamily members. Consistently, the 3/14 MEF cell lines that expressed very low (MEF 1) or undetectable OPN transcript levels (MEF 1H, 1J) expressed only 1 PEA3 subfamily member. Together these results showed that in the MEF cell lines, high OPN transcript levels correlated with the combined expression of at least two PEA3 subfamily members. To show that the PEA3 subfamily could upregulate OPN transcription activity, the mouse OPN promoter sequence was analyzed for PEA3 binding sites.

2. Determining PEA3-responsive regions in the OPN promoter

a. Regulatory elements in the OPN promoter

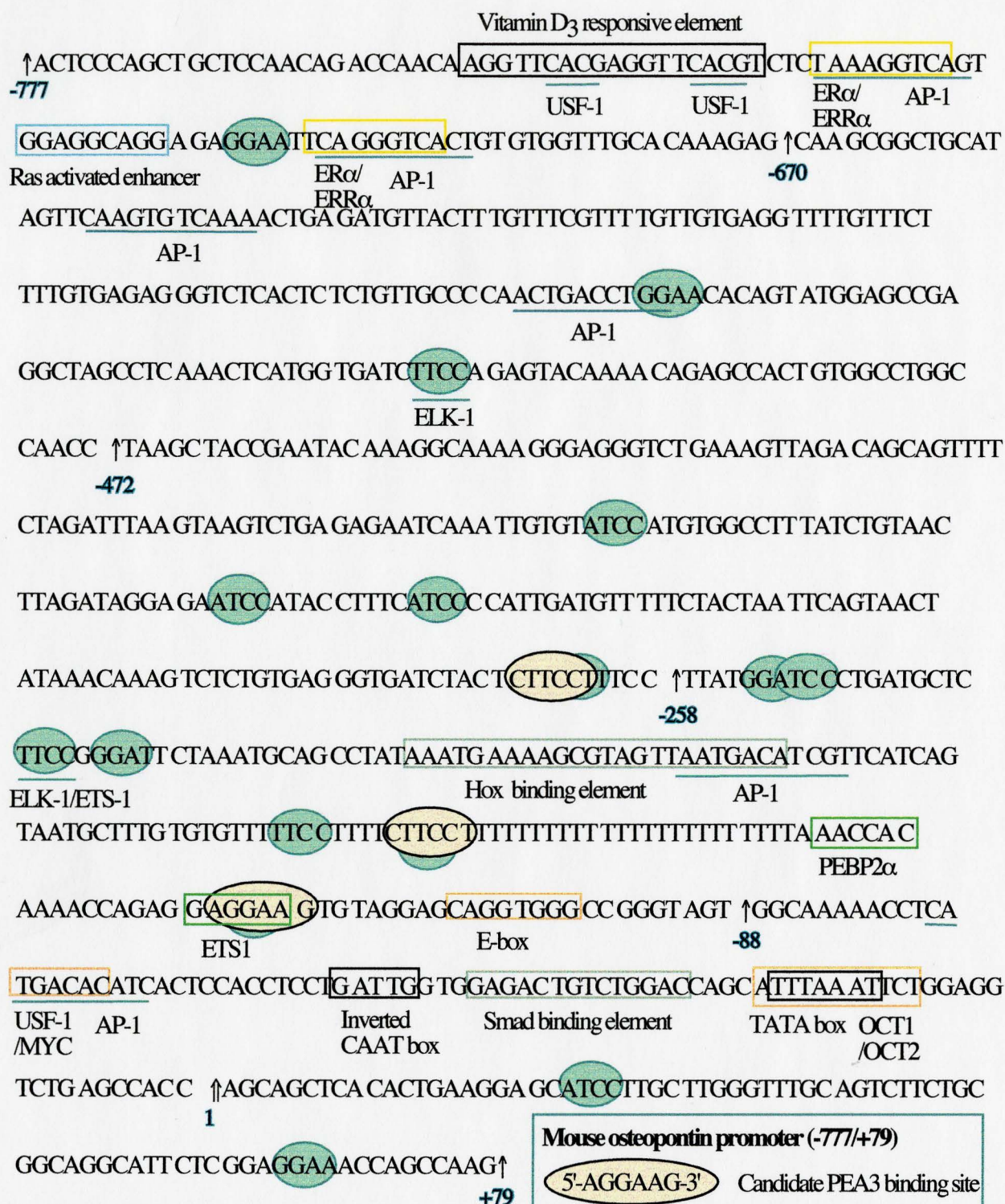
Positive regulation of the OPN promoter by the PEA3 subfamily of transcription factors would require the presence of candidate ETS binding sites in the OPN promoter. GST-PEA3 can bind the mouse OPN promoter between -716/-708 in a gel-shift assay (Guo et al., 1995). Other PEA3 binding regions in the OPN promoter may exist but have not yet been experimentally defined. Subsequently the longest OPN promoter construct used in transient transfection assays was -777 to +79, which included the RAE. Candidate and experimentally identified regulatory elements in the -777 to +79 mouse OPN promoter were illustrated in Figure 3.

The -777/+79 mouse OPN promoter has 16 candidate core ETS binding sites with sequence homology to 5'-GGA^A/_T-3', which can be recognized by the ETS domain of most Ets transcription factors (Wasylyk et al., 1993). There was at least one candidate core ETS binding site between every 5' endpoint in the series of OPN promoter deletion

mutants utilized in the subsequent transfection assays. There were 3 potential PEA3 binding sites 5'-AGGAAG-3' that conformed to the PEA3 recognition sequence in the polyomavirus enhancer (Martin et al., 1988) and is similar to the PEA3 recognition sequence 5'-AGGAAA-3' found in the uPA enhancer (Rorth et al., 1990). Comparison to the vertebrate Transfac database also identified 2 consensus Elk-1 binding sites, 1 of which overlaps a c-Ets-1 binding site, suggesting that these potential Ets binding sites could also be functional. Therefore, there were candidate ETS and PEA3 binding sites in the mouse OPN promoter that had the capacity to be recognized by the PEA3 subfamily.

The OPN promoter was assessed for consensus sequences recognized by transcription factors that could cooperate with Ets proteins. Sequences identified by homology to sequences in the Transfac database identified potential binding sites similar in sequence to known functional binding sites. There were no consensus sites for Sp1, SRF, NF κ B, Pax-5, Stat1, which have been shown to interact with Ets proteins to enhance transcriptional activity (Li et al., 2000). There were 2 consensus E-box sequences recognized by USF-1 between -777 and -670 of the OPN promoter. USF-1 can synergistically transactivate a luciferase reporter with Ets and E-box binding sites in the promoter, with either PEA3 (Greenall et al., 2001) or Ets-1, but not Elf-1 or PU.1 (Sieweke et al., 1998). There were 6 consensus sites for AP-1, which have been shown to interact with many Ets proteins (Wasylyk et al., 1993). It is noteworthy that there were 2 potential AP-1 sites between -777/-670 flanking the RAE and only candidate ETS binding site in this region, which could contribute to regulation of the OPN promoter by the PEA3 subfamily.

Figure 3. The mouse osteopontin promoter sequence from -777 to +79. Candidate core ETS binding sites and PEA3 binding sites were assessed by sequence analyses of the OPN promoter. Candidate binding sites regulatory sites of transcription factors that cooperate with Ets proteins were illustrated with an underline. Known regulatory sites of transcription factors that cooperatively regulate the OPN promoter were outlined with similarly coloured boxes. The 5' endpoints (-777, -670, -472, -258, -88) and the 3' endpoint (+79) of a series of 5' OPN promoter deletion mutant reporters utilized in transient transfection assays were indicated with an arrow. Between -777 and +79 there were 16 candidate core ETS binding sites, among which are 3 candidate PEA3 binding sites.



b. Assessing OPN transcriptional activity in response to PEA3

OPN expression was higher in the wildtype MEF 4 cell line than the PEA3 null MEF 1 cell line by Northern blot analysis and there were candidate PEA3 binding sites in the mouse OPN promoter, but there was no evidence that PEA3 transcriptionally upregulated OPN expression *in vivo*. An equally plausible explanation was that mRNA stability was increased in the wildtype MEF 4 cell line. To identify regions in the OPN promoter with functional PEA3 binding sites, a progressive series of 5' OPN promoter deletion mutants were cotransfected with PEA3 expression vectors.

OPN transcriptional activity was measured with a series of mouse 5' OPN promoter deletion constructs linked to a firefly luciferase reporter gene. The 5' OPN promoter deletion mutants -777/+79, -670/+79, -472/+79, -258/+79, and -88/+79, were previously cloned into the promoterless vector backbone pXP2-luciferase (Guo et al., 1995).

Relative light units (RLU) measuring luciferase activity was divided by the total protein (μg) to yield a normalized activity ($\text{RLU}/\mu\text{g}$) for each duplicate or triplicate sample. The normalized activities ($\text{RLU}/\mu\text{g}$) were then averaged and graphed with error bars indicating the average deviation between the samples.

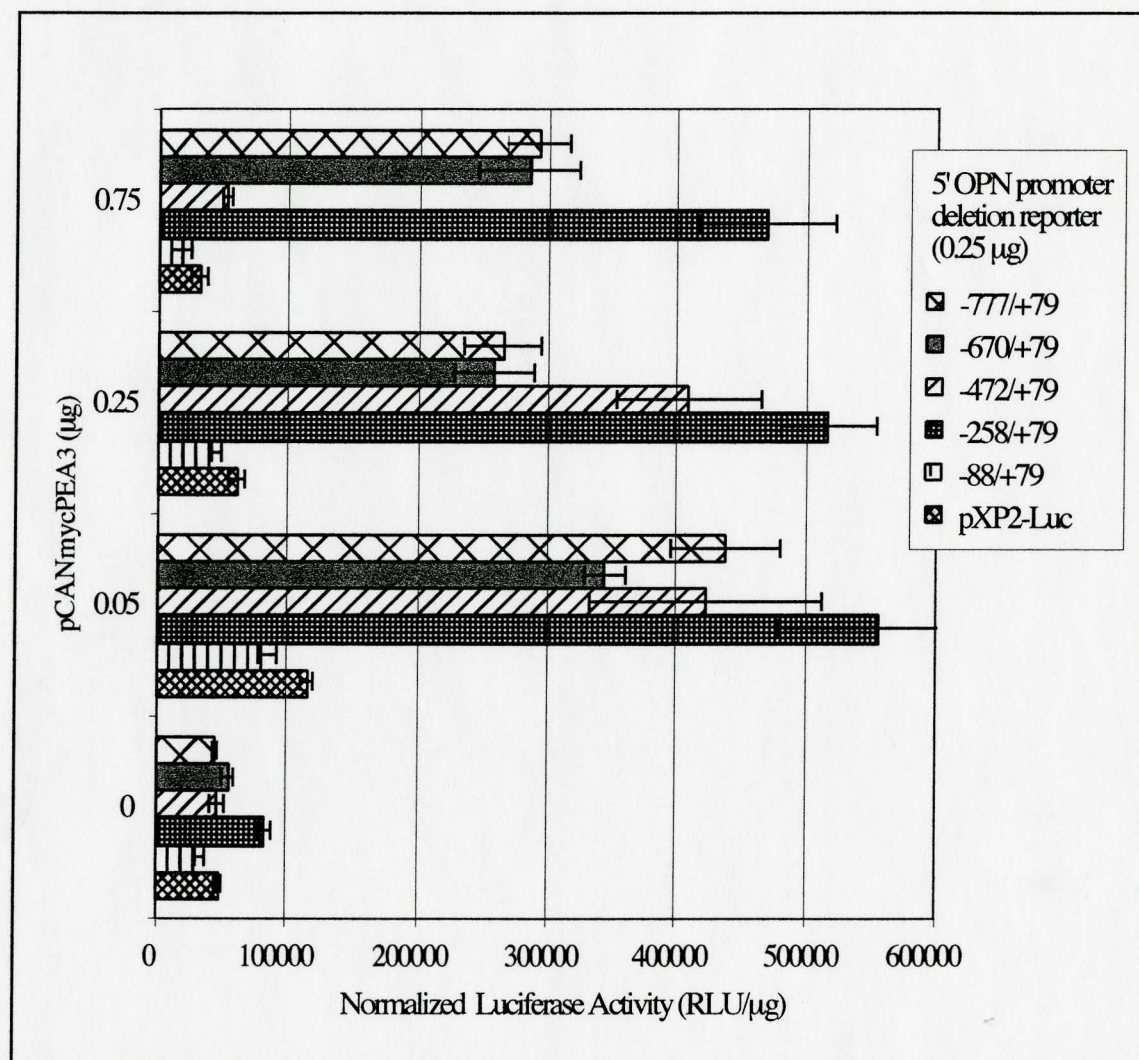
The human embryonic kidney cell line HEK 293-1C cell line expresses a stably transfected PEA3 expression vector that can be induced to express PEA3 mRNA and protein (Jason Peters, personal communication). To determine whether PEA3 affected OPN expression, PEA3 was induced in the HEK 293-1C cell line and assayed for OPN expression. OPN transcripts were not induced by PEA3 in the HEK 293-1C cell line by

Northern blot analysis (data not shown), but this may have been due to low activity levels of induced PEA3.

On the other hand, the -777/+79 OPN promoter reporter is upregulated by 5-fold in the parental HEK 293 cell line when transiently cotransfected with the orphan nuclear receptor ERR α (Vanacker et al., 1998). Therefore OPN promoter activity can be induced in the parental HEK 293 cell line. In addition, the low PEA3 (Jason Peters, personal communication) and low OPN transcript levels (data not shown) in the HEK 293-1C cell line permits the effect of transiently transfected PEA3 on the OPN promoter to be assessed. A preliminary titration from 0 to 0.75 μ g of the -777/+79 OPN reporter showed increasing levels of OPN promoter activity in the HEK 293-1C cell line (data not shown), so a subsaturating amount of 0.25 μ g of OPN promoter reporter was subsequently used to assay the effect of cotransfected PEA3.

To determine regions of PEA3-responsiveness in the OPN promoter, the HEK 293-1C cell line was transiently cotransfected with pCANmycPEA3 (0 to 0.75 μ g) and 0.25 μ g of a series of 5' OPN promoter deletion mutants (Figure 4). When the empty PEA3 expression vector pCANmyc was added (0 μ g pCANmycPEA3), the OPN promoter deletion mutants had similar activity to the promoterless reporter pXP2-luc, indicating that endogenous OPN expression was low in HEK 293-1C cell line. The

Figure 4. The OPN promoter was responsive to ectopic PEA3 in the HEK 293-1C cell line. A series of 5' OPN promoter deletion luciferase reporters (0.25 μ g) and the empty reporter vector pXP2-luc, were cotransfected in triplicate with pCANmycPEA3 (0, 0.50, 0.25, or 0.75 μ g) in the HEK 293-1C cell line. PEA3 expression plasmid DNA was balanced to 0.75 μ g with the empty expression vector pCANmyc. Relative luciferase units were normalized to total protein for each sample (RLU/ μ g) and averaged. An independent experiment with different DNA preparations yielded similar results, except for the decrease in -472/+79 OPN promoter activity with 0.75 μ g pCANmycPEA3, in comparison with 0.25 μ g pCANmycPEA3. When 0.75 μ g empty PEA3 expression vector pCANmyc was added (0 μ g pCANmycPEA3), the OPN promoter deletion mutants had similar activity to the promoterless reporter pXP2-luc. On the other hand, transiently cotransfected pCANmycPEA3 (0.05 to 0.75 μ g) increased the activity of any OPN promoter reporter construct longer than -88/+79 above pXP2-luc levels, verifying that PEA3 could upregulate OPN transcriptional activity in the HEK 293-1C cell line.



transcriptional activity of the -88/+79 OPN promoter was similar to pXP2-luc even with transient transfection of pCANmyc PEA3, suggesting that the -88/+79 region of the OPN promoter was not responsive to PEA3.

On the other hand, transiently cotransfected pCANmycPEA3 (0.05 to 0.75 μ g) increased the activity of any OPN promoter reporter construct longer than -88/+79 by more than 5-fold, verifying that OPN transcriptional activity was PEA3-responsive in the HEK 293-1C cell line (Figure 4). Slight decreases in OPN transcriptional activity with increasing amounts of cotransfected pCANmycPEA3 suggested that the number of PEA3 binding sites was saturated with 0.05 μ g pCANmycPEA3. The large decrease in -472/+79 transcriptional activity with 0.75 μ g pCANmycPEA3 in comparison to 0.50 μ g pCANmycPEA3 was not reproduced in another experiment and thus was attributed to a technical error in DNA measurement.

The region with the greatest difference in PEA3-responsive transcriptional activity was between -258 and -88 of the OPN promoter. For a given amount of cotransfected pCANmycPEA3, the -258/+79 OPN transcriptional activity increased more than 6-fold when divided by the -88/+79 OPN transcriptional activity (Figure 4). Therefore the region between -258 and -88 of the mouse OPN promoter has functional PEA3 responsive sites that are required for maximal OPN promoter activity.

When the OPN promoters were cotransfected with 0.05 μ g pCANmycPEA3, the -472/+79 OPN promoter was activated 8-fold and the -258/+79 OPN promoter was activated 6-fold in comparison to their transcriptional activity in response to pCANmyc. This suggested that there was another functional PEA3 binding site between -472 and

-258 of the mouse OPN promoter.

The OPN promoter is responsive to PEA3 in the HEK 293-1C cell line. The higher OPN transcript levels in the wildtype MEF 4 cell line compared to the PEA3 null MEF 1 cell line could be due to the responsiveness of the OPN promoter to the PEA3 subfamily in the MEF 4 cell line.

3. The PEA3 subfamily transcriptionally activated OPN

a. Higher OPN transcriptional activity in the MEF 4 cell line than the MEF 1 cell line

The previous transient cotransfection with pCANmycPEA3 and the OPN promoter deletion mutant reporters showed that the OPN promoter was responsive to PEA3 in the HEK 293-1C cell line. Thus upregulation of OPN expression by PEA3 could account for the greater than 10-fold higher OPN expression in the wildtype MEF 4 cell line compared to the PEA3 null MEF 1 cell line (Figure 1). However, it was still unclear whether the higher OPN transcript levels in the wildtype MEF 4 cell line was due to higher OPN transcriptional activity and/or increased transcript stability. The MEF 1 and MEF 4 cell lines were used to identify OPN as a potential PEA3 target gene and subsequently were used to assess the ability of the PEA3 subfamily to affect OPN transcription.

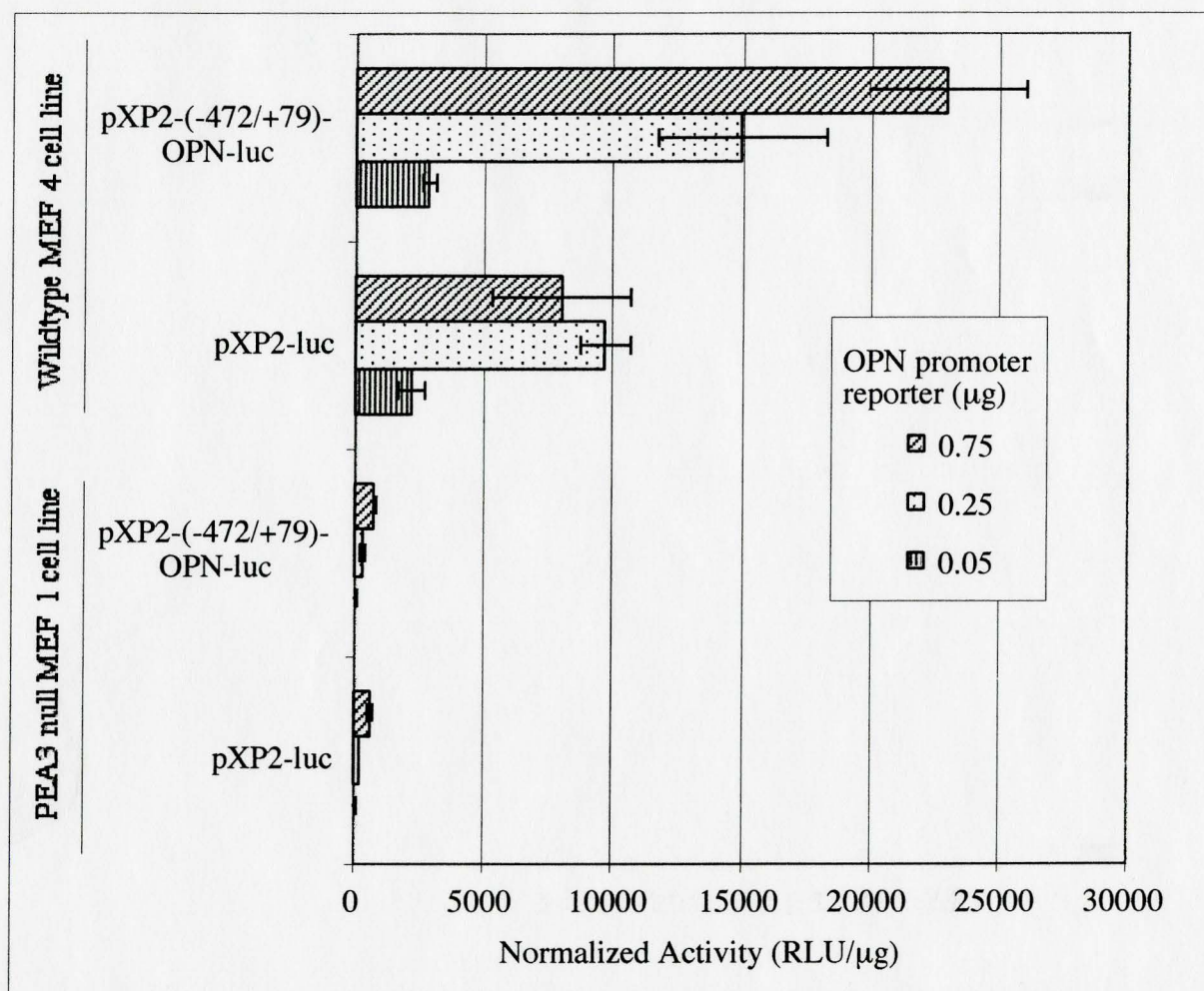
To test whether OPN transcriptional activity was higher in the wildtype MEF 4 cell line than the PEA3 null MEF 1 cell line, both cell lines were transfected with titrating amounts of the empty reporter vector pXP2-luc or the -472/+79 OPN promoter reporter (Figure 5). A preliminary transfection with a titrating amount of the 5' OPN promoter reporters (0.05 to 0.75 μ g) in the MEF 4 cell line showed that the -472/+79 OPN

promoter mutant had the highest activity (data not shown) and subsequently was used to assess the OPN transcriptional activity in the MEF 1 and MEF 4 cell lines.

The OPN transcriptional activity in the MEF 4 cell line increased in a dose-dependent manner up to 3-fold higher in comparison to the empty reporter vector pXP2-luc (Figure 5). This suggested that positive regulatory transcription factors responsible for OPN promoter activation in the MEF 4 cell line were not limited between 0.05 μ g and 0.75 μ g of the OPN promoter reporter. Subsequent experiments in the MEF 4 cell line therefore utilized a subsaturating 0.25 μ g amount of -472/+79 OPN promoter reporter.

In contrast to the MEF 4 cell line, the -472/+79 OPN promoter (0.25 μ g to 0.75 μ g) had similar transcriptional activities in comparison to the backbone vector pXP2-luc in the PEA3 null MEF 1 cell line, suggesting that the OPN promoter had no transcriptional activity in the PEA3 null MEF 1 cell line. Figure 5. OPN transcriptional activity was higher in the wildtype MEF 4 cell line than the PEA3 null MEF 1 cell line. To determine whether the higher OPN transcript levels in the MEF 4 cell line than the MEF 1 cell reflected differences in OPN transcriptional activity, a titrating amount (0.05, 0.25, and 0.75 μ g) of the OPN promoter luciferase reporter pXP2-(-472/+79)-OPN-luc and the empty reporter vector pXP2-luc were transiently transfected in the MEF 4 and the MEF 1 cell lines. Each sample was assayed in triplicate. The relative luciferase units were normalized to the total amount of protein and then averaged (RLU/ μ g) to determine the transcriptional activity. An independent transfection with different DNA preparations yielded similar results. The OPN transcriptional activity was maximally 3-fold higher with the OPN promoter reporter than the empty reporter in the MEF 4 cell line. In contrast, the OPN transcriptional activity

Figure 5. OPN transcriptional activity was higher in the wildtype MEF 4 cell line than the PEA3 null MEF 1 cell line. To determine whether the higher OPN transcript levels in the MEF 4 cell line than the MEF 1 cell reflected differences in OPN transcriptional activity, a titrating amount (0.05, 0.25, and 0.75 μ g) of the OPN promoter luciferase reporter pXP2-(-472/+79)-OPN-luc and the empty reporter vector pXP2-luc were transiently transfected in the MEF 4 and the MEF 1 cell lines. Each sample was assayed in triplicate. The relative luciferase units were normalized to the total amount of protein and then averaged (RLU/ μ g) to determine the transcriptional activity. An independent transfection with different DNA preparations yielded similar results. The OPN transcriptional activity was maximally 3-fold higher with the OPN promoter reporter than the empty reporter in the MEF 4 cell line. In contrast, the OPN transcriptional activity was similar to the empty reporter vector in the MEF 1 cell line. A comparison showed that the OPN transcriptional activity in the MEF 4 cell line was higher than in the MEF 1 cell line.



was similar to the empty reporter vector in the MEF 1 cell line. A comparison showed that the OPN transcriptional activity in the MEF 4 cell line was higher than in the MEF 1 cell line activity in the MEF 1 cell line. However, the lack of OPN promoter activity above backbone levels did not exclude the possibility that the amount of reporter used was insufficient to detect OPN transcriptional activity in the MEF 1 cell line.

When the levels of -472/+79 OPN promoter activity were compared between the wildtype MEF 4 cell line and the PEA3 null MEF 1 cell line, OPN transcriptional activity was greater in the MEF 4 cell line than the MEF 1 cell line with any amount of OPN reporter tested. Transcriptional activity of the -472/+79 OPN promoter reporter ranged from 7- to 20-fold higher in the MEF 4 cell line than the MEF 1 cell line (Figure 5). Thus the higher OPN mRNA expression in the wildtype MEF 4 cell line in comparison to the PEA3 null MEF 1 cell line, was due in part to greater OPN transcriptional activity in the MEF 4 cell line.

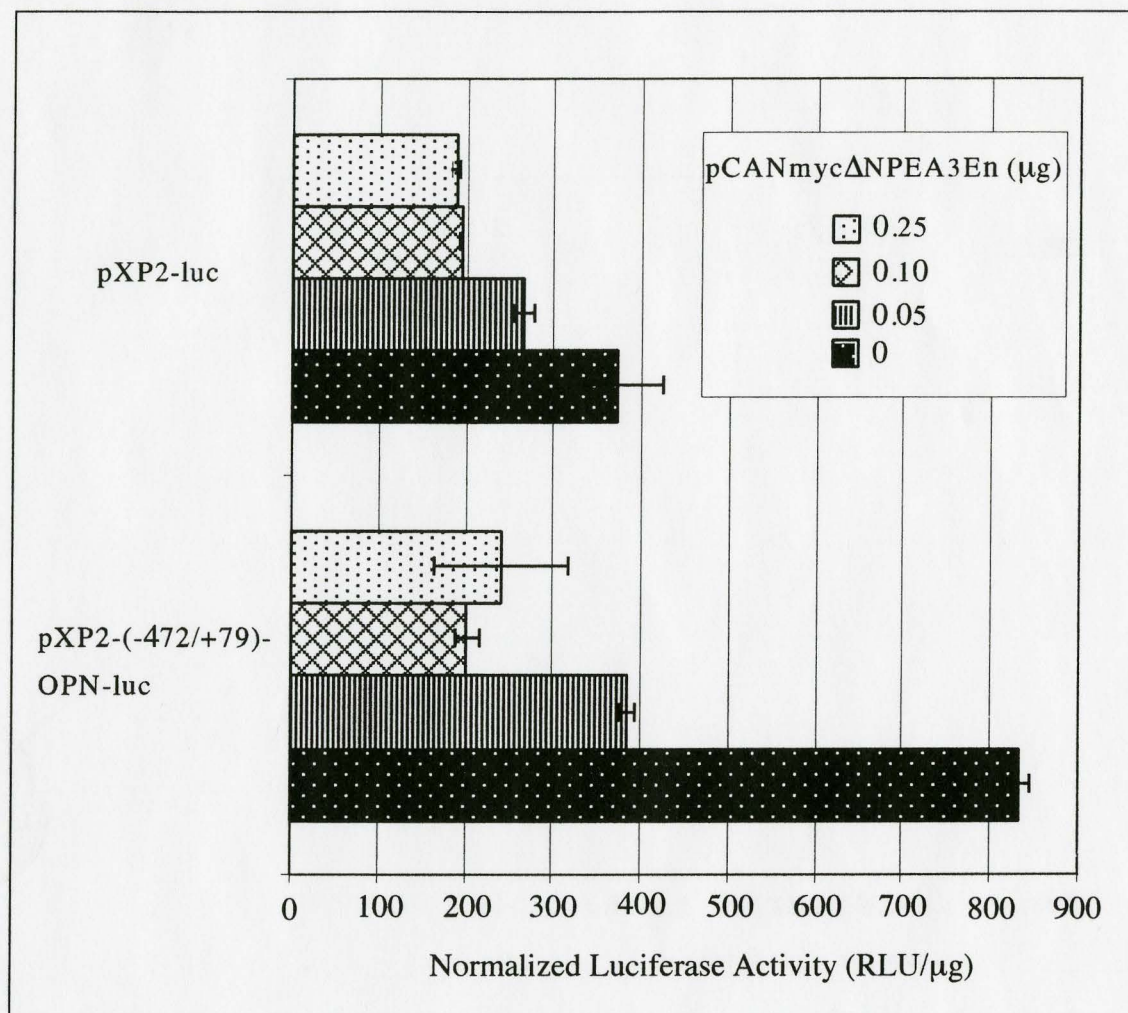
b. Ectopic dominant negative PEA3 decreased OPN transcriptional activity in the MEF 4 cell line:

The greater PEA3 subfamily transcript levels and OPN transcriptional activity in the MEF 4 cell line versus the MEF 1 cell line, suggested that the PEA3 subfamily regulated OPN transcriptional activity. To determine whether inhibition of endogenous PEA3, ERM, and ER81 could suppress OPN transcriptional activity, the dominant negative PEA3 vector pCANmycΔNPEA3En was cotransfected with an OPN promoter reporter in the wildtype MEF 4 cell line.

The expression vector pCANmyc Δ NPEA3En encodes an engrailed repression domain fused to the carboxy-end of PEA3 inclusive of the ETS domain, such that pCANmyc Δ NPEA3En can attach to PEA3 binding sites but does not activate transcription (Trevor Shepherd, personal communication). Thus pCANmyc Δ NPEA3En inhibits the effects of endogenous PEA3, ERM and ER81 by competing for PEA3 binding sites on promoters of target genes. Transient cotransfections with the -472/+79 OPN reporter showed that OPN transcriptional activity did not decrease further between 0.25 μ g and 0.75 μ g pCANmyc Δ NPEA3En (data not shown), so the maximum concentration of pCANmyc Δ NPEA3En was reduced 0.25 μ g.

Transient cotransfections of the -472/+79 OPN reporter (0.25 μ g) and a titration of pCANmyc Δ NPEA3En or its backbone (0 to 0.25 μ g) were assessed in the MEF 4 cell line (Figure 6). The OPN promoter had ~2-fold higher activity compared to the empty reporter vector, consistent with the fold-difference observed in Figure 5. OPN promoter activity was suppressed almost entirely with 0.05 μ g pCANmyc Δ NPEA3En (Figure 6). With 0.1 μ g or 0.25 μ g the transcriptional activity of OPN was reduced to the level of the reporter backbone. The suppression of OPN transcriptional activity in the MEF 4 cell line

Figure 6. Ectopic dominant negative PEA3 decreased OPN promoter activity in the MEF 4 cell line. To determine whether competitive inhibition of the PEA3 subfamily suppressed OPN transcriptional activity, a titrating amount (0.05, 0.10, and 0.25 μ g) of pCANmyc Δ NPEA3En was transiently cotransfected in the wildtype MEF 4 cell line with 0.25 μ g pXP2-(-472/+79)-OPN-luc or pXP2-luc. The amount of pCANmyc was kept constant at 0.25 μ g and the effect of pCANmyc Δ NPEA3En on OPN transcriptional activity was compared to the effect of 0.25 μ g pCANmyc (0 μ g pCANmyc Δ NPEA3En). The relative luciferase activity was normalized to total protein. Each bar represented an average of triplicate samples and the error bars showed the average deviations. A separate experiment showed similar results. With 0 μ g pCANmyc Δ NPEA3En, OPN transcriptional activity in MEF 4 cell line was modestly ~2-fold higher than pXP2-luc. OPN transcriptional activity was substantially reduced with 0.50 μ g and was further reduced to the level of the empty reporter vector with 0.10 μ g or 0.25 μ g pCANmyc Δ NPEA3En.



by transient cotransfection of a competitor for PEA3 subfamily binding sites suggested that OPN transcriptional activity could be attributed to endogenous PEA3, ERM, or ER81. Whether the entire PEA3 subfamily transcriptionally activated OPN or had differences in the specificity of OPN transcriptional activation was unknown.

c. Ectopic PEA3, ERM, and ER81 upregulated OPN transcriptional activity in the MEF 1 and COS-1 cell lines

Transient cotransfection of a dominant negative PEA3 expression vector in the MEF 4 cell line and an OPN promoter reporter provided evidence that inhibition of PEA3, ERM, and ER81 suppressed OPN transcriptional activity. To ascertain that the PEA3 subfamily members upregulated OPN transcription, their expression vectors were cotransfected with an OPN reporter in a PEA3 null cell line. OPN was initially identified as expressing substantially lower transcript levels in the PEA3 null MEF 1 cell line in comparison to the wildtype MEF 4 cell line, therefore the MEF 1 cell line was initially used to determine whether increased PEA3, ERM, or ER81 protein levels would upregulate OPN transcriptional activity.

The transcriptional activity was measured with the longest OPN reporter available, pXP2-(-777/+79)-OPN-luc, because it was the most similar to the endogenous OPN promoter and included the region of the promoter that binds purified PEA3 (Guo et al., 1995). Increased numbers of MEF 1 cells were transfected to allow sufficient protein to be harvested for both luciferase assays and Western blot analyses from the same transfection. The amount of expression vector was increased to a maximum of

1.75 µg to adjust for the increased number of cells transfected. The PEA3 subfamily in a common vector backbone was not available, so the effects of 1.75 µg pCANmyc and 1.75 µg pRSVmyc on the transcriptional activity of 0.25 µg pXP2-luc and the -777/+79 OPN reporter were measured.

Transient cotransfection of each PEA3 subfamily expression vector with the -777/+79 OPN reporter increased OPN promoter activity by over 3-fold in the MEF 1 cell line, in comparison to the transcriptional activity induced by the empty expression vector data not shown). Unfortunately, PEA3 but not ERM or ER81 expression vector protein was detectable in the MEF 1 cell line using an anti-myc antibody. Therefore the PEA3 subfamily upregulated OPN transcriptional activity in the PEA3 null MEF 1 cell line but could not be related to the amount of ERM or ER81 protein. Therefore the transient transfections were repeated under the same conditions in the COS-1 cell line.

Transient transfection of pCANmycPEA3, pCANmycERM, and pRSVmycER81 in the COS-1 cell line had previously yielded detectable amounts of protein by Western blot analyses using the anti-myc antibody (Trevor Shepherd, personal communication), probably due to higher transfection efficiency in the COS-1 cell line. The COS-1 cell line does not express PEA3 protein (Bojovic and Hassell, 2001), which makes it suitable to detect the effects of transfected PEA3 expression vectors. Consequently, the COS-1 cell line was assayed under conditions identical to those used in the MEF 1 cell line to assess the protein level and ability of each PEA3 subfamily member to upregulate OPN transcriptional activity.

The highest concentration of the PEA3 subfamily expression vectors transactivated pXP2-luc to similar levels, therefore their effects on the -777/+79 OPN promoter reporter were due to their effects on the OPN promoter and not the reporter backbone vector pXP2-luc (Figure 7a). The activity of pXP2 or the OPN reporter was higher with pRSVmyc than with pCANmyc, indicating that pCANmyc backbone suppressed OPN promoter activity more than the RSVmyc backbone.

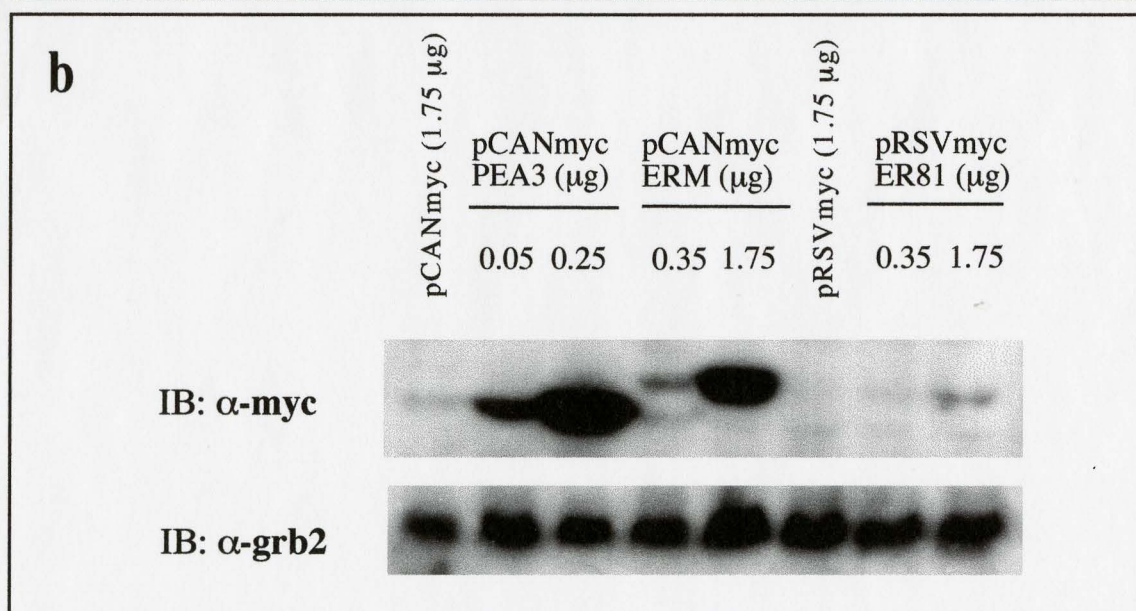
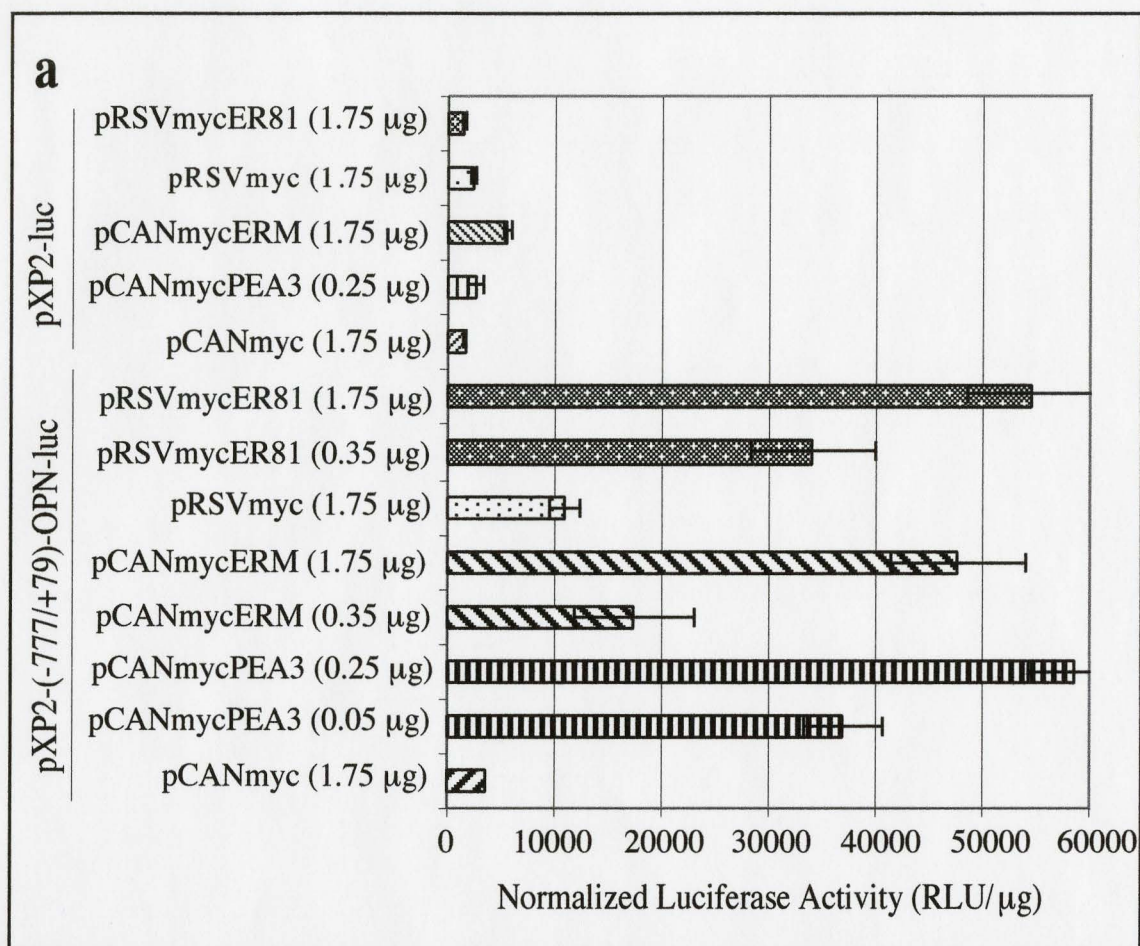
In the COS-1 cell line the activation of the -777/+79 OPN reporter was substantially less with the empty expression vectors than with transient cotransfection of PEA3 subfamily expression vectors. The transcriptional activity of the -777/+79 OPN promoter increased with cotransfection of pCANmycPEA3 (10- to 16-fold), pCANmycERM (5- to 13-fold), and pRSVmycER81 (3- to 5-fold) in comparison to the transcriptional activation of the -777/+79 OPN promoter with pCANmyc or pRSVmyc (Figure 7a). Therefore the PEA3 subfamily upregulated OPN transcriptional activity in both the MEF 1 cell line and the COS-1 cell line.

In the COS-1 cell line protein was visualized by Western blot analyses with an anti-myc antibody (Figure 7b). No protein was observed with empty expression vectors pCANmyc or pRSV, whereas higher pCANmycPEA3 and pCANmycERM protein levels were detected than the amount of pRSVmyc ER81 protein. The anti-grb2 Western blot showed that the proteins were equally loaded. Despite the differences in the expression protein backbone vectors, an increased amount of each PEA3 subfamily expression vector protein was detected in the COS-1 cell line that correlated with an upregulated

OPN transcriptional activity, suggesting that each member of the PEA3 subfamily upregulated OPN transcript levels in a dose-dependent manner.

Figure 7. Ectopic PEA3, ERM, and ER81 upregulated OPN transcriptional activity in the COS-1 cell line. (a) To determine whether transiently transfected PEA3 subfamily members upregulated OPN transcriptional activity, PEA3 subfamily expression vectors were transiently cotransfected with the -777/+79 OPN promoter reporter in the COS-1 cell line. The expression vectors pCANmycPEA3 (0.05 μ g, 0.25 μ g), pCANmycERM (0.35 μ g, 1.75 μ g), pRSVmycER81 (0.35 μ g, 1.75 μ g), and the empty expression vectors pCANmyc (1.75 μ g) and pRSVmyc (1.75 μ g) were transiently cotransfected with 0.25 μ g pXP2-(-777/+79)-OPN-luc or pXP2-luc. Luciferase activity was normalized to total protein for each duplicate sample and then averaged (RLU/ μ g). Two independent transfections yielded similar results. OPN transcriptional activity was increased by ectopic PEA3, ERM, and ER81 over the effect of the empty expression vectors.

(b) Western blot analysis determined the corresponding protein levels of the PEA3, ERM, and ER81 expression vectors. Pooled whole cell lysate (10 μ g) extracted from duplicate samples was assessed for expression vector protein levels by Western blot analysis using an anti-myc antibody. The Western blot was then stripped and reprobed with an anti-grb2 antibody to illustrate equal protein levels. The protein levels of each PEA3 subfamily member increased in correlation with the amount of expression vector transfected.



DISCUSSION

In this study, OPN was identified as a potential PEA3 target gene by screening a differential product library for genes that had higher transcript levels in the wildtype MEF 4 cell line than the PEA3 null MEF 1 cell line. Northern blot analysis showed that OPN transcript levels were over 10-fold higher in the wildtype MEF 4 cell line than the PEA3 null MEF 1 cell line, suggesting that PEA3 regulated OPN transcriptional activity.

To investigate whether the PEA3 subfamily regulated OPN, the correlation between PEA3, ERM, ER81, and OPN transcript levels were assessed by Northern blot analyses in 4 wildtype (100, 101, 104, 4) and 5 PEA3 null (1, 115, B5, B10, B12) MEF cell lines (Figure 1). OPN mRNA was expressed in the PEA3 null MEF cell lines and in cell lines that did not express ER81 transcripts, which indicated that PEA3 and ER81 were not required for OPN expression in the MEF cell lines. OPN transcripts were coexpressed with ERM transcripts in all of the MEF cell lines, although the MEF 1 cell line expressed substantially lower OPN transcript levels than the other MEF cell lines. Although there was no direct correlation between the mRNA levels of PEA3, ERM, ER81, and OPN, members of the PEA3 subfamily and OPN were coexpressed in the MEF cell lines in agreement with OPN being regulated by the PEA3 subfamily.

OPN transcripts were more correlated with ERM transcripts than with PEA3 or ER81 transcripts, suggesting that ERM was more likely to contribute to OPN transcriptional regulation than PEA3 or ER81 in the MEF cell lines. In 6 out of 8 MEF cell lines that expressed high OPN transcript levels there was coexpression of at least 2 PEA3

subfamily members. This suggested that the combined expression of the PEA3 subfamily members regulated OPN transcript levels. Northern blot analyses of the 9 MEF cell lines showed that the PEA3 subfamily had the potential to regulate OPN. It remained unknown whether ERM levels or the combined protein levels of the PEA3 subfamily regulated the level of OPN transcripts.

Northern blot analyses was assayed in 5 independent MEF 1 retransformant cell lines (C5-1, 1C, 1H, 1J, 1M) to determine whether an increased abundance of PEA3 subfamily transcripts upregulated OPN transcript levels, in comparison to the parental MEF 1 cell line (Figure 2). Two (1H, 1J) out of the 3 MEF 1 retransformant cell lines that expressed PEA3 but not ERM or ER81 transcripts did not express detectable OPN transcript levels. Hence an increased abundance of PEA3 protein was insufficient to upregulate OPN transcript levels relative to the MEF 1 cell line. Conversely, 2 MEF 1 retransformant cell lines (C5-1, 1M) that expressed PEA3, ERM, and ER81 transcripts coexpressed higher OPN transcript levels than the MEF 1 cell line. Therefore the mRNA expression of at least 2 PEA3 subfamily members correlated with high OPN transcript levels in the MEF 1 retransformant cell lines, which was in agreement with the Northern blot analyses of the 9 MEF cell lines (Figure 1).

Note that a longer exposure of the Northern blot probed with OPN cDNA (Figure 2) detected expression of OPN in the MEF 1 cell line but not in the MEF 1H or 1J cell lines (data not shown). The MEF 1 cell line expresses ERM but not PEA3 transcripts, whereas the MEF 1H and 1J cell lines express high PEA3 mRNA levels but no detectable ERM transcripts. The higher levels of OPN mRNA in the MEF 1 cell line than in the MEF 1H

and IJ cell lines are in agreement with a higher specificity of ERM than PEA3 to regulate OPN in MEF cell lines. An alternative explanation is that the bicistronic PEA3 protein may act differently than endogenous PEA3 in the MEF 1 retransformant cell lines, which could be tested by assaying the expression of other PEA3 target genes by Northern blot analyses.

The theory that ERM is more specific than PEA3 or ER81 in regulating OPN may not be mutually exclusive of the theory that the PEA3 subfamily additively regulates OPN transcriptional activity. In 8 out of 11 MEF cell lines that expressed high levels of OPN transcripts, transcripts of at least 2 PEA3 subfamily members were detected. All 3 PEA3 subfamily members may contribute to OPN regulation but their protein levels may be too low in the MEF cell lines to be individually sufficient in upregulating OPN expression. The relative amount of ERM protein in the MEF cell lines could be greater than that of PEA3 and ER81 protein, which could account for the positive correlation between ERM transcripts and OPN transcripts in 9 out of 11 cell lines. The mRNA profiles of the PEA3 subfamily and OPN in 9 MEF cell lines and 5 MEF 1 retransformant cell lines suggest that individual members of the PEA3 subfamily express insufficient protein levels to regulate OPN by themselves, ERM protein levels may be relatively higher than PEA3 or ER81, and that the PEA3 subfamily can additively regulate OPN.

Future Western blot analyses to assay relative PEA3 subfamily protein levels in the MEF cell lines would resolve whether there was more ERM than PEA3 or ER81 protein. The amount of PEA3 protein is substantially higher in the MEF 4 cell line than the MEF 1 cell line by Western blot analyses (Laing et al., 2000), but the PEA3 protein levels in

the other MEF cell lines is unknown. An ERM antibody was used to detect purified ERM protein in preliminary Western blot assays (data not shown), but endogenous ERM protein levels are unknown. Presently there are no purified ER81 antibodies available to detect endogenous ER81 protein in MEF cell lines, although the Northern blot analyses indicate that ER81 protein would be absent in most cell lines. The relative protein levels of PEA3 subfamily members remain to be clarified.

It is unclear why 3 out of the 11 MEF cell lines (B10, B12, 1C) that expressed high levels of OPN transcript coexpressed only 1 PEA3 subfamily member by Northern blot analysis. Perhaps OPN transcript levels were increased due to elevated expression levels of activating transcription factors such as Oct 1 or Oct 2 (Wang et al., 2000), or decreased expression levels of inhibitory transcription factors such as Hox-c-8 or Hox-c-9 (Shi et al., 1999, Shi et al., 2001; Hullinger et al., 2001). Alternatively, there may be increased levels of transcription factors that cooperate with the PEA3 subfamily to facilitate DNA binding, such as AP-1 (Bassuk and Leiden, 1997). The expression profile of other transcription factors in MEF cell lines is unclear but could account for the upregulated OPN expression in the MEF B10, B12, and 1C cell lines.

Analyses of the mouse OPN promoter indicated 16 candidate ETS core binding sites spread throughout the length of the -777/+79 OPN promoter (Figure 3). Three of these sequences are identical to the sequence motif functionally recognized by PEA3 in the polyomavirus enhancer (Martin et al., 1988) and is similarly recognized in gel-shift assays by ERM (Monte et al., 1994) and ER81 (Monte et al., 1995). Hence the OPN promoter has sequence motifs that can potentially bind the PEA3 subfamily.

Analysis of the OPN promoter also identified 6 consensus sites for AP-1, which is a dimeric binding site of Jun and Fos transcription factor families that is recognized by Jun/Fos or Jun/Jun (Karin et al., 1997). The PEA3 subfamily cooperates with AP-1 to upregulate transcription of the uPA enhancer (Nerlov et al., 1992), MMP-1 (Gutman and Wasylyk, 1990; Higashino et al., 1995), and MMP-9 (Gum et al., 1996). Interestingly, 2 potential AP-1 sites were located between -777 to -670 of the OPN promoter, flanking the RAE and the adjacent candidate ETS binding site (Figure 3). Competitive gel-shift assays showed that purified AP-1 protein did not compete effectively with proteins that interacted with a -740/-713 RAE probe, suggesting that AP-1 protein did not bind between -740 and -713 of the OPN promoter (Guo et al., 1995). However, other candidate AP-1 binding sites may be functional, such as the -707/-700 candidate AP-1 binding site adjacent to a candidate ETS binding site. It would be interesting to see whether transient cotransfection of the AP-1 family of transcription factors vectors would increase OPN reporter activity and if so, whether they would synergistically upregulate OPN promoter activity together with PEA3 expression vectors.

Upstream stimulatory factor 1 (USF-1) can synergistically transactivate an ETS binding site/E-box luciferase reporter with PEA3 (Greenall et al., 2001). Furthermore, USF-1 can bind PEA3 by gel-shift assays and increase the capacity of PEA3 to bind with DNA (Greenall et al., 2001). There were 2 consensus sequences between -777/-670 corresponding to an E-box recognized by the basic helix-loop-helix protein USF-1 (Figure 3). Competitive gel-shift assays with consensus USF-1 oligonucleotides showed that USF-1 could potentially bind 2 additional E-box motifs in the OPN promoter and

contribute to transcriptional regulation of OPN in human malignant astrocytoma cell lines (Wang et al., 2000). One of these E-boxes (-105/-98) is adjacent to a functional ETS binding site (-120/-115) recognized by Ets-1 and Ets-2 (Sato et al., 1998; Vary et al., 2000). USF-1 may cooperate with PEA3 (Greenall et al., 2001) in upregulating OPN in malignant astrocytomas. Opn mRNA is expressed in brain stem by in situ hybridization (Shin et al., 1999) and high levels of PEA3 subfamily transcripts are observed in brain tissue (Xin et al., 1992; Monte et al., 1994; Monte et al., 1995). Promoter mutation analysis showed that PEA3 and AP-1 contributed to higher uPA transcript levels in astrocytomas and glioblastomas relative to low-grade glioma and normal brain tissue (Lakka et al., 2001), suggesting that PEA3 is expressed in astrocytomas. OPN is also expressed in human malignant astrocytoma cell lines and may be transcriptionally regulated by USF-1 (Wang et al., 2000). The PEA3 subfamily may cooperate with USF-1 to upregulate OPN expression in the progression of astrocytomas. Whether OPN is cooperatively activated by both USF-1 and PEA3 could be determined by transient transfection assays.

Transient transfection of a series of 5' OPN promoter deletion mutants revealed a relatively uniform expression between the 5' OPN promoter deletion mutants and promoterless pXP2 vector in the HEK 293-1C cell line (Figure 4, 0 μ g pCANmycPEA3). The same promoter constructs fused to a β -galactosidase reporter in a NIH 3T3 cell line expressed a similarly uniform expression (Guo et al., 1995), suggesting that both the HEK 293-1C and NIH 3T3 cell lines have low endogenous OPN activity. A H-ras-transformed 3T3 cell line had a modest 2-fold higher OPN promoter activity than the

parental NIH 3T3 cell line for each promoter reporter (Guo et al., 1995). In the HEK 293-1C cell line each of the 5'OPN promoter luciferase reporters were also more active (~5-fold) with transiently cotransfected PEA3 except for -88/+79, although it also increased modestly with transfected PEA3 (Figure 4). Thus consistent with the regulation of PEA3 by ras/MAPK signal transduction pathway (Janknecht, 1996; Janknecht and Hunter, 1996), the OPN promoter reporter were upregulated in the H-ras-transformed 3T3 cell line relative to the parental NIH 3T3 cell line, as well as in the HEK 293-1C cell line when transiently transfected with PEA3.

In the HEK 293-1C cell line, the -258/+79 OPN promoter deletion mutant was responsive (6-fold) to transiently transfected PEA3 expression vectors, in comparison to the empty expression vector (Figure 4). Ets-1 similarly increases the activity of a -254/+66 mouse OPN promoter reporter 8-fold, in comparison to the empty expression vector in the NIH 3T3 cell line (Sato et al, 1998). The (-120/-115) core ETS binding site is functionally recognized by Ets-1 (Sato et al., 1998) and Ets-2 (Vary et al., 2000) by gel-shift and transient transfection assays. MMP-1, MMP-3, and uPA are regulated by both Ets-1 (Wasylyk et al., 1993) and PEA3 (Higashino et al., 1995; Kaya et al., 1996, Rorth et al., 1990). The Ets-1/Ets-2 binding site overlaps a candidate PEA3 binding site and could be a recognized by both the Ets-1/Ets-2 and the PEA3 subfamilies (Figure 3). Thus the same region of the OPN promoter is responsive to multiple Ets subfamilies.

The greatest decrease in PEA3 responsiveness was detected when the region between -258 and -88 of the OPN promoter was deleted. There are 7 core ETS binding sites between -258 and -88 in the OPN promoter that could account for PEA3 responsiveness

(Figure 3). The PEA3 responsiveness between -258 and -88 of the OPN promoter indicate that additional regions besides the -777/-670 promoter region (Guo et al., 1995) are functional in binding PEA3. There were 16 candidate core ETS binding sites in the OPN promoter and competitive gel-shift assays could be utilized to determine which sites bind PEA3. Purified PEA3 protein bound to a radiolabelled consensus PEA3 binding motif could be mixed with excess cold probes of the 16 candidate core ETS binding sites, fractionated on a native acrylamide gel, autoradiographed, and assessed for sites that competitively bind the PEA3 protein. Site-directed mutagenesis of PEA3 binding site probes and subsequent abolished competitive binding in a similar gel-shift assay would verify which sites in the OPN promoter bind PEA3 protein. Transient cotransfections of PEA3 and OPN promoter reporters mutated at a PEA3 binding site would show which sites are functional in PEA3 transcriptional activation of the OPN promoter. In short, the OPN promoter is responsive to ectopic PEA3 in the HEK 293-1C cell line in areas of the promoter that have core ETS binding sites and the identity of functional PEA3 binding sites remains to be elucidated.

To determine whether the OPN promoter could be responsive to PEA3 in MEF cell lines, OPN transcriptional activity was compared between the wildtype MEF 4 cell line and the PEA3 null MEF 1 cell line. OPN transcriptional activity was 7- to 20-fold greater in the MEF 4 cell line than the MEF 1 cell line (Figure 5), comparable to the 10-fold higher OPN transcript levels in the MEF 4 cell line than the MEF 1 cell line detected by Northern blot analysis (Figure 1). Thus the higher OPN mRNA expression in the wildtype MEF 4 cell line in comparison to the PEA3 null MEF 1 cell line was attributed

to higher OPN transcriptional activity in the MEF 4 cell line. The greater OPN transcriptional activity also reflected the greater number of PEA3 subfamily members expressed in the MEF 4 cell line (Figure 1), supporting the theory that the PEA3 subfamily additively regulated OPN transcriptional activity.

An alternative explanation was that the transfection efficiency was higher in the MEF 4 cell line than the MEF 1 cell line, as suggested by the higher activity of the empty reporter vector pXP2-luc in the MEF 4 cell line than the MEF 1 cell line (Figure 5). However, other unidentified transcription factors may differ between the two cell lines that affect the expression of pXP2-luc. Furthermore, the transfection efficiency between the MEF 4 cell line and the MEF 1 cell line has previously been reported to be similar (Laura Hastings, personal communication). The transfection efficiencies of the expression vectors were also not measured, although independent experiments with different DNA preparations reproduced the higher OPN transcriptional activity in the MEF 4 cell line than the MEF 1 cell line. Future transient transfection assays measuring another reporter vector would be required to confirming equal transfection efficiencies between expression vectors.

To determine whether inhibition of PEA3 subfamily activity suppressed OPN transcriptional activity, dominant negative PEA3 was transiently cotransfected in the wildtype MEF 4 cell line that expressed high transcript levels of PEA3 and OPN. The -472/+79 OPN promoter was ~2-fold higher in activity compared to the reporter backbone vector pXP2-luc, but the fold-activation was reduced with increasing amounts of pCANmycΔNPEA3En to the level of pXP2-luc (Figure 6). Therefore competitive

inhibition of PEA3, ERM, and ER81 protein resulted in the suppression of OPN transcriptional activity, suggesting that the PEA3 subfamily contributed to endogenous OPN expression in MEF 4 cell line. Although the expression of other Ets family members may also have been inhibited, the expression of other Ets proteins was not been investigated in the MEF cell lines.

To address whether individual members of the PEA3 subfamily increased OPN transcriptional activity, PEA3 subfamily expression vectors and a -777/+79 OPN reporter were transiently cotransfected in the COS-1 cell line. OPN transcriptional activity increased 10- to 16 fold with pCANmycPEA3, 5- to 13-fold with pCANmycERM and 3- to 5-fold with pRSVmycER81 in comparison to empty expression vectors in the COS-1 cell line (Figure 7a). Furthermore, increased expression vector concentrations correlated with increased protein levels of all PEA3 subfamily expression vectors by Western blot analysis (Figure 7b). Therefore increased OPN transcriptional activity correlated with increased PEA3 subfamily expression vector protein levels in the COS-1 cell line, suggesting that each PEA3 subfamily member activated the OPN promoter in a dose-dependent manner and are functionally redundantly in activating OPN transcription. This is consistent with the observation that the transcripts of at least 2 PEA3 subfamily members are correlated with high OPN transcript levels (Figure 1 and 2).

The greater amount of pCANmycERM and pCANmycPEA3 protein than pRSVmycER81 protein suggested that the pCANmyc driven by a cytomegalovirus promoter was more stable or had a higher efficiency of transcription or translation than the Rous Sarcoma Viral LTR in pRSVmyc in the COS-1 cell line. The increased stability

of transcriptional or translational efficiency of pCANmyc in the COS-1 cell line may account for the ~3-fold higher activation of pCANmycPEA3 and pCANmycERM in the COS-1 cell line than in the MEF 1 cell line. In contrast, the fold-activation of pRSVmycER81 remained similar between the MEF 1 cell line and the COS-1 cell line. Cloning ER81 in pCANmyc would make comparisons between the effects of the PEA3 subfamily members on OPN transcriptional activity more effective.

The regulation of OPN by the PEA3 subfamily is of particular interest in the progression of mammary adenocarcinomas, because both PEA3 (Benz et al., 1997) and OPN (Tuck et al., 1998) are overexpressed in human primary breast cancers. All primary and metastatic tumors in MMTV-Neu mice overexpressed PEA3 (Trimble et al., 1993), ERM and ER81 (Trevor Shepherd, private communication) by Northern blot analyses or RNase protection. Similarly, OPN mRNA and protein is upregulated in the mammary tumors of MMTV-v-H-ras or MMTV-c-myc mice, in comparison to normal mammary glands (Rittling and Novick, 1997). It is unknown whether MMTV-Neu mice overexpress OPN. Therefore both the PEA3 subfamily and OPN are overexpressed in breast tumors and it is possible that PEA3 subfamily overexpression in MMTV-Neu adenocarcinomas results in upregulated OPN expression.

Elevated OPN levels increase the tumor number (Gardner et al., 1994; Rittling and Novick, 1997; Wu et al., 2000) and metastatic behaviour of H-ras-transformed cells (Chambers et al., 1992; Behrend et al., 1994). Subsequently, increased OPN expression in MMTV-Neu mammary tumors may accelerate tumor progression. Future assessments of OPN and the PEA3 subfamily expression in MMTV-Neu mammary adenocarcinomas

could be assessed to determine whether OPN played a role in Neu-induced tumor progression.

This study shows for the first time that OPN is regulated by the PEA3 subfamily in MEF cell lines. The ability of the PEA3 subfamily to transcriptionally activate OPN is consistent with the role of the PEA3 subfamily as transcriptional activators (Higashino et al., 1995; Kaya et al., 1996). PEA3 activates multiple proteins involved in degrading major components of the extracellular matrix including MMP-1 (Higashino et al., 1995), MMP-3 (Higashino et al., 1995), MMP-9 (Kaya et al., 1996), MMP-7 (Crawford et al., 2001), and urokinase plasminogen activator enhancer (Rorth et al., 1990). The subsequent activation of PEA3 target genes can lead to metastatic behaviour of tumor cells (Kaya et al., 1996; Hida et al., 1997). Elevated OPN levels also cause benign tumor cells to progress to malignant cells (Oates et al., 1996; Chen et al., 1997). However, while other PEA3 target genes digest extracellular matrix proteins to mediate metastases, OPN increases the migration of cells by increasing recognition by cell adhesion receptors such as integrin $\alpha_5\beta_3$ (Oldberg et al., 1986). The ability of the PEA3 subfamily to upregulate the transcription of both MMPs and OPN produce complementary routes in which tumour cells can attach and migrate.

CONCLUSIONS

OPN was identified as a candidate transformation-associated PEA3 target gene that was expressed more in the wildtype MEF 4 cell line than the PEA3 null MEF 1 cell line. The relative PEA3 subfamily and OPN transcript levels in 5 PEA3 null MEF, 4 wildtype MEF, and 5 MEF 1 retransformant cell lines suggested that the PEA3 subfamily additively regulates OPN expression, although OPN may be a specific target gene of ERM in the MEF cell lines. The relative PEA3 subfamily member protein levels in MEF cell lines have yet to be determined. Transient transfection of 5' OPN promoter deletion mutants and ectopic PEA3 showed that the -258 to -88 OPN promoter region was required for maximal PEA3 responsiveness in HEK 293-1C cells. There are 16 candidate ETS binding sites and future studies include determining functional PEA3 binding sites in the OPN promoter. OPN promoter activity was higher in the wildtype MEF 4 cell line than the PEA3 null MEF 1 cell line, consistent with the higher OPN mRNA expression in the MEF 4 cell line than the MEF 1 cell line. Ectopic dominant negative PEA3 suppressed OPN promoter activity in the MEF 4 cell line. Conversely, ectopic PEA3, ERM, and ER81 each transcriptionally activated the OPN promoter in the MEF 1 and COS-1 cell lines. Thus this study shows for the first time that OPN is transcriptionally activated by the PEA3 subfamily. Potentially tumor cells overexpressing the PEA3 subfamily could upregulate OPN expression and subsequently increase the potential for malignant transformation.

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