PEA3 EXPRESSION REGULATION & ITS EFFECT ON CD1 TRANSCRIPTION

UP- AND DOWN-STREAM OF PEA3:

REGULATION OF PEA3 EXPRESSION AND ITS EFFECT ON CYCLIN D1 TRANSCRIPTION

By

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ABSTRACT

PEA3 is a member of the expanding Ets family of transcription factors. In the adult mouse, *pea3* mRNA is expressed at highest levels in the brain, epididymis and at lower levels in the mammary gland, testes, ovary and uterus. *PEA3* is overexpressed in 93% of all *HER2/Neu* positive human breast tumors and in 77% of mouse multiple intestinal (Min) tumors. Many of these tumors have disruptions in the Ras/MAPK and Wnt-signaling pathways. Analysis of the influence of these pathways on *pea3* promoter activity revealed that effectors of both pathways increased transcription from this promoter. Deletion mutations of the *pea3* promoter linked to a luciferase reporter gene were used to localize the DNA sequences that are responsible for the effect of the Ras/MAPK pathway on its expression. A Ras-responsive element (RRE), composed of an ETS and an AP-1 binding site, was identified between sequences –247 and -227 and its importance was confirmed through mutational analysis.

CYCLIN D1 is a potent oncogene involved in different types of tumors. The *CYCLIN D1* gene is amplified in 20% of human mammary carcinomas, and its mRNA is overexpressed in 50% of human breast cancers. The *CYCLIN D1* (*CD1*) promoter was shown to be responsive to PEA3 transactivation and to dominant-negative PEA3 inhibition in co-transfection experiments in Cos-1 cells. Of the 4 Ets-binding sites (EBS) in the *CD1* promoter, one site was shown to be important for the activity of the promoter and for its capacity to respond to PEA3 transactivation. It was also determined that

PEA3, β-catenin, Lef-1 and c-Jun cooperated synergistically to activate the *CD1* promoter. PEA3 was absolutely required for the manifestation of this synergy among these transcription factors. These findings collectively illustrate the key role of PEA3 as an effector of multiple oncogenic signaling pathways.

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CONTRIBUTIONS FROM OTHERS

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ABBREVIATIONS

AP-1	Adaptor protein complex 1
ATP	adenosine triphosphate
bp	base pair
CD1	cyclin D1
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dTTP	deoxythymidine triphosphate
EBS	Ets binding site
ETS	E26 transformation-specific or E twenty-six
kb	kilobase
luc	luciferase
mRNA	messenger RNA
PCR	polymerase chain reaction
PEA3	Polyomavirus enhancer activator 3
RNA	ribonucleic acid
RRE	Ras-responsive element
Taq	*Thermophile aquaticus*
tRNA	transfer RNA
U	unit

INTRODUCTION

1. *PEA3*, a Member of the *ETS* Oncogene Family

Polyomavirus enhancer activator 3 (PEA3) is a protein capable of binding the PEA3 motif ($^{5'}$AGGAAG$^{3'}$) in the polyomavirus enhancer (Martin *et al.* 1988). *pea3* was originally identified from mouse 3T6 cells (Martin *et al.* 1988) and later cloned from FM3A mammary tumor cells as a PEA3 motif-binding protein and named Pea3 (Xin *et al.* 1992). The cDNA encodes a protein of 480 amino acids, which migrates with an apparent molecular mass of 66kDa on denaturing polyacrylamide gels.

Analysis of the *pea3* cDNA sequence reveals the presence of an ETS domain, a DNA-binding domain comprising ~85 amino acids, placing PEA3 in the family of transcription factors founded by v-ETS (Leprince *et al.*, 1983). The term ETS (**E**26 **t**ransformation-**s**pecific or **E** **t**wenty-**s**ix) is given to the DNA binding domain of cellular proteins that exhibit strong amino acid sequence conservation with the DNA binding domain of the v-Ets protein. Over the past 20 years, a large number of different Ets-related proteins have been discovered in species such as human, mouse, chicken, pufferfish, xenopus and *Drosophila* (Reviewed in Oikawa and Yamada, 2003). The mouse ETS family is today comprised of 27 members and is further divided into sub-families according to sequence homology within as well as outside the ETS domain (Graves and Petersen, 1998). All Ets proteins bind to 9 to 15 base pair sequence elements; a common feature of such Ets-binding sites is a central 5'-GGA,A/T-3' motif

(Wasylyk *et al.* 1993). Specificity for binding of particular Ets proteins is conferred by sequences flanking this core.

PEA3 is the founding member of a sub-family of Ets proteins including ER81 (Brown and McKnight, 1992; Monté *et al.*, 1995; Jeon *et al.*, 1995) and ERM (Monté *et al.*, 1994). The ETS domains of these three proteins are 97% identical and they share additional sequence similarity over their entire length. All three family members are co-expressed in several tissues and organs, which suggests that their transcription is influenced in part by a common pathway(s) (Xin *et al.*, 1992; Monté *et al.*, 1994; Monté *et al.*, 1995). *pea3* expression occurs mainly in the brain and epididymis, but lower levels of *pea3* mRNA are also found in the kidney, skeletal muscle, hair follicles, spinal cord, intestine, testis and mammary gland (Xin *et al.*, 1992). The expression profiles of the *pea3* subfamily members, which overlap during early embryonic development and subsequently become more unique (Chotteau-Lelievre *et al.*, 1997, Laing *et al.*, unpublished data), suggest that these three genes may play both distinct and similar roles. Definition of the cellular expression profiles of these genes may clarify the degree of overlap between their functions.

2. ETS Proteins and Oncogenesis

Ever since the identification of *v-ets-1* as a cause for erythroleukemias in chicken (Leprince *et al.*, 1983), 27 members of the ETS family of transcription factors have been shown to be involved in oncogenesis in mice and humans. In humans, Ewing's sarcomas are the result of chromosomal translocations that fuse a variety of ETS DNA-binding

domains with the N-terminal domain of *EWS*. The majority of these tumors are characterized by a t(11;22)(q24;q12) translocation that fuses the *EWS* gene on chromosome 22 to *FLI-1* on chromosome 11, thereby producing a chimeric protein that acts as a more potent transcriptional activator than FLI-1 (Bonin *et al.*, 1993; May *et al.*, 1993). The resulting increase in expression of the FLI-1 target genes likely contributes to tumor formation (Ohno *et al.*, 1993; Bailly *et al.*, 1994). PEA3 has also been identified as an EWS fusion in Ewing family of tumors (Kaneko *et al.*, 1996).

ETS proteins can be activated indirectly in oncogenesis. Many have been shown to be downstream targets of constitutively activated non-nuclear oncoproteins. Overexpression of *HER-2/neu*, a gene encoding a receptor tyrosine kinase of the epidermal growth factor receptor (EGFR) family, is found in 20-30% of all breast carcinomas. These tumors have a higher incidence of metastasis and result in a poor prognosis for the patient (Slamon *et al.*, 1987). Interestingly, *PEA3* mRNA is overexpressed in 93% of tumors that overexpress *HER-2/neu* (Benz *et al.*, 1997). There is no evidence to suggest that the *PEA3* gene is amplified in these tumors. This strongly suggests that the increased expression is controlled at the level of transcription. PEA3 has been shown to regulate expression of *HER-2/neu* and its own expression, which would suggest the presence of a positive feedback loop leading to overexpression of PEA3 target genes and any gene downstream of *HER-2/neu* (Benz *et al.*, 1997).

PEA3 is a transcriptional activator of genes whose products are involved in degradation of the extracellular matrix (Benbow and Brinckerhoff, 1997). This can in turn lead to increased invasiveness and metastasis of tumors over-expressing *PEA3*. In

transient transfection assays, PEA3 was shown to transactivate the promoters of collagenase (*MMP-1*), stromelysin (*MMP-3*), gelatinase (*MMP-9*), (Higashino, 1995) and matrilysin (*MMP-7*) (Crawford *et al.*, 1999). PEA3 binding sites also occur in the promoters of urokinase type plasminogen activator (Rorth *et al.*, 1990; Nerlov *et al.*, 1992), stromelysin-2 (*MMP-10*) and stromelysin-3 (*MMP-11*) (Crawford and Matrisian., 1996). In addition, exogenous expression of PEA3 in MCF-7 cells, a non-metastatic breast cancer cell line, increases the cells' invasive and metastatic potentials (Kaya *et al.*, 1996). Recently, experiments involving overexpression of antisense *PEA3* in human mammary epithelial cells (184B5) showed that PEA3 was required to observe a HER-2/Neu-induced increase in *COX-2* promoter activity (Subbaramaiah *et al.*, 2002). This is a significant observation since the enhanced synthesis of prostaglandins by COX-2 was shown to favor tumor growth by stimulating cell proliferation (Sheng *et al.*, 2001), promoting angiogenesis, (Tsujii *et al.*, 1998), increasing invasiveness (Dohadwala *et al.*, 2001) and inhibiting apoptosis (Sheng *et al.*, 1998). Overall, PEA3 appears to be an important player in a number of different oncogenic processes.

2. Signaling Pathways that Influence Gene Expression

a. The Mitogen Activated Protein Kinase Pathway

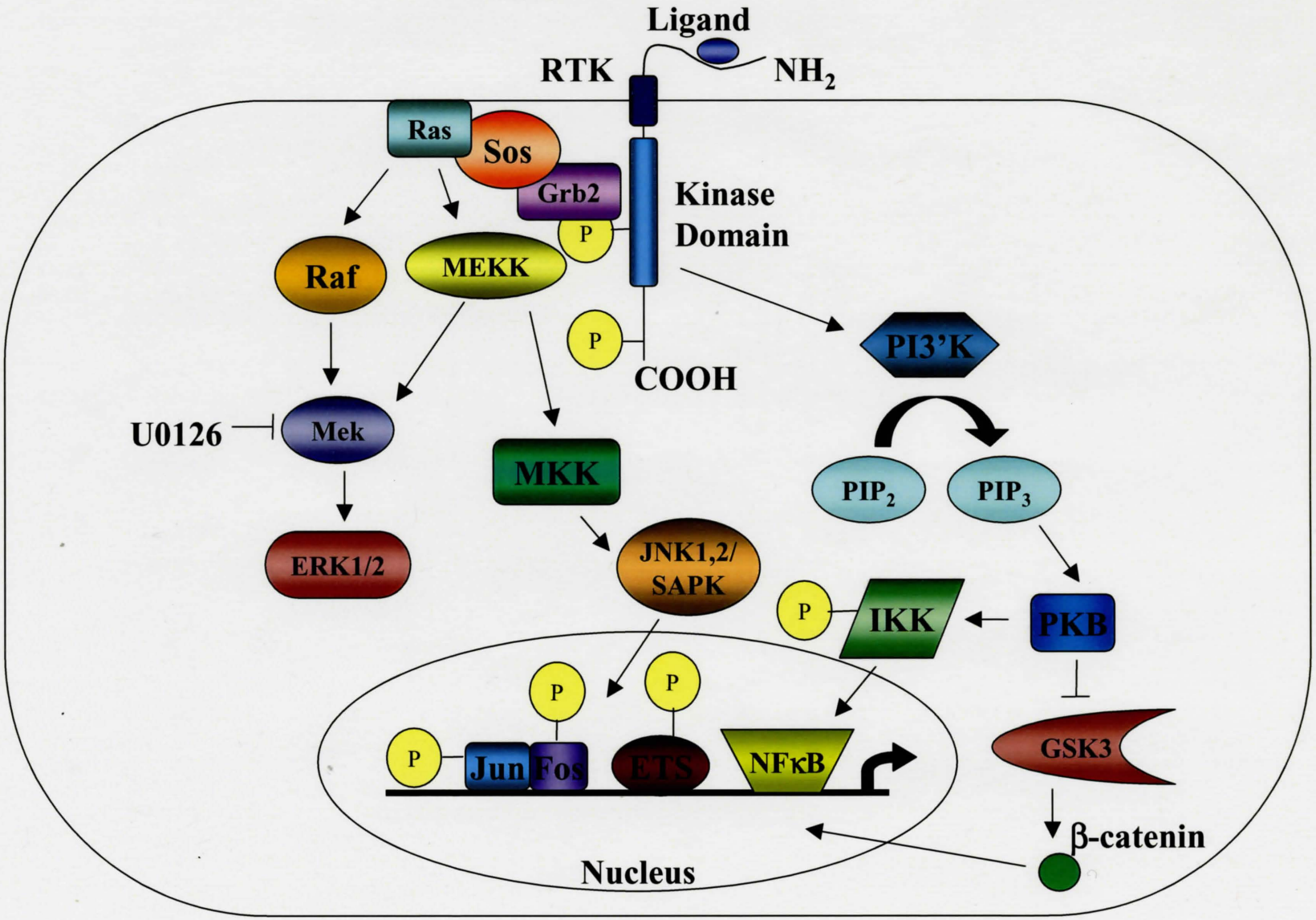
One of the most widely studied growth factor signaling pathways is the **m**itogen **a**ctivated **p**rotein **k**inase (MAPK) pathway (Figure 1). This pathway is initiated by the binding of a growth factor to its receptor, which is generally a receptor tyrosine kinase (RTK). Activation of the RTK can lead to activation of Ras (Chardin *et al.*, 1993; Boriack-Sjodin *et al.*, 1998), which in turn mediates activation of Raf, initiating a kinase

cascade that includes MEK (see Figure 1). MEK activates MAPK (also called ERK for extracellular-signal regulated kinase). Activated MAPK then translocates to the nucleus where it phosphorylates members of the AP-1 family of transcription factors (Cooper, G.M., 1995), such as Jun and Fos, and proteins of the ETS family such as ETS-1 and ETS-2 (Yang *et al.*, 1996), allowing them to turn on their target genes.

There is a strong correlation of elevated transcript levels of *HER2/Neu* RTK and *pea3* sub-family members in mammary tumors (Benz *et al.*, 1997; Shepherd and Hassell, 2001). Recent studies present evidence that *pea3* and *erm* are also regulated by members of the fibroblast growth factor (FGF) family of signaling molecules (Raible and Brand, 2001), which are receptor tyrosine kinases with high sequence similarity to the HER2/Neu protein, especially in the kinase domain, which suggests that their downstream effectors might be common (Raible and Brand, 2001). There is a strong correlation between the patterns of expression of *fgf-8* and *fgf-3* and those of *pea3* and *erm* during zebrafish embryonic development (Roehl and Nusslein-Volhard, 2001, Raible and Brand, 2001). Interference with FGF signaling by an FGF inhibitor (SU5402) leads to absence of *erm* and *pea3* transcripts in the affected tissues. Moreover, ectopic FGF signals can induce *pea3* and *erm* transcription in concentration gradients characteristic of the diffusion pattern in which these signaling molecules exert their function (Raible and Brand, 2001). Overall, it seems likely that activation of the MAPK pathway could lead to increased PEA3 expression.

Figure 1. **Growth Factor Signaling Pathways**

This figure illustrates the major players involved in signaling initiated by receptor tyrosine kinase (RTK) ligands, which lead to cellular mitosis. The Ras/MAPK pathway is depicted on the left, and part of the PI3'K pathway is shown on the right. Many players that are involved in other cellular functions, such as apoptosis and cell migration, were omitted for clarity.


Ligand
RTK
NH2
Ras
Sos
Grb2
P
Kinase
Domain
Raf
MEKK
P
COOH
PI3'K
U0126
Mek
MKK
PIP2
PIP3
ERK1/2
JNK1,2/
SAPK
P
IKK
PKB
P
P
P
Jun
Fos
ETS
NFκB
GSK3
β-catenin
Nucleus

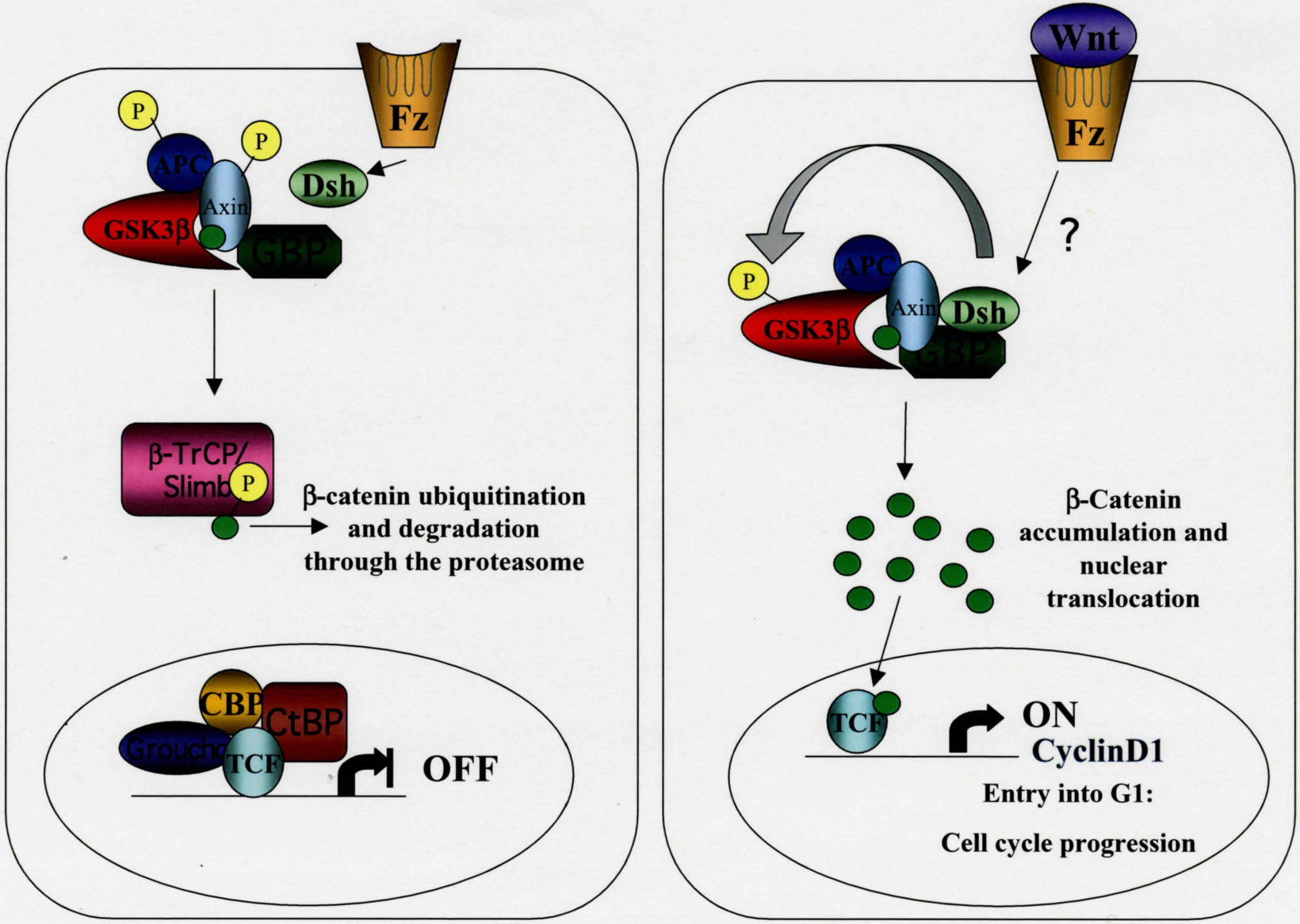
b. The Wnt-signaling Pathway

The Wnt-signaling pathway was first studied in Drosophila and involves a sequence of protein:protein interactions that is triggered by the binding of Wnt ligands to their cognate receptor. Figure 2 illustrates the essentials of this pathway (reviewed in van Noort and Clevers, 2002 and in Peifer and Polakis, 2000). One of the final events of this pathway is the activation of β-catenin through its release from the APC (**a**denomatous **p**olyposis **c**oli) complex. This allows β-catenin protein levels to rise since, in the absence of Wnt signal, binding of APC potentiates β-catenin phosphorylation by GSK3 on serine residues in its carboxy-terminal tail, targeting it for degradation by the proteosome system. Free to enter the nucleus, β-catenin can associate with its co-factor and DNA binding partner, Lef-1/TCF. β-catenin acts as an activation domain for Lef-1/TCF (reviewed in van Noort and Clevers, 2002 and in Peifer and Polakis, 2000). Several target genes containing consensus binding sites for Lef-1/TCF have been implicated in cell proliferation and metastasis, including *CYCLIN D1* (Rimerman *et al.*, 2000) and several matrix metalloproteinases (*MMP*s) (Crawford *et al.*, 1999).

Overexpression of *pea3* was observed in mammary tumors arising in MMTV-Wnt-1 transgenic (Howe *et al.*, 2001) and in intestinal tumors of Min (**m**ultiple **i**ntestinal **n**eoplasia) mice, which have a mutation in the *apc* gene (Wilson et *al.*, 1997). This mutation is also common in colon carcinoma cell lines (reviewed in Grady and Markowitz, 2002), such as SW480, which express relatively high levels of PEA3 (Crawford *et al*, 2001).

Figure 2. **The Wnt-Signaling Pathway**

The major players in the Wnt-signaling pathway are illustrated. The "OFF" state is depicted on the left, in the absence of Wnt. The "ON" state is shown on the right. Wnt binding to the family of receptors Frizzled (Fz) leads to activation of Dishevelled (Dsh). Dsh function includes inhibiting GSK3β kinase activity. This prevents phosphorylation of adenomatous polyposis coli (APC) protein, which is then unable to form a complex with axin, GSK3β and β-catenin. It is believed that formation of this complex is necessary for regulation of β-catenin levels in the cell. Phosphorylation of β-catenin on serines in its carboxy-terminal tail, targets it for degradation by the proteosome system. Accumulation of β-catenin allows it to translocate to the nucleus where it binds to members of the Lef-1/TCF family of transcription factors and induces transcription from target genes' promoters. In the absence of Wnt signaling, Lef-1/TCF is free to associate with transcription co-repressors, such as Groucho and CtBP.


Fz
P
APC
P
Axin
Dsh
GSK3β
GBP
β-TrCP/
Slimb
P
β-catenin ubiquitination
and degradation
through the proteasome
CBP
CtBP
Groucho
TCF
OFF
Wnt
Fz
?
P
APC
Axin
Dsh
GSK3β
GBP
β-Catenin
accumulation and
nuclear
translocation
TCF
ON
CyclinD1
Entry into G1:
Cell cycle progression

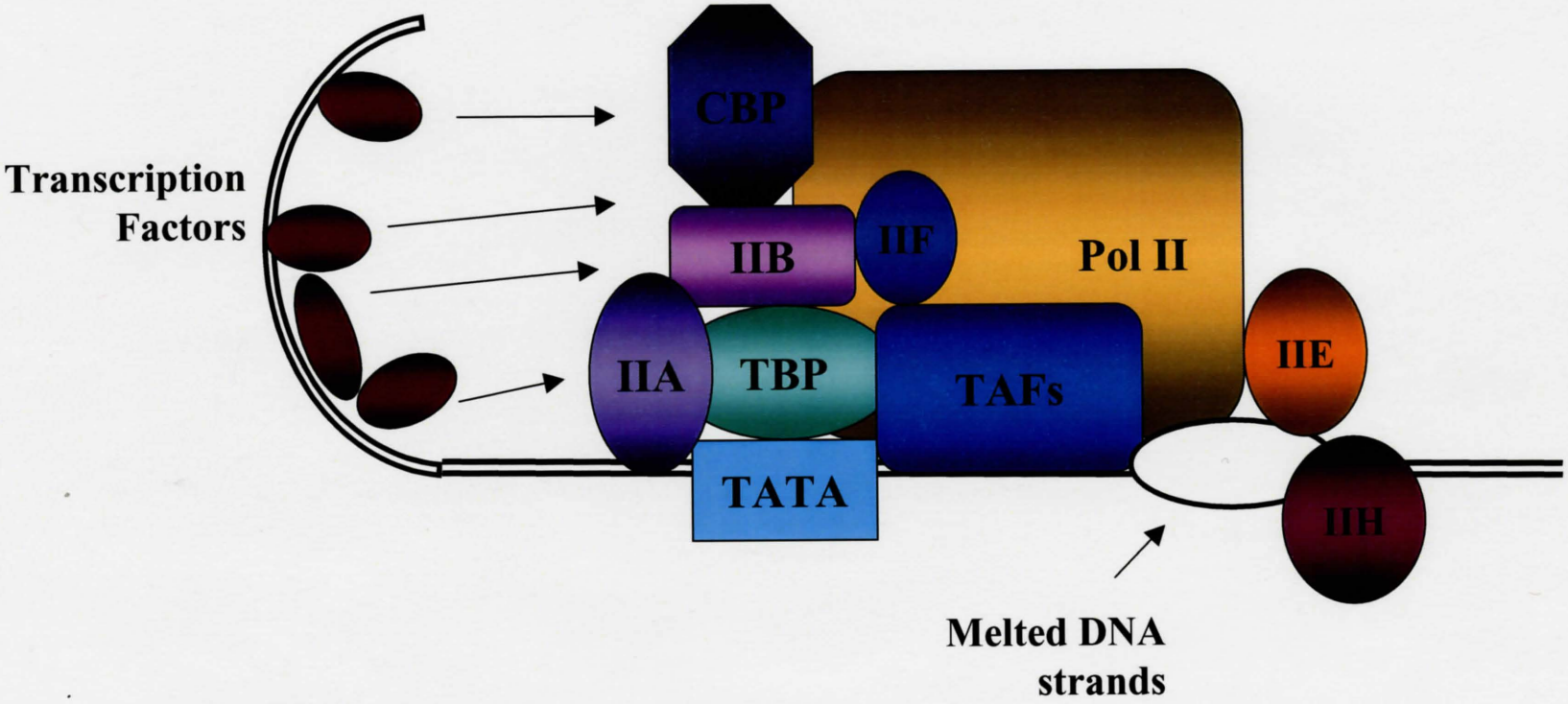
An analysis of the influence of the MAPK and Wnt-signaling pathways on the PEA3 promoter would provide one more piece in the puzzle of oncogenesis, bringing us one step closer to seing the entire picture. It is likely that the Wnt-signaling pathway and/or the MAPK pathway upregulate expression of *PEA3*, which may in turn act to upregulate target genes involved in tumor formation and progression.

4. The *pea3* promoter

A promoter is defined as "a binding site in a DNA chain at which RNA polymerase binds to initiate transcription of messenger RNA by one or more nearby structural genes" (Merriam-Webster online dictionary). Commonly, promoters are considered to also be the region of DNA on either side of this binding site; most promoters are located mainly upstream of the transcription start site, but sequences 3' of the transcriptional initiation site have also been implicated in the regulation of expression of a number of genes (Carey and Smale, 1999). More than half of all the known promoters have a short stretch of DNA ($^{5'}$TATAAA$^{3'}$), which is normally located ~25bp upstream of the transcription start site, also called a TATA box (Carey and Smale, 1999). This is bound by TATA binding protein (TBP), which is believed to be the first event in the assembly of the pre-initiation complex (Carey and Smale, 1999) The *pea3* promoter is a TATA-less promoter (Barrett, 1997; Kann, 1999). The *pea3* promoter has a putative initiator (Inr) element ($_{-3}$CTCACAACT$_{+6}$) (Bucher, 1990) with one mismatch to the Inr consensus sequence, PyPyANT/APyPy, with the N being at the +1 position (Smale and Baltimore, 1989).

Figure 3. **Mechanism of Transcription Initiation**

The pre-initiation complex is shown here as it is believed to be right before promoter release and transcription initiation. TATA-binding protein (TBP) binds to the TATAA sequence of the promoter and recruits the other factors and RNA polymerase II (Pol II) to the promoter. The DNA strands are melted and stabilized by TFIIE and TFIIH. Many transcription factors can help stabilize the complex through DNA:protein and protein:protein interactions.


Transcription Factors
CBP
IIB
IIF
Pol II
IIA
TBP
TAFs
IIE
TATA
IIH
Melted DNA strands

The mechanism of initiation of TATA-less promoters is still obscure, although it is believed that the transcription machinery is recruited to the start site in a similar way to the TATA box-containing promoters (Butler and Kadonaga, 2002). The assembly of the pre-initiation complex on TATA box-containing promoters is fairly well characterized (reviewed in Azizkhan *et al.*, 1993 and Smale S.T., 1997). The process of transcription initiation is illustrated in Figure 3.

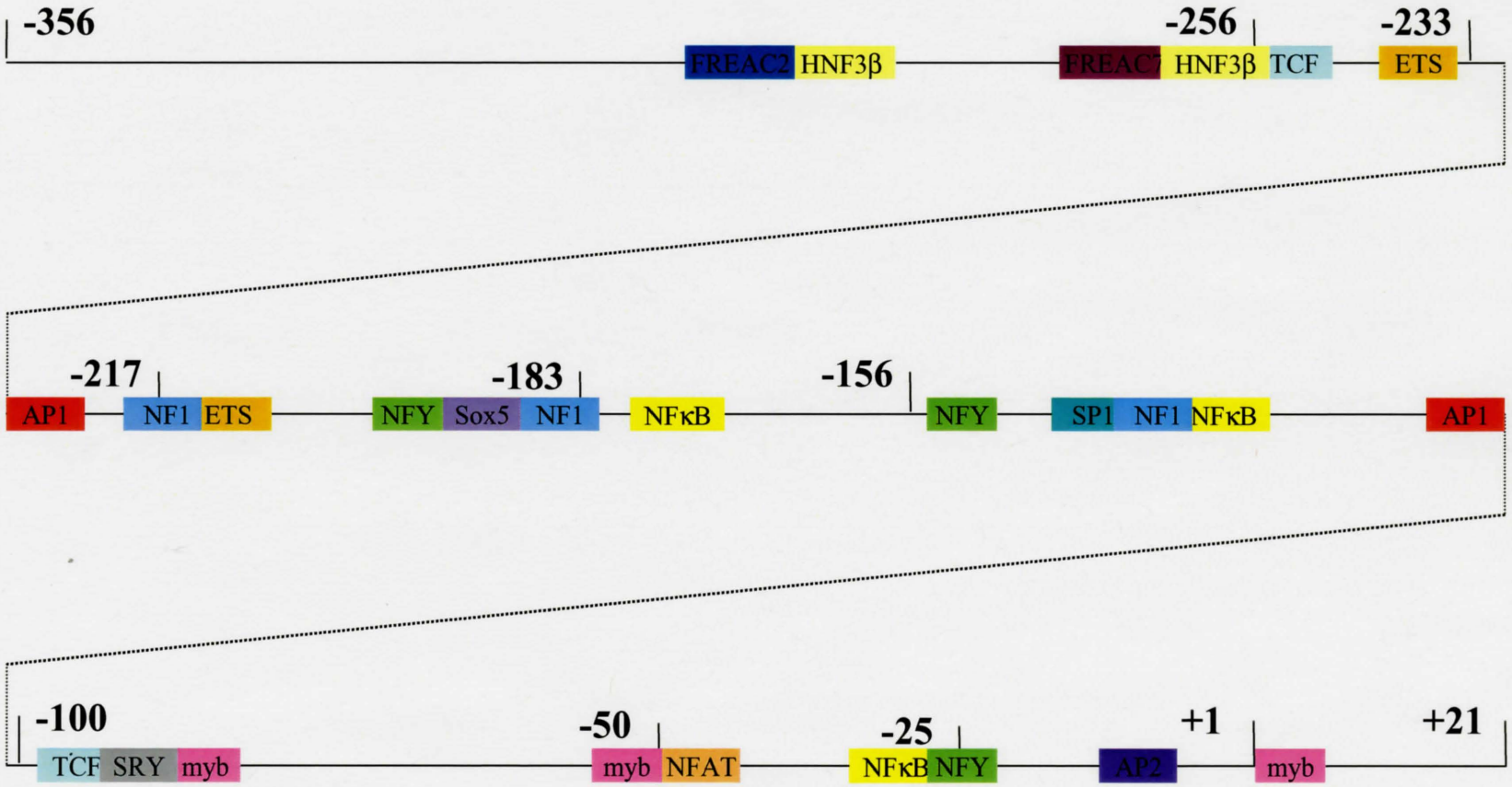
In addition to the binding sites for the components of the basal transcription machinery, promoters usually contain DNA sequence elements that can be bound by transcription activators and repressors. The latter are responsible for increasing or decreasing, respectively, the rate of transcription of a given gene by the basal transcription machinery. The *pea3* promoter contains multiple putative transcription factor binding sites, as determined by scanning the TRANSFAC database (Quandt *et al.*, 1995; Wingender *et al.*, 1996; Wingender *et al.*, 1997). Figure 4 illustrates the putative binding sites that show at least 80% consensus with the optimal transcription factor binding sites and that are at least 80% conserved in sequence and position relative to the transcription start site among multiple species: human, mouse, chicken and/or pufferfish (Kann, 1999).

Finding out what genes lie downstream of transcription factors such as PEA3 may be just as important as finding how their expression is regulated. A lot of work is currently being done to try and identify target genes for transcription factors, using microarray analyses, reporter gene assays, knockout animal models and electromobility shift assays. There is a strong correlation between elevated levels of *pea3*

Figure 4. **Structure of the Mouse *pea3* Promoter**

The 5' end of the mouse *pea3* gene was analyzed for the presence of conserved transcription factor consensus sequences using MatInspector® software. Putative recognition sequences matching 80% of the consensus, and 80% conserved between mouse and human, are labeled. The sequence is numbered relative to the major transcription start site, +1.

AP1: activator protein 1 (Piette *et al.*, 1988); AP2: activator protein 2 (Hermann and Doerfler, 1991); ETS: E twenty- six or E26 transformation specific (Watson *et al.*, 1985); FREAC2: forkhead box protein F2 (Pierrou *et al.*, 1994); FREAC 7: forkhead box protein F7 (Pierrou *et al.*, 1994); HNF3β: hepatocyte nuclear factor 3 β (Mincheva *et al.*, 1997); myb: myeloblast viral transforming gene (Beug *et al.*, 1979); NF1: nuclear factor 1 (Santoro *et al.*, 1988); NFAT: nuclear factor of activated T-cells (Shaw *et al.*, 1988); NFκB: nuclear factor κB (Ghosh *et al.*, 1990); NFY: nuclear transcription factor Y (Tronche *et al.*, 1991); Sox5: transcription factor Sox5 (Wunderle *et al.*, 1996); SP1: transcription factor SP1 (Dynan and Tjian, 1983); SRY: sex determining region Y (Sinclair *et al.*, 1990); TCF/Lef: transcription factor/lymphoid enhancer factor (Oosterwegel, *et al.*, 1991; Travis *et al.*, 1991).


-356
FREAC2
HNF3β
-256
FREAC7
HNF3β
TCF
-233
ETS
-217
AP1
NF1
ETS
-183
NFY
Sox5
NF1
NFκB
-156
NFY
SP1
NF1
NFκB
AP1
-100
TCF
SRY
myb
-50
myb
NFAT
-25
NFκB
NFY
AP2
+1
myb
+21

and *MMP-7* (Crawford *et al.*, 2001) and *COX-2* (Howe *et al.*, 2001; Subbaramaiah *et al.*, 2002) mRNA in breast and intestinal tumors. Results of *in vitro* studies of the effects of PEA3 on their promoters, suggest that *MMP-7* and *COX-2* are PEA3 target genes (Crawford *et al.*, 2001; Howe *et al.*, 2001). These are but a few examples of the possible PEA3 target genes that are currently being identified. Another potential PEA3 target gene is the *CYCLIN D1* gene.

5. CYCLIN D1 and the Cell Cycle

CYCLIN D1 is one of the most extensively studied cell cycle regulators, and was cloned both as an oncogene-encoded protein (Hinds *et al.*, 1994) and through its ability to rescue G1 cyclin-deficient yeast mutants (Lew *et al.* 1991). Normal, quiescent cells can be induced to undergo mitosis by addition of growth factors to the culture media. The signaling cascade that is induced by these growth factors turns on a first set of genes, called immediate-early genes, such as *c-fos*. These in turn work to turn on so-called delayed-response genes. One such gene is *cyclin D1*, which was demonstrated to be expressed approximately three hours after addition of growth factors and was sensitive to cycloheximide protein synthesis inhibition (Matsushime *et al.*, 1991).

In normal cells, CYCLIN D1 is an essential G1 cyclin (see Figure 5). It is a component of the CDK2 and CDK4 kinase/cyclin complexes (reviewed in Ekholm and Reed, 2000). These kinases have been shown to phosphorylate the Rb protein, thereby relieving its repression activity and allowing the cell cycle to progress through G1 to S (reviewed in Sherr and Roberts, 1999). CYCLIN D1 levels fluctuate very little

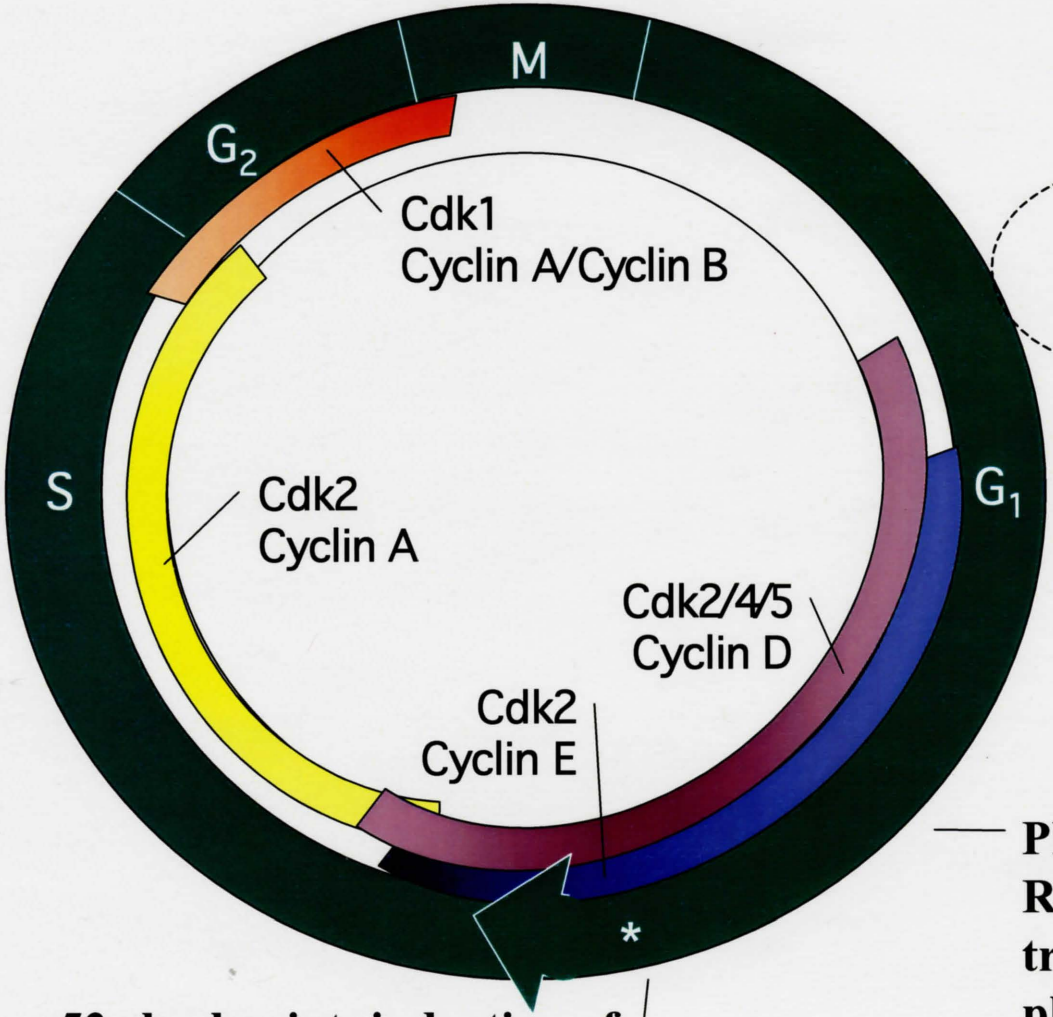
throughout the cell cycle, which would imply that any increase or decrease in protein levels is likely to have a significant effect on the cell's decision to proceed through to mitosis. These facts alone make D-type cyclins very potent proto-oncogenes, since any upregulation in their expression patterns could quickly lead to cancer. It is also interesting to note that interference with *CYCLIN D1* expression (by antisense RNAs) or function (by antibodies), has been shown to block DNA synthesis in growth factor stimulated cells (Baldin *et al.*, 1993).

Several examples of CYCLIN D1 involvement in cancer have been described. The *CYCLIN D1* gene is amplified in 20% of human breast cancers (Dickson *et al.*, 1995), and the CYCLIN D1 protein is overexpressed in over 50% of human mammary carcinomas (Bartkova *et al.*, 1994a). Transgenic mice that overexpress *cyclin D1* from the MMTV (**m**ouse **m**ammary **t**umor **v**irus) promoter develop, and die from, breast cancer (Wang *et al.*, 1994). It was also shown recently that *cyclin D1*$^{-/-}$ mice are resistant to breast cancers induced by the r*as* and *neu* oncogenes (Yu *et al.*, 2001). Studies also show that *cyclin D1* is essential for transformation of Rat-1 cells by activated *Neu* (Lee *et al.*, 2000). Hence, Cyclin D1 is not only an important cell cycle regulator in normal cell growth, it is also one of the key elements leading to tumor formation in mammary cells.

Given the strong evidence that both *PEA3* and *CYCLIN D1* are involved in breast tumors, it seems apparent that a better understanding of the mechanisms involved in the regulation of both their promoters will shed light on the process of mammary tumorigenesis. These findings in turn could lead to the development of new treatments or

Figure 5. **Overview of the Major Players in Cell Cycle Regulation**

A cell deprived of growth factors exits the cell cycle and rests in the G0 state. Upon growth factor stimulation, the cell re-enters the cell cycle in G1, where cyclins (mainly D and E) are produced. These cyclins than associate with their partner cyclin-dependent kinases (Cdk) and stimulate their activity. Cdks then phosphorylate target proteins, including Rb. Phosphorylation of Rb allows it to release E2F, which becomes free to enter the nucleus and stimulate transcription of genes necessary for S phase. At the G1/S phase transition is a checkpoint involving p53. If DNA damage occurs, p53 is phosphorylated and activates transcription of the p21 gene, which is a cdk inhibitor. Inhibition of cdk function stops the cell at the G1/S boundary, allowing for DNA repair. Once the cell progresses through to S phase, it is committed to go through the entire cell cycle, including G2 and M (mitosis).


M
G2
Cdk1
Cyclin A/Cyclin B
G0
Growth
factor
S
Cdk2
Cyclin A
G1
Cdk2/4/5
Cyclin D
Cdk2
Cyclin E
Phosphorylation of
Rb; E2F-induced
transcription of S
phase genes
*
p53 checkpoint: induction of
p21 repressor in case of DNA
dammage

means of earlier detection of breast cancer in humans, thereby stricking a strong blow to one of the leading causes of death among women.

6. Characterization of the Factors Influencing the Activity of the PEA3 Promoter

The main objective of this project was to determine the influence of the Ras/MAPK signaling pathway on the *pea3* promoter and to identify the sequence elements responsible for its effect on *pea3* expression. To this end, a series of luciferase reporter plasmids containing deletion and point mutations of the *pea3* promoter were constructed. These reporter plasmids were used in transfection/induction experiments in a mouse NIH3T3 fibroblast cell line, which is engineered to express an activatable form of Raf-1, ΔRaf-1:AR (McCarthy *et al.*, 1997). The relative *in vivo* activities of the different promoter regions were then evaluated and the levels obtained with or without Raf-1 activation were compared. The *pea3* promoter contains a number of putative ETS binding sites (EBS), as well as binding sites for numerous other transcription factors (Barrett, 1997, see also Figure 4). In the region of –240 relative to the major transcription start site, a putative **R**as-**R**esponsive **E**lement (RRE) was identified. An RRE comprises an ETS protein binding site and an AP-1 binding site (Gutman, and Wasylyk, 1990). This type of sequence element has been shown to act as a Ras/MAPK pathway-responsive element in a number of promoters including the collagenase gene (Gutman and Wasylyk, 1990) and the polyomavirus enhancer (Wasylyk *et al.*, 1988). The presence of an RRE in the *pea3* promoter further suggests that its promoter may be a target of this pathway.

Also, this provided the grounds for investigating the role of these sequences in overall promoter activity and responsiveness to Raf-1 activation.

The same reporter plasmids were also used in co-transfection experiments to determine the effect of transcriptional activators on the *pea3* promoter. The effect of c-Jun was assessed since it is one of the major effectors of the Ras/MAPK pathway (Binetruy et al., 1991).

By gaining a better understanding of the mechanisms of regulation of the pea3 promoter, it may be possible to shed light on yet another player frequently involved in breast tumor formation. Moreover, this information may lead to the design of treatment or detection tools against mammary tumorigenesis.

7. Characterization of the Factors Influencing the Activity of the CYCLIN D1 promoter

The secondary objective of this project was to determine the influence of PEA3 on the *CYCLIN D1* promoter. There are at least four putative Ets binding sites in the *CYCLIN D1* promoter (Figure 6). The influence of the MAPK pathway and of the Wnt-signaling pathway on the *CYCLIN D1* promoter was also established by determining the effects of their downstream effectors, and their possible cooperation on the *CYCLIN D1* promoter. Reporter analyses were used to test these hypotheses in co-transfection assays in Cos-1 cells.

CYCLIN D1 gene expression has been shown to be regulated by a number of pathways, including the MAPK pathway (Lavoie *et al.*, 1996) and the Wnt-signaling

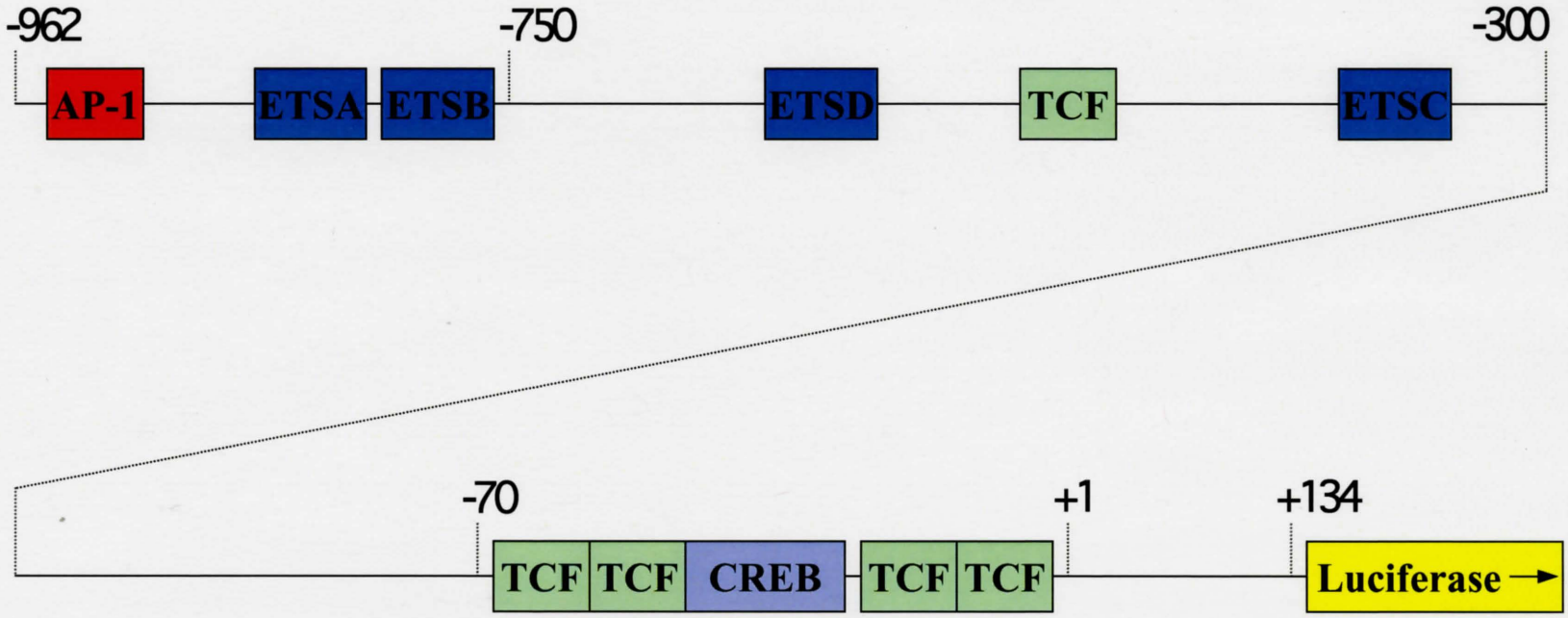
pathway (Tetsu and McCormick, 1999). Studies even show cooperation between these two pathways (Rimerman *et al.*, 2000). Similarly, analysis of the *matrilysin* promoter revealed a cooperative effect of β-Catenin, LEF-1, c-Jun and PEA3 family members to provide a large activation in HEK293 cells (Crawford *et al.*, 2001). Since both the *CD1* and the *matrilysin* genes are involved in mammary tumors, it was hypothesized that both their promoters could be regulated in a similar fashion. Previous studies of the *CD1* promoter have identified putative binding sequences for the Lef/TCF, AP-1 and ETS transcription factors (Tetsu and McCormick, 1999) (Figure 6).

A better understanding of the regulation of CYCLIN D1 expression is critical to the unearthing of how this important cell cycle regulator becomes overexpressed and promotes tumor formation. Similarly, discovery of a direct link between PEA3 subfamily members and CYCLIN D1 expression could reveal one of the possible roles of PEA3, ERM and ER81 in tumor formation.

Figure 6. **Structure of the *CYCLIN D1* Promoter**

The structure of the *CYCLIN D1* promoter and the major transcription factors that are involved in its regulation are illustrated here. Some of the TCF sites have been shown to be essential for β-catenin induced expression of this promoter (Tetsu and McCormick., 1999).

AP-1: activator protein 1 (Piette *et al.*, 1988); Ets: E twenty-six or E26 transformation specific (Watson *et al.*, 1985); TCF/Lef-1: transcription factor/lymphoid enhancer factor (Oosterwegel *et al.*, 1991); CREB: cyclic-AMP response element binding protein (Montminy and Bilezikjian, 1987).


-962
-750
-300
AP-1
ETSA
ETSB
ETSD
TCF
ETSC
-70
+1
+134
TCF
TCF
CREB
TCF
TCF
Luciferase

OBJECTIVES

1. To amplify 14 defined regions of the sequences upstream of the major transcription start site in the mouse *pea3* promoter and clone these promoter sequences into the pGL3 basic luciferase vector.
2. To assess the relative activity of these promoter luciferase constructs in the presence or absence of Raf-1 activation in McMA cells.
3. To generate 6 site-directed mutants of the *pea3* promoter using PCR and clone these promoter sequences into the pGL3 basic luciferase vector.
4. To identify the DNA sequences responsible for the activation of the mouse *pea3* promoter by Raf-1.
5. To confirm the importance of these sequences using co-transfection experiments in McMA cells.
6. To determine the influence of PEA3 on the *CYCLIN D1* promoter using co-transfection experiments in Cos-1 cells and confirm this effect using DN-PEA3.
7. To determine the effect of PEA3, β-catenin, Lef-1 and c-Jun on the *CYCLIN D1* promoter using co-transfection experiments in Cos-1 cells.
8. To generate a site-directed mutant at the EBS D in the *CYCLIN D1* promoter and clone this promoter sequence into the pGL3 basic luciferase vector.
9. To assess the relative *in vivo* promoter activity of four EBS mutants of the *CYCLIN D1* promoter, and their responsiveness to PEA3, β-catenin, Lef-1 and c-Jun in co-transfection experiments in Cos-1 cells.

MATERIALS

All restriction endonucleases were purchased from Invitrogen Life Technologies, Burlington, Ontario, Canada, (formerly Gibco BRL Life Technologies), or from Roche Diagnostics, Laval, Quebec, Canada, and were used according to manufacturer's specifications. Agarose, DNA polymerase I, deoxynucleotides (dATP, dGTP, dCTP, dTTP), *Taq* DNA polymerase, 1 kb DNA ladder, 100 bp DNA ladder and lipofectAMINE™ transfection reagent were also obtained from Invitrogen Life Technologies. SuperFect® transfection reagent was obtained from QIAGEN, Mississauga, Ontario, Canada. T4 DNA ligase was purchased from New England BioLabs, Ltd., Mississauga, Ontario, Canada. R1881 androgen analog was obtained from Sigma-Aldrich Canada Ltd, Oakville, Ontario, Canada. MAX Efficiency® DH5α™ Competent Cells were also obtained from Invitrogen Life Technologies. Ingredients used in the production of bacterial media (trypticase-peptone, yeast extract and granulated agar) were purchased from Becton Dickinson, Cockeysville, MD, USA. The cell culture media (Dubelcco's Modified Eagle Medium [DMEM] and DMEM without phenol red) were purchased from Gibco-Invitrogen. Ingredients to supplement these media (fetal bovine serum, penicillin-streptomycin, Geneticin, Fungizone® and non-essential amino-acids) were also purchased from Gibco-Invitrogen. Blastocidin was obtained from Invitrogen Life Technologies.

The pGL3 luciferase vector was purchased from Promega Corporation, Madison, Wisconsin, USA. The pCan-myc vector that was used as expression vector was obtained from Invitrogen. The 5X reporter lysis buffer and luciferase reagent were purchased from Promega Corporation, Madison, Wisconsin, USA.

All oligonucleotide primers and probes used for polymerase chain reaction and electromobility shift assays, respectively, were synthesized by Dinsdale Gooden (The Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University, Hamilton, Ontario, Canada).

METHODS

1. PCR Amplification of the Putative Promoter Region of Mouse *pea3* and *cyclin D1*

A directional cloning strategy was used to clone the deletion mutants of the promoter region of the mouse *pea3* and human *CYCLIN D1* genes. Primers corresponding to the desired end-points of the promoter were designed to include restriction endonuclease recognition sequences for cloning of the polymerase chain reaction (PCR) products into the pGL3 vector. All 5' and 3' primers contained the *Bgl*II and *Hind*III recognition sequences, respectively. These sequences are located at positions 36 and 53 respectively in the pGL3 vector multiple cloning region. This primer design strategy allowed molecular cloning of the PCR product in the desired orientation. Table 1 and Table 2 list all the primers that were used for the amplification of putative promoter regions of the mouse *pea3* and *CYCLIN D1* genes, respectively.

PCR reactions contained 2.5U of *Taq* DNA polymerase, 100ng of template DNA, 1mM dNTPs, 50mM KCl, 20mM Tris-HCl pH8.4 and 1.5 to 2.0 mM $MgCl_2$ and 100 pmol of each primer. Each reaction had a volume of 100µl and amplification was performed using 7 cycles of the following protocol: denaturation of DNA strands at 94°C for 30 seconds, annealing of primers at 48°C for 30 seconds and DNA strand synthesis at 72°C for 30 seconds. The annealing temperature was calculated for the primers used, omitting the restriction endonuclease recognition sequences. The amplification was then

Table 1. **Oligonucleotide Primers for Amplification of the Mouse *pea3* Promoter**

Each oligonucleotide primer sequence is given in the 5' to 3' orientation. Upstream primers possess the *Bgl*II endonuclease restriction sequence. Downstream primers possess the *Hind*III restriction sequence. Primers used for site-directed mutagenesis are also included here. The name of each *pea3* promoter/luciferase construct represents the region of the sequence that has been amplified and cloned into the pGL3 basic vector. Three of the constructs were previously generated by Jane M. Barrett in our laboratory (marked by *). –156+21luc and –3+21luc were generated by restriction enzyme digestion and therefore oligonucleotide primers were not used.

Construct Name	5' Primer	Identifier	3' Primer	Identifier	Template
-1029+21 luc	GGAAGATCTCCAGCACCAGTCTGACACAGC	AB8490	CCCAAGCTTCCGGGCGCAGCAGACAGTTGT	AB28124	-1341+21 luc
-926+21 luc	GGAAGATCTATCACCAAGTCACTTGGGTTTC	AB8489	CCCAAGCTTCCGGGCGCAGCAGACAGTTGT	AB28124	-1341+21 luc
-756+21 luc	GGAAGATCTTGAGCCAGTTAAATTTACTGAG	AB8488	CCCAAGCTTCCGGGCGCAGCAGACAGTTGT	AB28124	-1341+21 luc
-556+21 luc	GGAAGATCTGCTCGCAGCACCACGTTATGG	AB8486	CCCAAGCTTCCGGGCGCAGCAGACAGTTGT	AB28124	-1341+21 luc
-356+21 luc	GGAAGATCTCCCAAAACCCAGGTTGGAACCCGTGG	AB28123	CCCAAGCTTCCGGGCGCAGCAGACAGTTGT	AB28124	*
-256+21 luc	GGAAGATCTTCTTATTTTTTTATGAATGGAAGTCC	AB29202	CCCAAGCTTCCGGGCGCAGCAGACAGTTGT	AB28124	-356+21 luc
-233+21 luc	GGAAGATCTAAAAAGTGAATGAAGCCAGGAGC	AB26196	CCCAAGCTTCCGGGCGCAGCAGACAGTTGT	AB28124	-356+21 luc
-217+21 luc	GGAAGATCTCAGGAGCCAGCCCCTACTTTC	AB26197	CCCAAGCTTCCGGGCGCAGCAGACAGTTGT	AB28124	-356+21 luc
-183+21 luc	GGAAGATCTTGGCTGGGAAACTCCTCCCTC	AB26619	CCCAAGCTTCCGGGCGCAGCAGACAGTTGT	AB28124	-356+21 luc
-156+21 luc	N/A	N/A	N/A	N/A	*
-100+21 luc	GGAAGATCTAAGTCAATGAAACAAAGGGAA	AB8495	CCCAAGCTTCCGGGCGCAGCAGACAGTTGT	AB28124	-356+21 luc
-50+21 luc	GGAAGATCTAACGGAGGCCAAGGCAAAGGA	AB8494	CCCAAGCTTCCGGGCGCAGCAGACAGTTGT	AB28124	-356+21 luc
-25+21 luc	GGAAGATCTCACCAATCAGCTGCTCCCCCG	AB8493	CCCAAGCTTCCGGGCGCAGCAGACAGTTGT	AB28124	-356+21 luc
-3+21 luc	N/A	N/A	N/A	N/A	*
-356+21 Ets mt	TTTTGGACTTTGAATTCATAAAAAAATAAACTCCT	AB26718	TTTTTTGAATTCAAGTCCAAAAAGTGAATGAAG	AB26717	-356+21 luc
-356+21 Ap1 mt	CTGGCTTCGATATCTTTTTGGACTTCCATTCA	AB26720	CAAAAAGATATCGAAGCCAGGAGCCAGCC	AB26719	-356+21 luc
-356+21 Db mt	TAGAATTCAAGTCCAAAAAGATATCGAAGCCAGG	AB28193	TCCTGGCTTCGATATCTTTTTGGACTTGAATTCAT	AB28194	-356+21 luc
-256+21 Ets mt	GGAAGATCTTCTTATTTTTTTATGAATTCAAGTCCA	AB29980	CCCAAGCTTCCGGGCGCAGCAGACAGTTGT	AB28124	-356+21 luc
-256+21 Ap1 mt	GGAAGATCTTCTTATTTTTTTATGAATGGAAGTCC	AB29202	CCCAAGCTTCCGGGCGCAGCAGACAGTTGT	AB28124	-356+21 Ap1 mt
-256+21 Db mt	GGAAGATCTTCTTATTTTTTTATGAATTCAAGTCCA	AB29980	CCCAAGCTTCCGGGCGCAGCAGACAGTTGT	AB28124	-356+21 Db mt

Table 2. **Oligonucleotide Primers for Amplification of the *CYCLIN D1* Promoter**

Each oligonucleotide primer sequence is given in the 5' to 3' orientation. The name of each *CD1* promoter/luciferase construct represents the region of the sequence that has been amplified and cloned into the pGL3 basic vector. Upstream primers possess the *Bgl*II endonuclease restriction sequence. Downstream primers possess the *Hind*III restriction sequence. Primers used for site-directed mutagenesis are also included here.

Construct Name	Primer Direction	Primer Sequence (5' to 3')	Identifier
-962CD1 luc	Upstream	GCGCCTCAGGGATGGCTTTTGG	AB19578
	Downstream	GCAAGCTTTGGGGAGGGCTGTGGGTC	AB19579
ETS D mt	Upstream	CAAGTTTCTAGACGGCGCACAGGGGCGTCG	AB25035
	Downstream	CCTGTGCGCCGTCTAGAAACTTGCACAGGGGTT	AB25036

pursued for a further 25 cycles of the following protocol: denaturation of DNA strands at 94°C for 30 seconds, annealing of the primers at 55°C for 30 seconds and DNA strand synthesis at 72°C for 30 seconds. The annealing temperature was calculated for the primers used, including the restriction endonuclease recognition sequences, to promote amplification of the newly synthesized products containing these sequences. For each set of reactions, a negative control reaction containing no DNA template was performed.

For site-directed mutagenesis, two oligonucleotides (overlap primers) containing the desired mutation and complementary to each other over the site of the mutation were designed for PCR purposes. Each segment was amplified separately, with one end primer and the overlap primer from the complementary strand, using the same conditions as above. Each of the two segments were gel purified (see below) and used in a subsequent PCR reaction, this time using both end primers. The same PCR conditions were used once again. Table 1 and Table 2 include the overlap primers used to generate mutations in the *pea3* and *CYCLIN D1* promoter regions, respectively.

2. Purification and Modification of PCR Amplified DNA

To ensure efficient cloning, PCR generated products were purified by electrophoresis at 100-120V for up to two hours in a 1-1.5% agarose gel. The size of the obtained fragments was confirmed by comparison to an appropriate DNA ladder marker. The DNA was then extracted from the gel using QIAquick Spin kit from QIAGEN, following the manufacturers instructions. The DNA was eluted from the columns in 34µl of sterile water and subjected to digestion with *Bgl*II and *Hind*III restriction

endonucleases in the following conditions: 50mM Tris-HCl, pH 8.0, 10mM $MgCl_2$, 50mM NaCl, for 20hrs at 37°C.

3. Preparation of pGL3 vector DNA

The two cloning sites that were chosen for the cloning of the PCR generated fragments of the putative promoter were such as to avoid the polylinker upstream of the luciferase gene. 10 µg of the pGL3-basic reporter plasmid were linearized by digestion with 10U of *Hind*III and 10U of *Bgl*II endonucleases in the following conditions: 50mM Tris-HCl, pH 8.0, 10mM $MgCl_2$, 50mM NaCl for two hours at 37°C. The linearized vector was purified by electrophoresis at 80V for four hours in a 0.7% agarose gel. The appropriate fragment was excised from the gel and extracted from the agarose using the QIAquick Spin kit from QIAGEN following manufacturer's instructions.

4. Ligation Reaction

Putative promoter fragments were ligated into the pGL3 reporter vector using the following conditions: approximately 350ng linearized pGL3 vector and a four-fold molar excess of the PCR fragments were incubated with 200U of T4 ligase at room temperature for 16 hours, in 50mM Tris-HCl, pH 7.5, 10mM $MgCl_2$, 10mM dithiothreitol, 1mM ATP and 25µg/ml bovine serum albumin.

5. Transformation

The ligation products were transformed into MAX Efficiency® DH5α™ Competent Cells, following the manufacturers instructions with the following

modifications: 50 to 60µl of competent cells suspension was used and combined with 7µl of the ligation mixture. The entire transformation mixture was centrifuged in a microfuge following the one-hour incubation at 37°C and the bacterial pellet resuspended in 100µl of media. The entire volume was plated onto an LB Agar plate containing 100µg/ml of ampicillin and incubated for 16 hours at 37°C.

6. Restriction Endonuclease Analysis of Positive Clones

Colonies that grew on ampicillin plates were transferred with a sterile pipette tip to separate 15ml polypropylene tubes containing 3.5ml of LB medium containing 100µg/ml of ampicillin. After a 16-hour incubation at 37°C with gentle shaking, small-scale plasmid isolation was performed to isolate plasmid DNA (Sambrook *et al.* 1989). The purified DNAs were digested with *Hind*III and *Bgl*II endonucleases as described above. The resulting fragments were separated by electrophoresis on a 1% agarose gel at 100V and compared to a DNA ladder marker to confirm their sizes.

7. DNA sequencing with GL2 and RV3 primers

The DNA sequence and orientation of the inserts in the chimera were determined by automated sequence analysis using primers upstream (RV3, $^{5'}$CTAGCAAAATAGGC TGTCCC$^{3'}$) and downstream (GL2, $^{5'}$CTTTATGTTTTTGGCGTCTTCC$^{3'}$) of the cloning site. Sequencing reactions were performed in the Central Facility at the Institute for Molecular Biology and Biotechnology and analyzed with an ABI PRISM 3100 automated DNA sequencing apparatus.

8. Preparation of Luciferase Constructs and Expression Constructs

For all transient transfections, the DNA was isolated from 50 ml or 100 ml overnight cultures of bacteria (100 μg/ml ampicillin in LB media) and isolated following the protocol for QIAGEN Plasmid Midi or Maxi kits, respectively.

9. Transfection of McMA Cells

A mouse NIH3T3 fibroblast cell line stably transfected with a GFP-ΔRaf-1:AR construct (McMA), was used in transfection/induction experiments. The GFP-ΔRaf-1:AR construct comprises of a constitutively active form of Raf-1 kinase, ΔRaf-1 (Stanton *et al.*, 1989) fused to the hormone binding domain of human androgen receptor (AR). GFP fusion was added for easy detection of transfected cells. The fusion protein has been shown to have inducible Raf-1 activity by addition of testosterone to the culture media (Weinstein-Oppenheimer *et al.*, 2001). McMA cells were grown in Dubelcco's modified Eagle's medium (DMEM) without phenol red, supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin (100 U/100 μg/ml), and fungizone® (amphotericin, 0.5 μg/ml). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. Cells were periodically and sequentially selected in blastocidin (12 μg/ml) and G418 (400 μg/ml) to ensure the retention of the ΔRaf-1:AR expressing plasmid.

Cells were plated at a density of 7 x 10^5 cells/plate in 60 mm Petri dishes 48 hours prior to transfection. For each transfection, 0.5 μg of reporter DNA and the indicated amounts of expression plasmids (where applicable) were used. The total amount of DNA

was kept constant through the addition of sheared salmon sperm DNA to 5 µg. The DNA mixture was mixed with 8 µl of SuperFect® reagent in a final volume of 150 µl of serum-free DMEM without phenol red supplemented with 1 mM non-essential amino-acids solution and incubated for 15 minutes at room temperature to allow DNA-SuperFect® complex formation. The volume was then increased to 2 ml with DMEM without phenol red and the mixture was transferred to the cells. The absorption of the DNA-SuperFect® complex was allowed to proceed for two hours before the total volume was brought to 5 ml by the addition of 3 ml DMEM without phenol red. The cells were incubated for 24 hours before the induction step was performed.

10. Induction of Raf-1:AR in McMA cells

24 hours after transfection of the cells, each 60 mm Petri dish was trypsinized with 0.6 ml of 0.625 mg/ml trypsin solution in versene, and the cells were resuspended in a final volume of 4ml in DMEM without phenol red. 1 ml of suspension was transferred to each of two 15 mm plates. 0.57 µl of a 0.02 mg/ml solution of R1881 (20 nM final concentration) was added to the remaining 2 ml, and 1 ml transferred to each of two 15 mm plates. The cells were allowed to adhere and recover for 24 hours before cell extracts were prepared as described below.

11. Transfection of McMA cells (without later induction of the Raf-1:AR protein)

Cells were plated at a density of 6.5 x 10^4 cells/plate in 21 mm Petri dishes 24 hours prior to transfection. The DNA mixture, comprising the luciferase reporter construct of the *pea3* or *CD1* promoters and a combination of expression vectors, for a

total of 3 μg (for three plates), as outlined in the figure legends, was mixed with 6 μl of Superfect® reagent in a final volume of 255 μl of serum-free DMEM, supplemented with 1 mM of non-essential amino acids, and incubated 15 minutes at room temperature to allow complex formation. The volume was then increased to 600μl with DMEM without phenol red and 200 μl of the mixture was transferred to each of three wells, already containing 300 μl of DMEM without phenol red. The absorption of SuperFect®-DNA complex was allowed to proceed for 2 hours before the total volume was brought to 1ml by the addition of 0.5ml DMEM without phenol red. The cells were incubated for 48 hours and then cell extracts were prepared.

12. Transfection of Cos-1 Cells

The Cos-1 cell line was grown in Dubelcco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin (100U/100μg/ml), and fungizone® (amphotericin, 0.5μg/ml). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

Cells were plated at a density of 6 x 10^4 cells/plate in 21 mm Petri dishes or 1.2 x 10^5 cells/well in 35 mm Petri dishes 24 hours prior to transfection. The DNA mixture, comprising the luciferase reporter constructs bearing the *pea3* or *CD1* promoters and a combination of expression vectors, for a total of 1μg (2μg in the case of 35 mm dishes), as outlined in the figure legends. The total amount of DNA was kept constant using empty pCan expression plasmid. This DNA mixture was combined with 4μl of lipofectAMINE™ (6μl in the case of 35 mm dishes) in a final volume of 200μl of serum-

free DMEM, supplemented with 1mM of non-essential amino-acids. The lipofectAMINE^{TM}-DNA mixture was incubated 30 minutes at room temperature to allow lipid-DNA complexes to form. The volume was then increased to 500µl with serum-free DMEM and the mixture was transferred to the cells. For 35 mm dishes, the volume was increased to 800µl. The absorption of DNA-lipid complexes by the cells was allowed to proceed for four hours before the media on the cells was aspirated and replaced with 1ml of DMEM. The final volume for 35 mm dishes was 2ml. The cells were incubated for 48 hours and then cell extracts were prepared as described below.

13. Preparation of Cell Extracts for Luciferase Assays

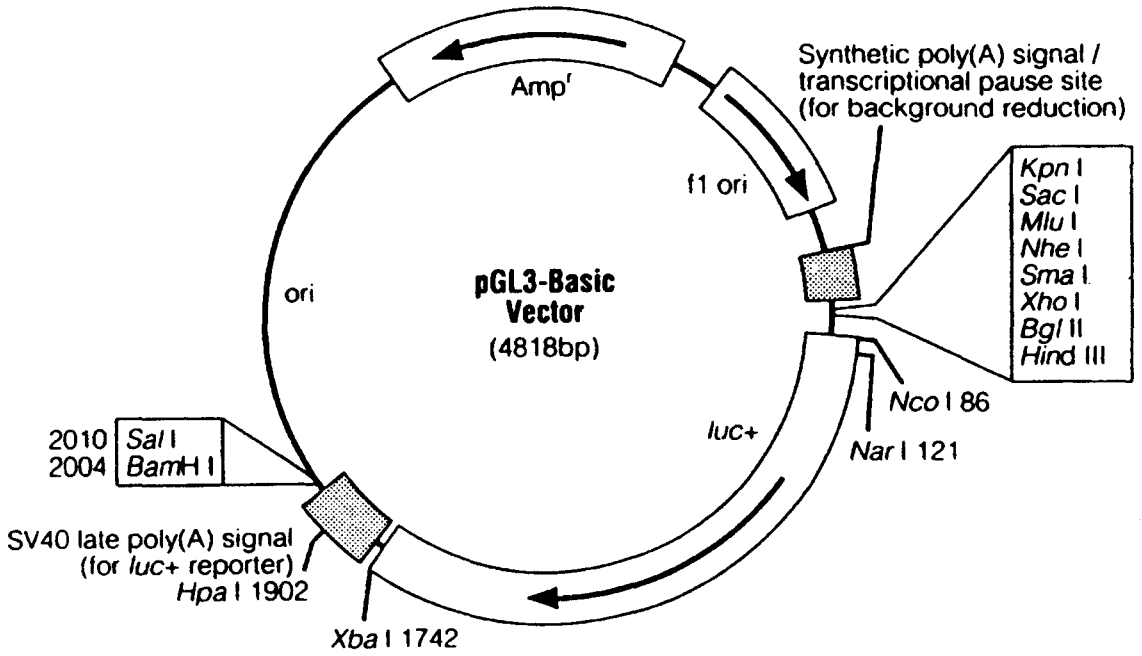
To isolate cell extracts for all cell lines, cells growing on 21 mm or 15 mm Petri dishes were washed twice with cold 1X phosphate buffered saline (PBS) followed by the addition of 100µl of 1X Promega Reporter Lysis Buffer. In 35 mm dishes, 200µl of 1X Promega Reporter Lysis Buffer was used. The cells were incubated for 15 minutes at room temperature with gentle shaking and scraped off the plate with a plastic cell scraper or a rubber policeman. The mixture was transferred to a microfuge tube and centrifuged 15 seconds at 13,000 rpm to pellet cellular debris. The clear liquid phase was transferred to a fresh microfuge tube and either assayed immediately or stored at -20°C for later use.

14. Luciferase Assay

The pGL3 basic vector from Promega (Figure 7) provides a useful tool for studying promoter activity. The North American firefly (Photinus pyralis) luciferase gene

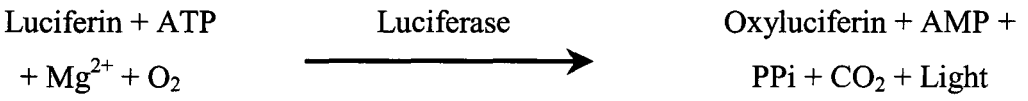
Figure 7. **The pGL3 Reporter Vector**

The cloning site is illustrated in the box at the right. The luciferase is illustrated by luc+ followed by the SV40 late poly(A) signal. The vector also possesses a synthetic poly(A) signal upstream of the luciferase gene, along with a transcriptional pause site, to reduce background luciferase expression. ori, origin of replication; Amp^r, β-lactamase gene for ampicillin resistance; f1 ori, f1 origin.


Amp^r
f1 ori
ori
pGL3-Basic
Vector
(4818bp)
luc+
Synthetic poly(A) signal /
transcriptional pause site
(for background reduction)
Kpn I
Sac I
Mlu I
Nhe I
Sma I
Xho I
Bgl II
Hind III
Nco I 86
Nar I 121
2010 Sal I
2004 BamH I
SV40 late poly(A) signal
(for luc+ reporter)
Hpa I 1902
Xba I 1742

coding sequence is preceded by a multiple cloning site, allowing for easy cloning of a promoter fragment. Several modifications have been made to the luciferase gene in order to make it a more efficient reporter gene in mammalian cells (Sherf and Wood, 1994). The peroxisomal translocation signal has been removed, preventing localization to the peroxisomes, thereby allowing the enzyme to be completely cytoplasmic and thus eliminating any compartmentalization issues. The other major modification that was made involves codon usage. Since efficiently expressed genes usually utilize the most abundant tRNA isoforms, the luciferase coding sequenc was altered to convert particularly infrequent codons to highly frequent ones, thereby increasing translation efficiency. Two consensus glycosylation sites were also eliminated to ensure that the enzyme would not be glycosylated in the endoplasmic reticulum or the Golgi apparatus, since the luciferase protein produced by the firefly does not contain any post-translational modifications. The pGL3 basic vector also contains a poly adenylation signal upstream from the cloning site to reduce background "read-through" transcription.

Firefly luciferase is the most widely used bioluminescent reporter because its enzyme activity is proportional to the protein levels and the luminescence assay is rapid, sensitive and convenient. The assay provides linearity over a 100 million-fold enzyme concentration range and is sensitive to as little as 10^{-20} moles of enzyme. The reaction by which this 61kDa monomeric enzyme produces light is as follows (Wood, 1998):



The luciferase assay reaction occurs in the dark chamber of a luminometer, which counts the photons emitted by the reaction over a pre-determined length of time and displays the value on the screen. To perform the assay, a 5-20µl aliquot of the cell extract was placed in the luminometer tube and inserted into the reading chamber. The automatic injector of the Berthold lumat 9501 luminometer dispensed 100µl of the buffered luciferase reagent containing luciferin, ATP, coenzyme A and Mg^{2+}. The light signal was measured for ten seconds. For some of the assays, the Luminoskan Ascent (Labsystems) was used instead. This instrument allows for an even faster assay as it automatically injects luciferase reagent and reads the light signal for each of the wells of a 96-well plate.

Luciferase activity for each sample was calculated as follows:

Raw luciferase value – Background value = Relative Light Units (RLUs)

$$\frac{\text{(RLUs)}}{\mu\text{g protein}/\mu\text{l}} = \text{Normalized luciferase value (RLUs/}\mu\text{g protein)}$$

15. Protein Assay

To determine the protein concentration in each sample, a Bradford assay was performed against a BSA standard curve. 5µl of BSA solutions with concentrations of

100µg/ml, 200µg/ml, 400µg/ml, 500µg/ml, 600µg/ml, 800µg/ml and 1mg/ml were aliquoted in duplicate in 96-well Petri dishes. 2µl (for McMA cell lysates) or 5µl (for Cos-1 cell lysates) of cell lysates were also aliquoted in duplicate and mixed with 200µl of a 1:5 dilution of the Bradford reagent from Bio-Rad. The binding of the Coomassie blue dye to the proteins was allowed to proceed for 5-10 minutes before the samples were read at 570nm in a EL 340 BioKinetics Reader from Bio-Teck Instruments (Mandel Scientific Co. Ltd.) and analyzed using the KC3 software, version 1.5 (also from Bio-Teck Instruments), which plots the standard absorbances, calculates the trendline and uses it to determine unknown concentrations and multiplies by the dilution factor. Parameters were set to obtain values of µg protein/µl for each sample.

All transfection and transfection/induction experiments were repeated at least once, using freshly plated cells and separatly prepared DNA mixtures, and assayed independently.

RESULTS

CHAPTER 1: THE *pea3* PROMOTER

1. *pea3* Transcripts are Elevated Following Raf-1 Induction

The mechanisms of regulation of *pea3* expression *in vivo* had not been previously analyzed in detail. It was unclear which of the different steps involved in the production of PEA3 protein was the main determinant of its expression levels, and what other cellular elements were involved in regulating this process. Since the levels of a protein in the cell can be regulated at the level of protein stability or at multiple levels of gene expression, it was necessary to determine the means by which *pea3* levels are regulated. A large number of genes are regulated, at least in part, at the level of transcription initiation. The observation that *pea3* mRNA is overexpressed in 93% of *HER2/Neu* positive breast tumors (Benz *et al.*, 1997) increased the probability that *pea3* is also transcriptionally regulated. This also led to the hypothesis that the Ras/MAPK pathway, which is induced by HER2/Neu, may regulate the *pea3* promoter.

One way to test this hypothesis was to determine whether *pea3* mRNA levels were increased following activation of the MAPK pathway. To do this, a NIH 3T3 cell line (McMA) engineered to express a ΔRaf-1:AR construct (McCarthy *et al.*, 1997, see methods) was used. This cell line allows the MAPK pathway to be turned on, through Raf-1 kinase activation, by the addition of an androgen analog (R1881) to the culture

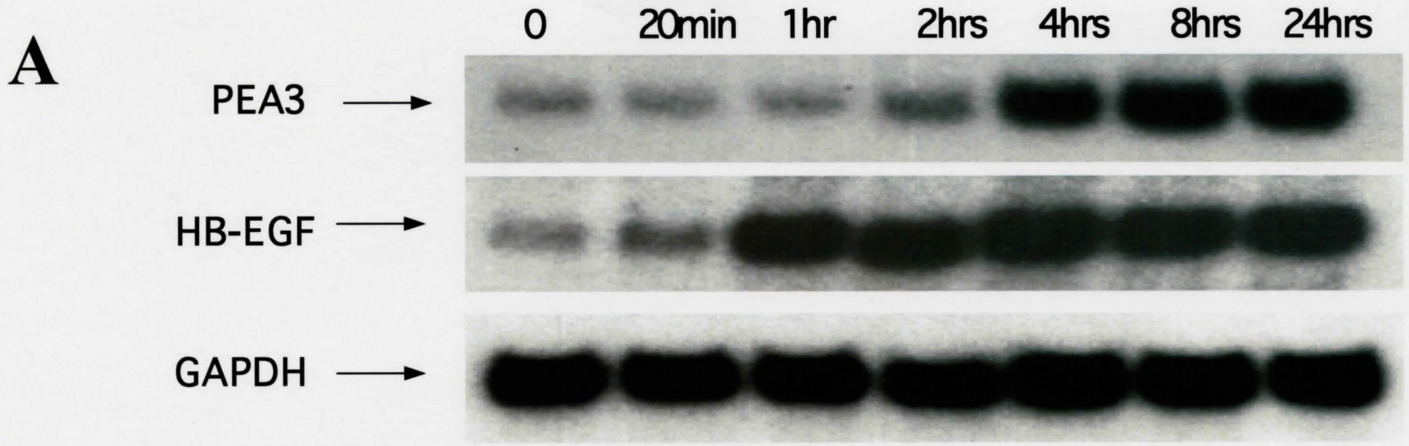
media. Northern analysis was performed using RNA extracted from these cells prior to and post induction with R1881 or serum. As can be observed from Figure 8a, the abundance of *pea3* mRNA was increased upon Raf-1 kinase activation (Xin J.H., unpublished data). The time delay of 2 to 4 hours required to first observe induction of the mRNA suggests that *pea3* is not a direct target of the MAPK pathway and may require production of an intermediate protein to induce expression of its mRNAs. Probing for *HB-EGF* showed the results that are expected in the case of an immediate-early gene, a gene that is directly influenced by a signaling pathway. An increase in the levels of *HB-EGF* mRNA was detected as soon as 20 minutes after addition of R1881 to the media, as previously shown (McCarthy *et al.*, 1997). The house keeping gene glyceraldehyde phosphate dehydrogenase (*GAPDH*) was used as a loading control.

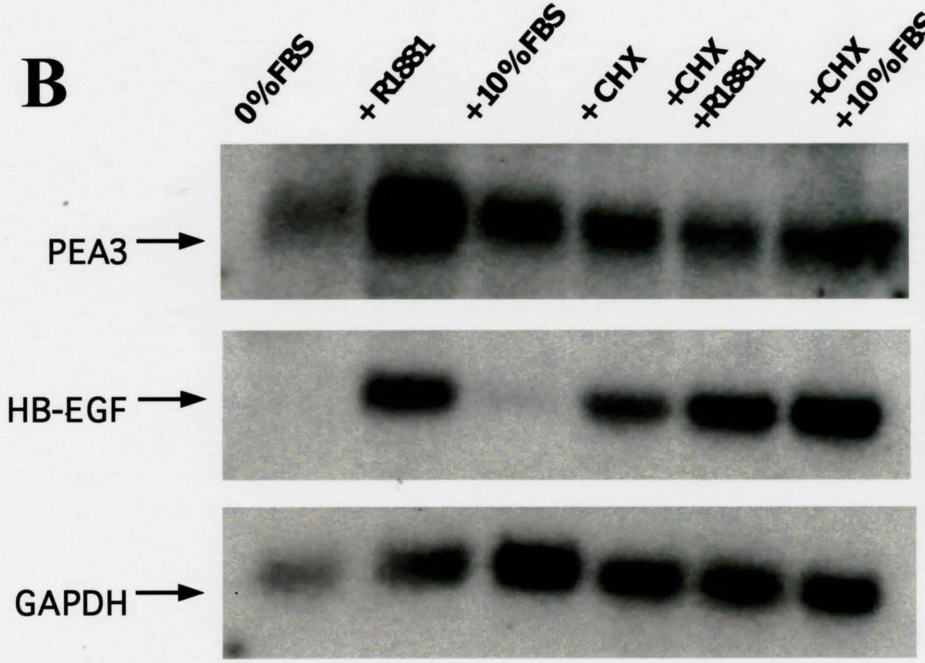
To determine whether protein synthesis is required to achieve *pea3* induction, cells were treated with cycloheximide (CHX) prior to induction of Raf-1 kinase activity. As illustrated in Figure 8b, cycloheximide reduced the induction of *pea3* mRNA by R1881 from 5.2-fold to less than 2-fold (Xin, J.-H., unpublished data), suggesting that protein synthesis is required for increased expression of *pea3* through the MAPK pathway. Interestingly, addition of serum alone seems insufficient to induce *pea3* mRNA expression, even in the absence of cycloheximide. The efficiency of the serum was verified by its induction of *HB-EGF* by almost 40-fold. This suggests that although serum was sufficient to induce *HB-EGF* expression, it lacked an element essential to induce *pea3* expression. Another interesting observation stems from the fact that *HB-EGF* induction is unaffected by addition of cycloheximide to the culture media, confirming

Figure 8. **Upregulation of *pea3* mRNA Levels Following Stimulation of Raf-1 Kinase Activity**

A. Total RNA was isolated from McMA cells before induction with 20nM of R1881 or after 20 minutes, 1, 2, 4, 8 or 24 hours. A Northern bolt, probed multiple times with the different probes indicated on the left is shown here. The kinetics of the three *pea3* subfamily members is similar, with *er81* being slightly delayed. *HB-EGF* probing was performed as a control, as it was previously shown to be induced quickly upon addition of R1881 to the culture media (McCarthy *et al.*, 1997). *GAPDH* was used as a control for RNA loading.

B. McMA cells were serum starved for 16 hours and then induced with 20nM R1881 or 10% serum, as indicated, for 8 hours before harvesting total RNA. Some samples were treated with cycloheximide (CHX) one hour prior to induction. Relative mRNA abundance, after normalizing to RNA loading control (GAPDH), is indicated in the table on the right.





Relative mRNA Abundance

	0% serum	+R1881	+10% serum	+CHX	+CHX +R1881	+CHX +10% serum
PEA3	1.0	5.2	1.2	1.9	1.9	1.3
HB-EGF	1.0	860	37.7	850	1750	1840

that it is an immediate-early gene of the MAPK pathway. *GAPDH* was once again used as a loading control.

The above results strongly suggest that *pea3* is a target of the MAPK pathway. However, it seems that production of one or more intermediate protein(s) is required to achieve induction, explaining the time delay observed before mRNA induction, pointing to an indirect effect of the MAPK pathway on the *pea3* promoter. Importantly, the increase in mRNA levels could be due to increased mRNA stability or to increased transcription initiation or elongation. To test the possibility of regulation at the level of transcription initiation, different regions of the *pea3* promoter were fused to a luciferase reporter gene and these constructs were tested in the McMA cell line in the presence or in the absence of Raf-1 kinase activity.

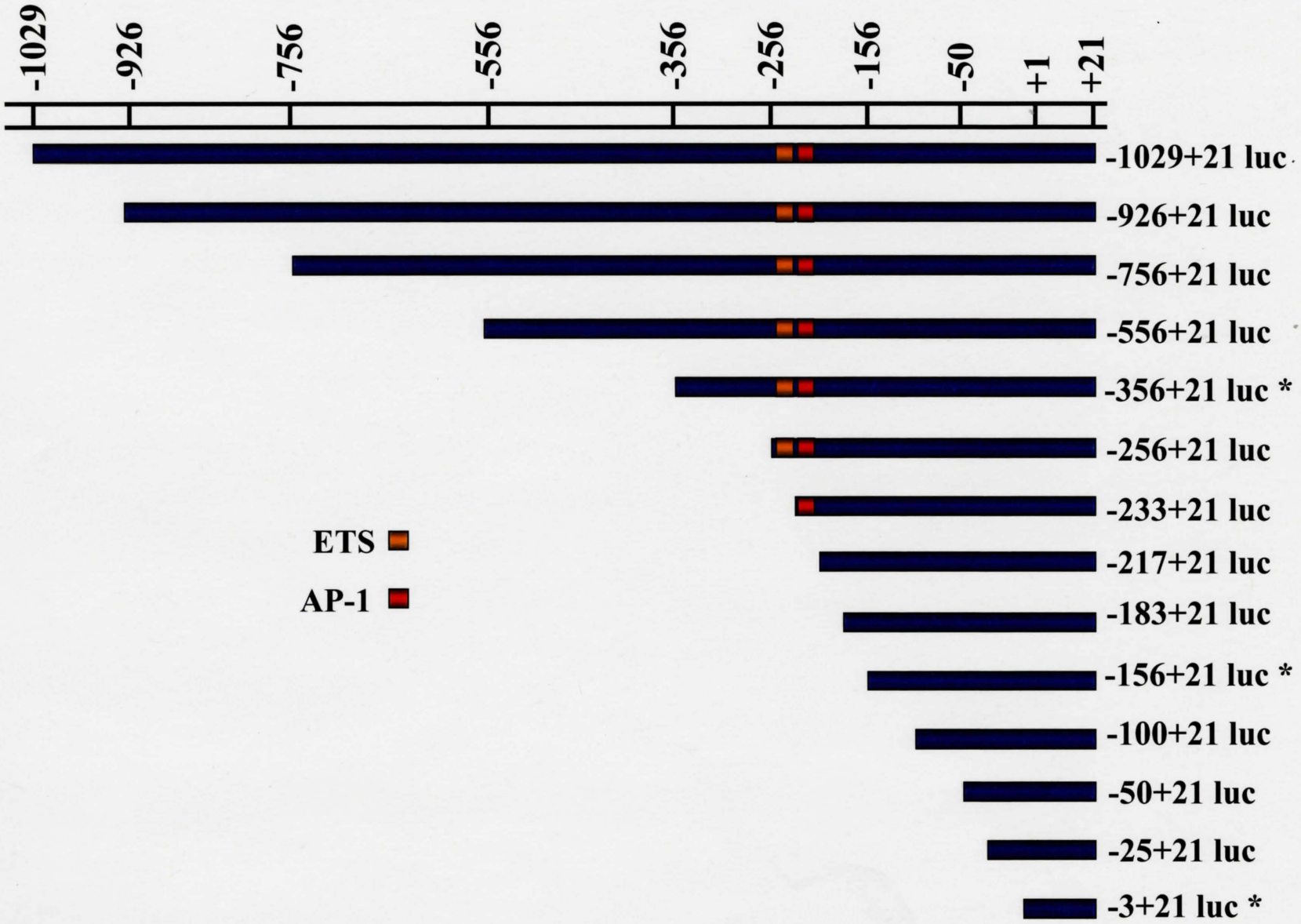
2. Generation of *pea3* Promoter Luciferase Reporter Constructs

Sequences flanking the putative promoter of the mouse *pea3* gene were amplified by PCR and cloned upstream of the luciferase reporter gene (Figure 9). The name of each construct represents the region of the *pea3* promoter relative to the transcription start site that was amplified.

Once the size of the PCR fragments was verified by agarose gel electrophoresis, the fragments were ligated in the proper orientation in the pGL3 plasmid (Figure 7). Positive clones were identified by restriction endonuclease analysis and confirmed by sequencing, using the RV3 and GL2 primers described by Promega (see Methods) for sequencing inserts in the pGL3 vector. All the constructs that were used in subsequent experiments

Figure 9. **Regions of the *pea3* Promoter Cloned in the pGL3 Reporter Vector**

The region of the promoter contained in each construct is indicated by a bar with the name of each construct shown on the left. The numbers above the bar represent the nucleotide position relative to the major transcription start site. The positions of the ETS and AP-1 binding sites part of the putative RRE are indicated by an orange and a red box respectively. The constructs marked with a * were generated by Jane M. Barrett in our laboratory.


-1029
-926
-756
-556
-356
-256
-156
-50
+1
+21
-1029+21 luc
-926+21 luc
-756+21 luc
-556+21 luc
-356+21 luc *
-256+21 luc
-233+21 luc
-217+21 luc
-183+21 luc
-156+21 luc *
-100+21 luc
-50+21 luc
-25+21 luc
-3+21 luc *
ETS
AP-1

are depicted in Figure 9. A few of the constructs that were used were previously made by Jane M. Barrett in our laboratory and are designated by an *.

3. Influence of Raf-1 Kinase on the *pea3* Promoter

To gain a better understanding of the role of the MAPK pathway on expression from the *pea3* promoter, transfection/induction experiments were performed to determine the responsiveness of each fragment of the *pea3* promoter to activated Raf-1 *in vivo*. Figure 10 illustrates that the *pea3* promoter responded to Raf-1 activation, as displayed by the increased activity in the presence of the R1881 inducer (Fig. 10a, induced 20nM R1881) when compared to the promoter activity in the absence of Raf-1 activation (Fig. 10a, uninduced). Deletion of sequences in the *pea3* promoter up to –256 had little effect on basal promoter activity or responsiveness to Raf-1 activation. Further deletion of 100bp (to –156) led to an increase in basal activity (Fig. 10a) and a 3-fold decrease in Raf-1 responsiveness (Fig. 10b). These results suggest the presence of DNA elements within this region that confer the *pea3* promoter responsiveness to Raf-1. The –50+21 luc construct, much like the pGL3 basic vector control, had little activity and was not affected by Raf-1 activation.

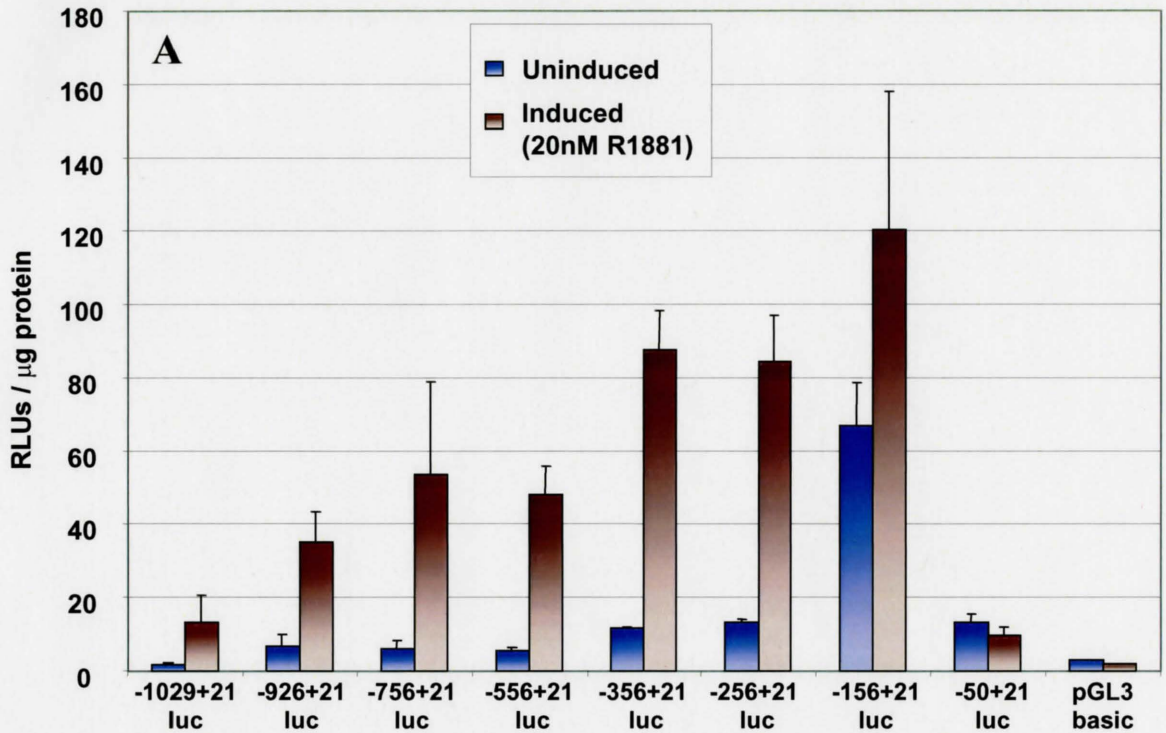
4. Identification of the Raf-Responsive Element in the *pea3* Promoter

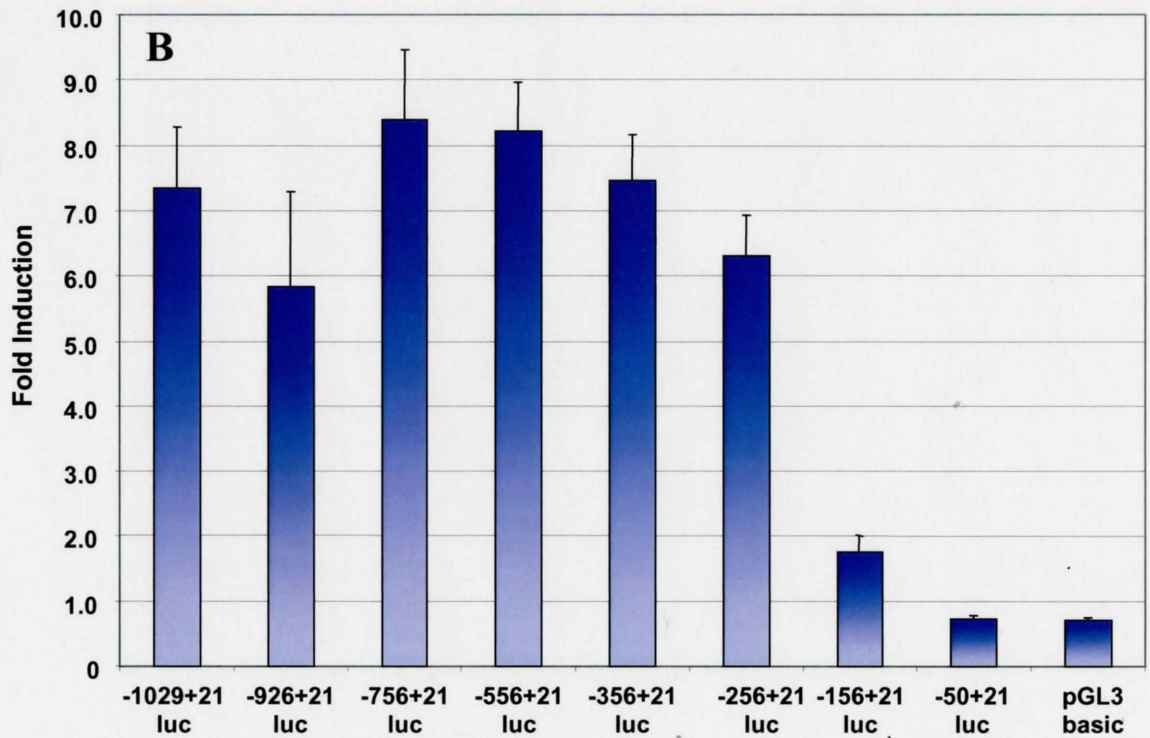
From the observation that deletion of 100bp between –256 and –156 in the *pea3* promoter led to a significant decrease in Raf-1 responsiveness, an analysis of the promoter sequence led to the identification of a putative **R**as-**R**esponsive **E**lement (RRE)

Figure 10. **Raf Activation Increased Expression of Luciferase from a *pea3* Promoter-Luciferase Construct**

A. Luciferase reporter constructs containing sequences upstream of the major transcription start site were assayed for their activity and responsiveness to Raf-1 in NIH 3T3 cells. 0.5μg of each reporter was transfected in 60mm plates and split into four wells of a 24-well plate 24 hours post-transfection. Two of the wells were mock induced with ethanol vehicle and two were induced with 20nM R1881. This graph is the average result of two separate preparations of each construct. This experiment was repeated with similar results.

B. The ratio of the activity of the promoter in the induced samples to that of the same promoter in the mock-induced samples is illustrated here.


A
Uninduced
Induced
(20nM R1881)
RLUs / μg protein
180
160
140
120
100
80
60
40
20
0
-1029+21 luc
-926+21 luc
-756+21 luc
-556+21 luc
-356+21 luc
-256+21 luc
-156+21 luc
-50+21 luc
pGL3 basic


B
Fold Induction
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
-1029+21 luc
-926+21 luc
-756+21 luc
-556+21 luc
-356+21 luc
-256+21 luc
-156+21 luc
-50+21 luc
pGL3 basic

within the region of the deletion (Fig. 4 and Fig. 9). RREs have been intensely studied and consist of an ETS binding site juxtaposed to an AP-1 binding site (Gutman and Wasylyk, 1990). Finer deletions were introduced in this region in attempt to determine the influence of the RRE on the ability of the promoter to be induced by Raf-1.

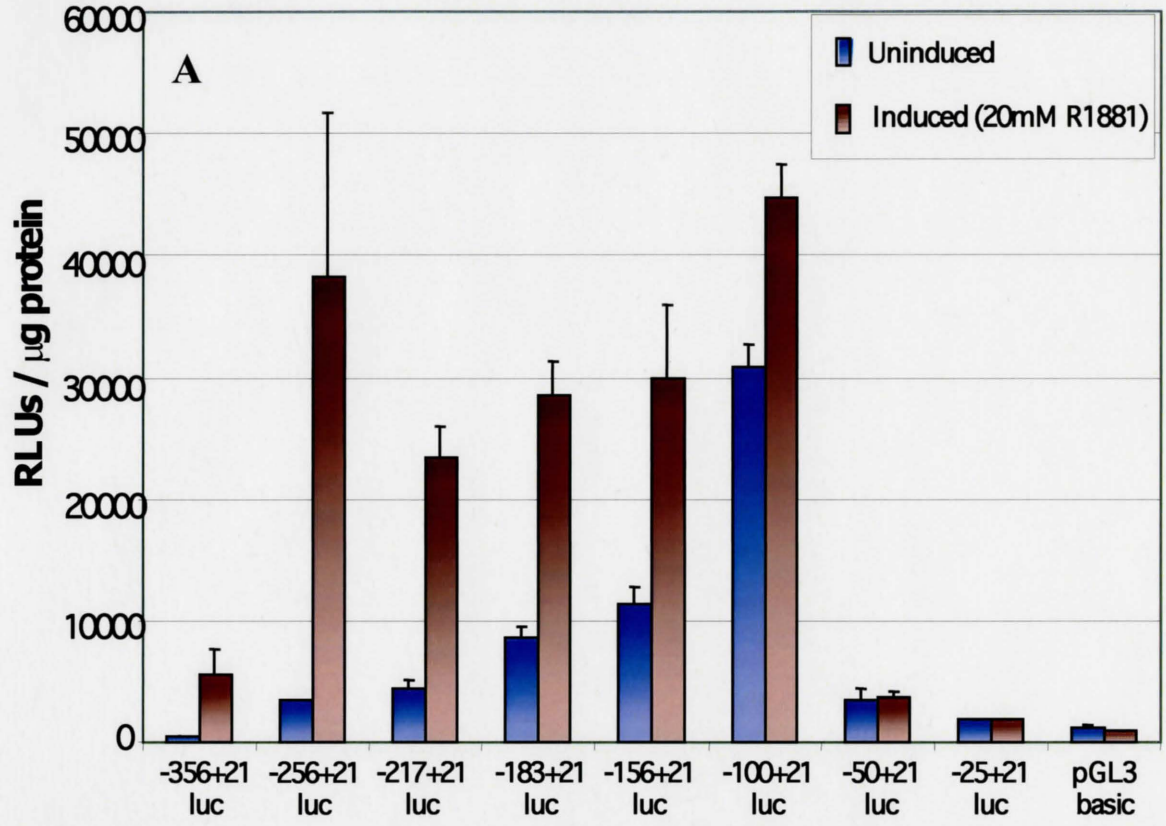
As can be observed from Figure 11, the results of the transfection/induction experiments involving these reporter constructs were puzzling. The basal level of activity of the different promoter fragments (Fig. 11a, blue series) increased progressively with increasing deletion size, up to -100. However, the level of induction of the promoters' activity from Raf-1 activation decreased steadily (Fig. 11b). Deletion of the **E**ts-**B**inding **S**ite (EBS) between –256 and –233 reduced the Raf-1 activation from over 8-fold to 4-fold, whereas deletion of the AP-1 binding site between –233 and –217 further decreased Raf-1 activation to 2-fold. This strongly suggests that this DNA element is responsible, at least in part, for the ability of the *pea3* promoter to respond to Raf-1 activation.

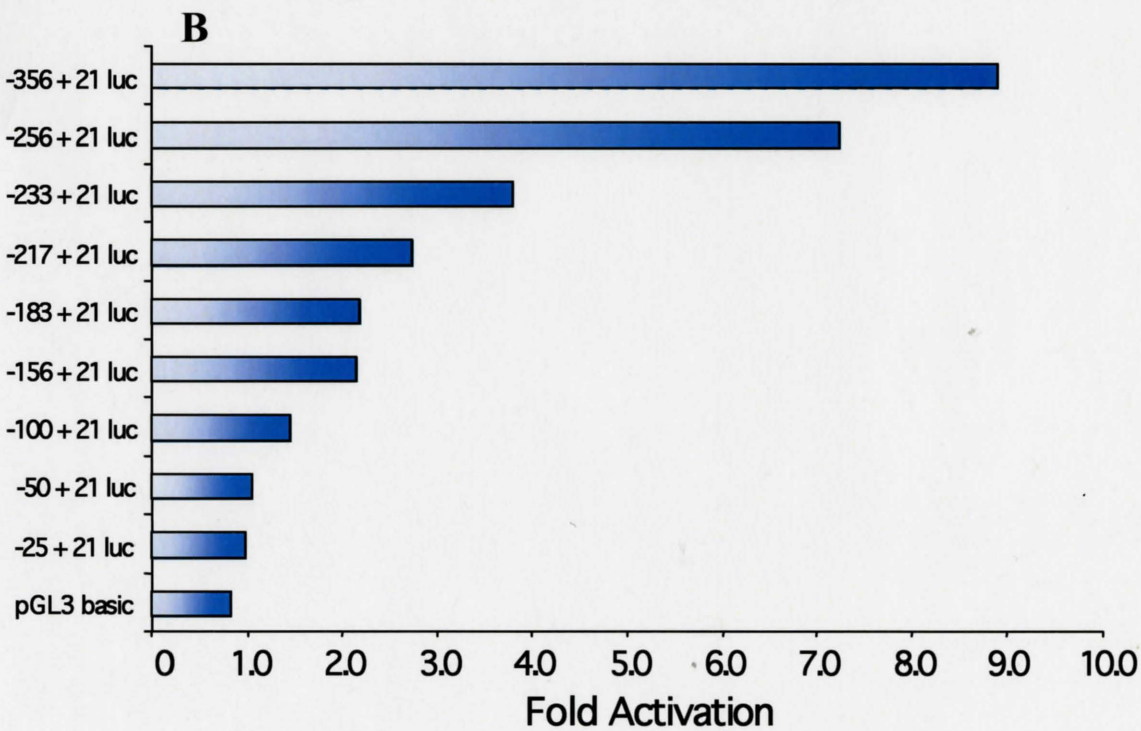
The increase in basal activity of the luciferase constructs with increasing deletion size could be caused by the deletion of negative regulatory elements in the promoter sequence. Also, it is possible that the increase in basal activity of each of the shorter constructs is due to positive regulatory sequences, located in the luciferase reporter plasmid, being brought progressively closer to the transcription start site at +1.

Figure 11. A Raf-1 Responsive Element is located between –256 and –183

A. 0.5μg of each reporter construct was transfected/induced as described in Figure 12. As progressive deletion mutations are introduced at the 5' end of the mouse *pea3* promoter, the level of basal activity of the promoter increases (blue series). Even though the level of activity in the presence of Raf activation (red series) increases as well, the overall effect on the promoter activity is decreased (see Figure 13b). This graph is representative of three experiments that gave similar results.

B. The ratio of the activity of the promoter in the induced samples to that of the same promoter in the mock-induced samples is illustrated here.


A
Uninduced
Induced (20mM R1881)
RLUs / µg protein
60000
50000
40000
30000
20000
10000
0
-356+21 luc
-256+21 luc
-217+21 luc
-183+21 luc
-156+21 luc
-100+21 luc
-50+21 luc
-25+21 luc
pGL3 basic


B
-356 + 21 luc
-256 + 21 luc
-233 + 21 luc
-217 + 21 luc
-183 + 21 luc
-156 + 21 luc
-100 + 21 luc
-50 + 21 luc
-25 + 21 luc
pGL3 basic
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
Fold Activation

5. The Promoter Activity Increase is Not Due to Sequences in the pGL3 plasmid

The observation in Figure 11 of the increase in basal activity of the reporter constructs with increasing deletions in the promoter can possibly be explained by a contribution of sequences close to the multiple cloning site in the pGL3 vector (Figure 7). To test this hypothesis, three of the reporter constructs, –256+21 luc, -183+21 luc and –100+21 luc, were inverted, as illustrated in Figure 12a, so that different vector DNA sequences would be upstream of the promoter fragments.

These three new constructs were tested in transfection/induction experiments in parallel with the constructs from which they were derived. The results of these experiments are displayed in Figure 12b and c. Even though the overall activity of the reverse constructs differed slightly from that of the regular constructs (Fig 12b), the fold induction by Raf-1 was identical in both cases (Fig 12c). Whereas this experiment disproved the hypothesis of pGL3 vector sequences influencing the activity of the promoter, it strengthened the argument of an RRE in the –256 to – 156 region, and confirmed the influence of the Ras/MAPK pathway on the *pea3* promoter. The fact that both sets of constructs responded the exact same way to Raf-1 activation (Fig 12c) strongly suggests that the increase in basal activity of the promoter constructs with increasing deletion size (Fig 11a, blue series) is not due to sequences in the pGL3 vector.

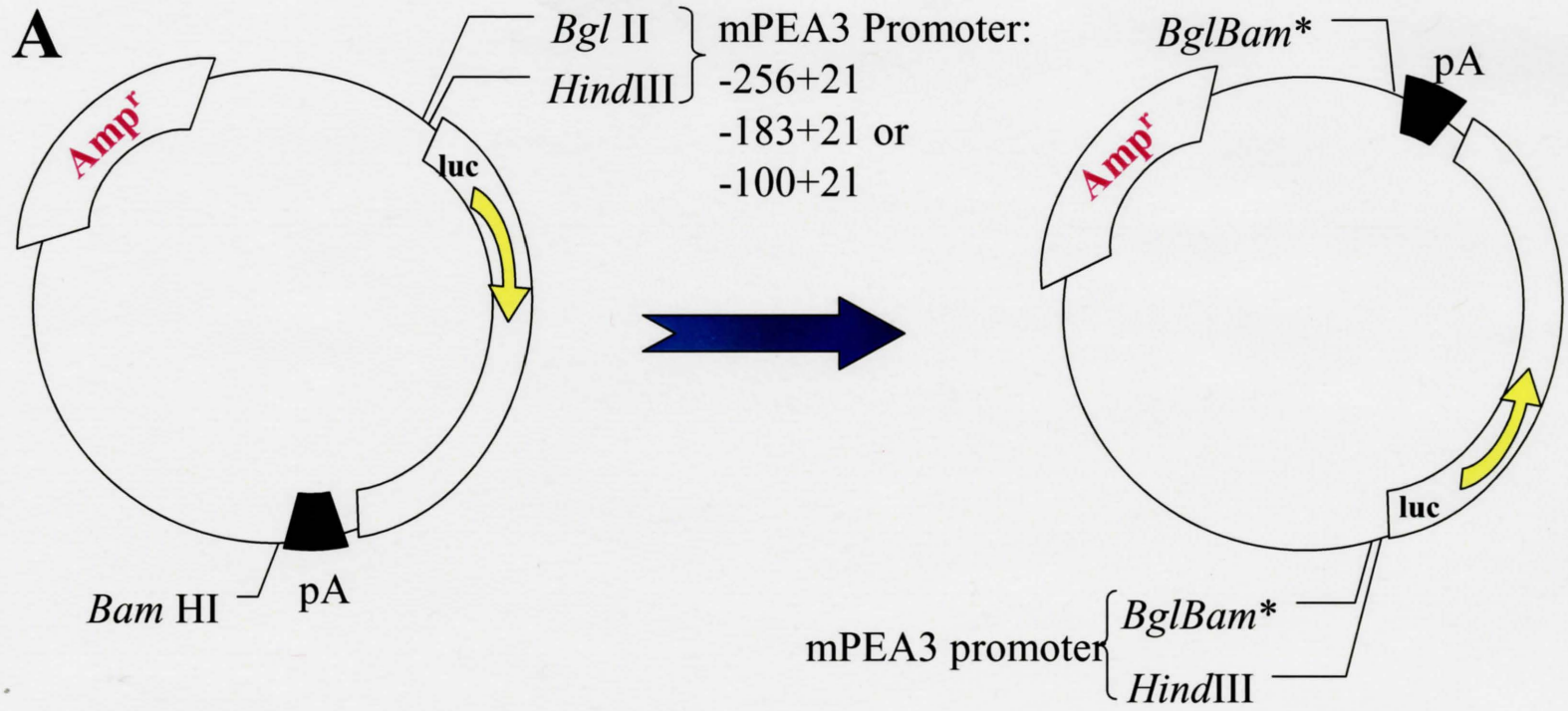
Figure 12. **The Increase in Basal Activity with Promoter Deletion is not a Plasmid Artifact**

A. ***Cloning Strategy to Design Reverse Constructs***

Three of the *pea3* promoter reporter constructs, -256+21, -183+21 and –100+21, were digested with *Bgl* II and *Bam* H1, which yield compatible sticky ends. The ligation product was then digested with *Bgl* II and *Bam* HI again, to rid the mixture of the fragments that were religated in the same orientation. The product of this digestion was transformed into competent bacteria and positive clones were identified by restriction endonuclease analysis.

B. 0.5μg of each of the reporter constructs was transfected/induced as before. The sequences for the -256+21, -183+21 and -100+21, along with the luciferase gene that follows them were inverted (reverse) and assayed in parallel with the regular clones. This graph is representative of three experiments that gave similar results.

C. The ratio of the activity of the promoter in the induced samples to that of the same promoter in the mock-induced samples is illustrated here.

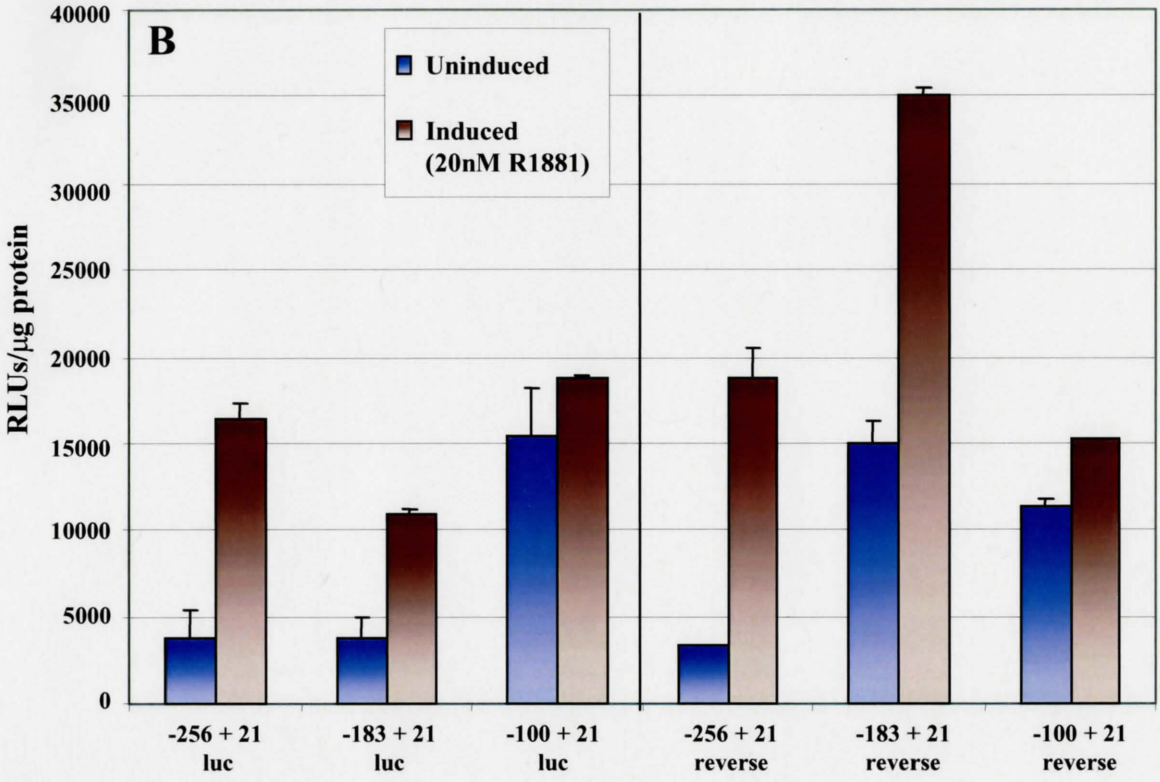


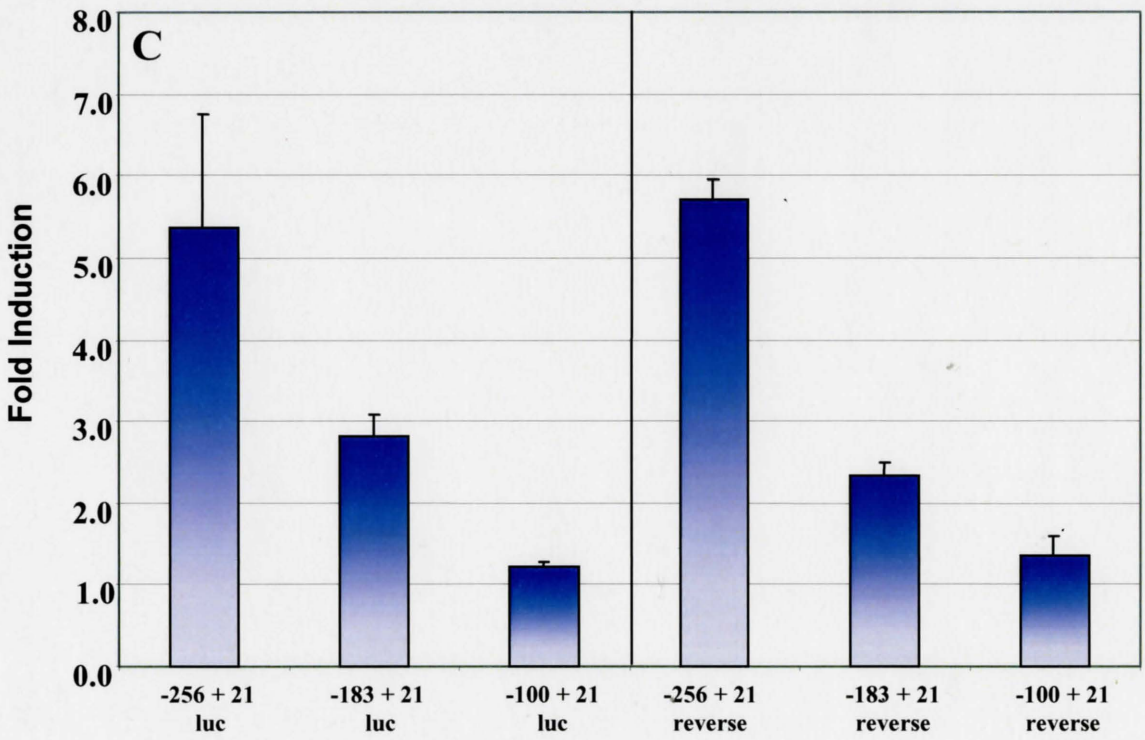
Digestion with *Bgl* II and *Bam*HI yielded sticky ends that can be ligated and are then resistant to cleavage by either *Bam*HI or *Bgl* II:

***Bam*HI : G GATC C**
C CTAG G

***Bgl* II : A GATC T**
T CTAG A

→ ***BglBam*: G G A T C T**
C C T A G A


B
Uninduced
Induced
(20nM R1881)
RLUs/μg protein
40000
35000
30000
25000
20000
15000
10000
5000
0
-256 + 21 luc
-183 + 21 luc
-100 + 21 luc
-256 + 21 reverse
-183 + 21 reverse
-100 + 21 reverse


C
Fold Induction
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
-256 + 21 luc
-183 + 21 luc
-100 + 21 luc
-256 + 21 reverse
-183 + 21 reverse
-100 + 21 reverse

6. The Putative Ets and AP-1 Binding Sites Function as an RRE

The results of the deletion mutation analyses provided evidence for a role for the putative Ets and AP-1 binding sites in *pea3* promoter responsiveness to Raf-1 activation. To further explore the importance of these two particular DNA elements in this context, site directed mutagenesis was used to create mutations in each of these sites. Each mutation was individually introduced into the –356+21 luc and the –256+21 luc reporter constructs. Double mutants were also generated, which contain both mutations. Figure 13a illustrates in detail the nature of these mutations.

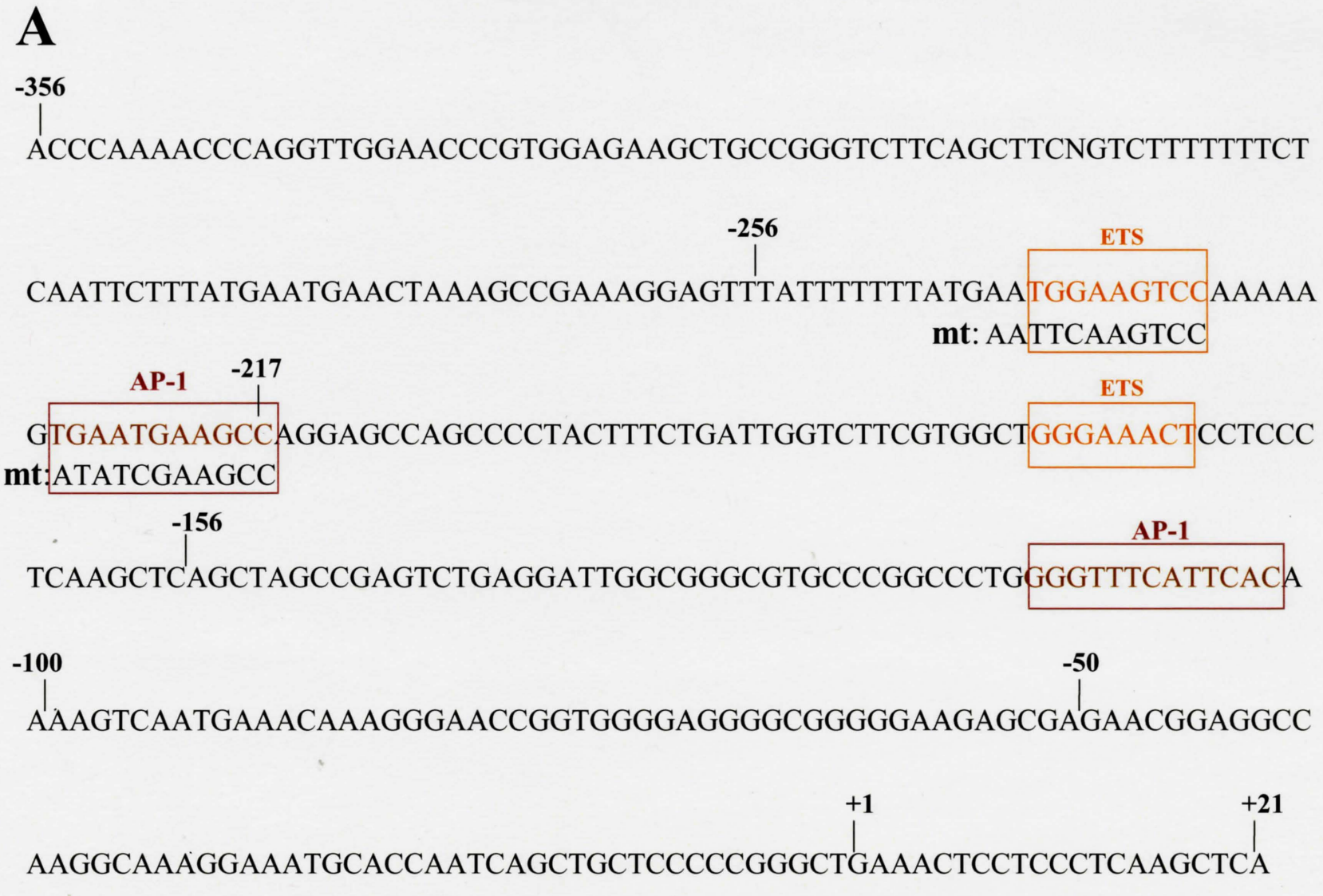
Each set of mutants was then tested in transfection/induction experiments in comparison with the wild type constructs. The wild-type –356+21 luc and –256+21 luc constructs had comparable levels of basal activity and were both induced approximately 14-fold by Raf-1 activation (Figure 13b and c). This result differs slightly from those obtained previously (Figure 11), but the level of induction by Raf-1 activity was similar for both constructs (Fig. 11b). As can be observed from Figure 13b, each of the mutations tested had little effect on the activity of the promoter in the absence of Raf-1 activity. Mutation of both the EBS and the AP-1 binding site in the –256+21 luc construct mirrors the effect of the deletion mutation (compare Fig. 11b, -256+21 luc and –217+21 luc with Fig. 13c, -256+21 luc and –256+21 Db mt luc). Also, mutation of each of the ETS or AP-1 binding site individually decreases the capacity of the *pea3* promoter to respond to Raf-1 activation (Fig. 13c), both in the context of the –356+21 luc or the –256+21 luc constructs. Introduction of both mutations in the same promoter fragment further decreased the ability of Raf-1 to induce expression of the reporter gene. These results

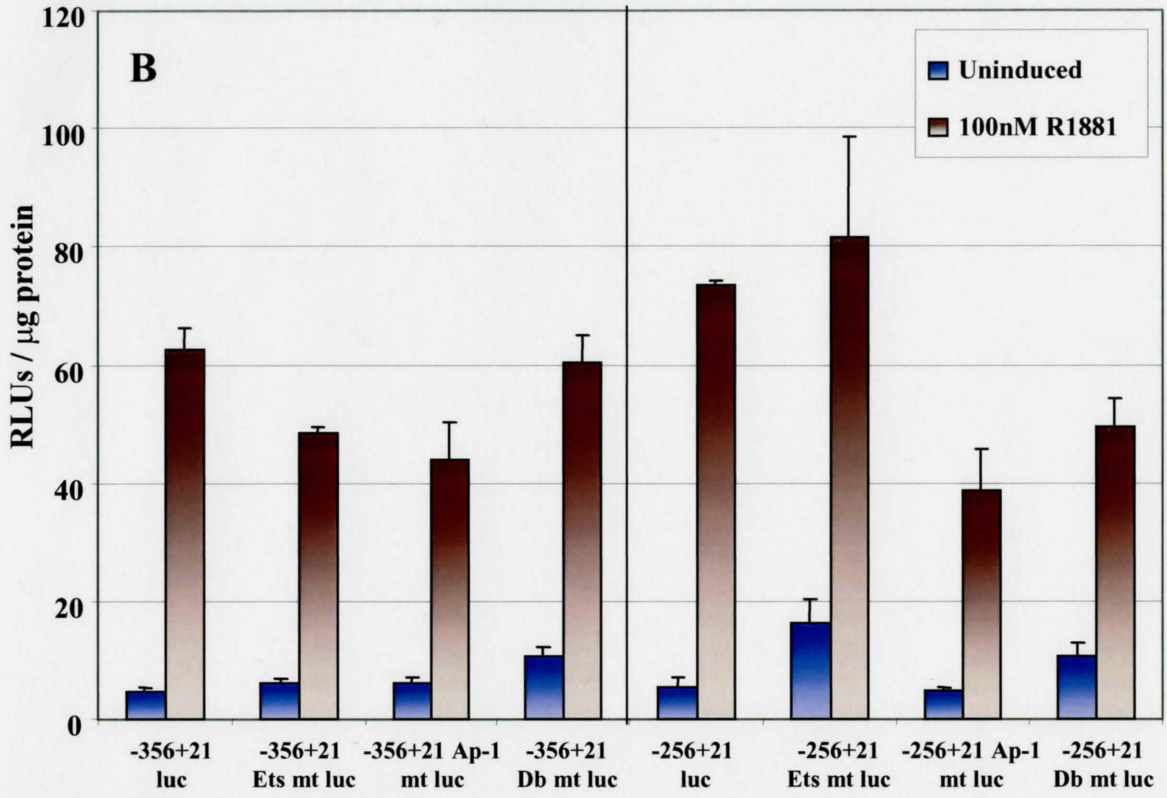
Figure 13. **The Putative Ets and AP-1 Binding Sites Function As a Raf-Responsive Element**

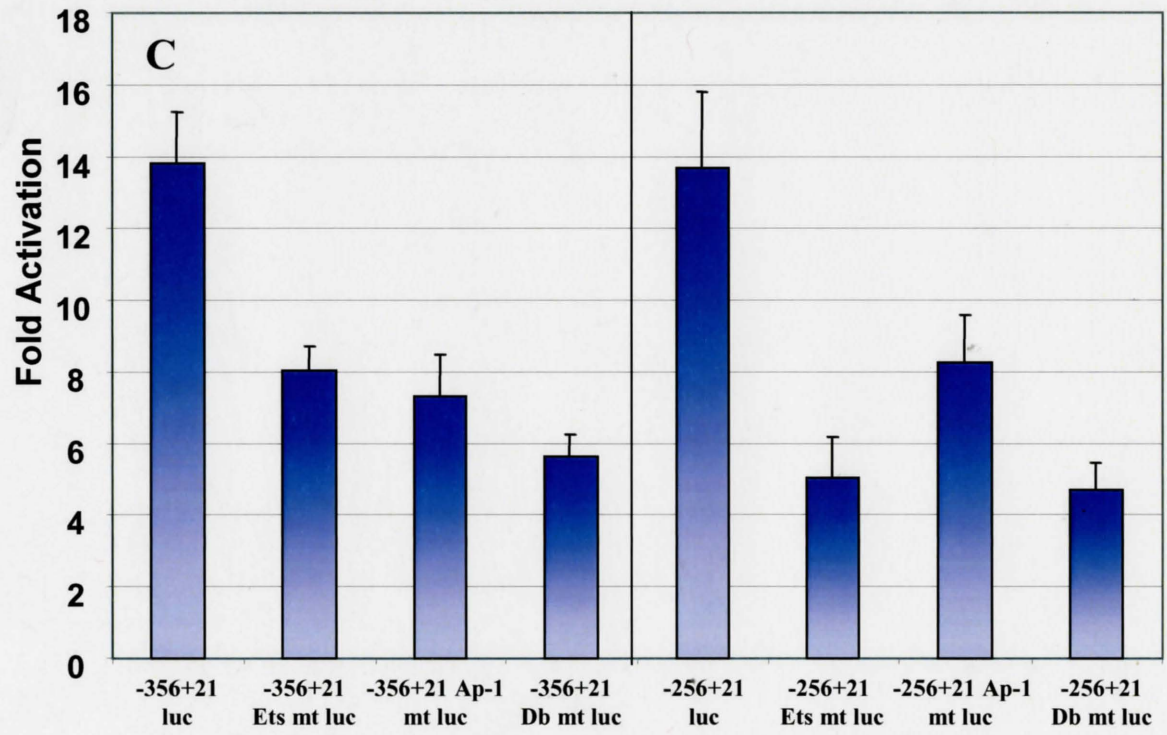
A. Sequence of the *pea3* promoter from –356 + 21 and illustration of the putative ETS and AP-1 binding sites, as well as the mutations that were introduced (mt) in the RRE. Numbers above the sequence refer to the position relative to the transcription start site (+1).

B. Point mutations were introduced in the putative Ets and AP-1 binding sites of the mouse *pea3* promoter, in the context of the –356+21 and –256+21 luciferase reporter construct. All the constructs were tested in parallel to determine the effect of each individual mutation, and the double mutation, on the capacity of the promoter to respond to Raf-1 activation in NIH 3T3 cells. 0.5μg of each construct was transfected/induced as before. This experiment was performed in triplicate and was repeated three times with similar results.

C. The ratio of the activity of the promoter in the induced samples to that of the same promoter in the mock-induced samples is illustrated here.


A
-356
ACCCAAAACCCAGGTTGGAACCCGTGGAGAAGCTGCCGGGTCTTCAGCTTCNGTCTTTTTTTCT
-256
ETS
CAATTCTTTATGAATGAACTAAAGCCGAAAGGAGTTTATTTTTTTATGAATGGAAGTCCAAAAA
mt: AATTCAAGTCC
AP-1
-217
ETS
GTGAATGAAGCCAGGAGCCAGCCCCTACTTTCTGATTGGTCTTCGTGGCTGGGAAACTCCTCCC
mt:ATATCGAAGCC
-156
AP-1
TCAAGCTCAGCTAGCCGAGTCTGAGGATTGGCGGGCGTGCCCGGCCCTGGGGTTTCATTCACA
-100
-50
AAAGTCAATGAAACAAAGGGAACCGGTGGGGAGGGGCGGGGGAAGAGCGAGAACGGAGGCC
+1
+21
AAGGCAAAGGAAATGCACCAATCAGCTGCTCCCCCGGGCTGAAACTCCTCCCTCAAGCTCA


B
Uninduced
100nM R1881
RLUs / µg protein
120
100
80
60
40
20
0
-356+21 luc
-356+21 Ets mt luc
-356+21 Ap-1 mt luc
-356+21 Db mt luc
-256+21 luc
-256+21 Ets mt luc
-256+21 Ap-1 mt luc
-256+21 Db mt luc


C
Fold Activation
18
16
14
12
10
8
6
4
2
0
-356+21 luc
-356+21 Ets mt luc
-356+21 Ap-1 mt luc
-356+21 Db mt luc
-256+21 luc
-256+21 Ets mt luc
-256+21 Ap-1 mt luc
-256+21 Db mt luc

suggest that these two DNA elements form a functional RRE, conferring the *pea3* promoter part of its responsiveness to Raf-1 activation. An interesting observation stems from the residual 3- to 6-fold induction, by Raf-1 activation, of the *pea3* promoter with a deletion or a mutation, respectively, of the RRE. A plausible explanation for this effect can be formulated by examining the rest of the *pea3* sequence. There are a large number of transcription factor binding sites outside of the sequences encompassing the RRE, including another EBS (-177) and another AP-1 binding site (-107, see Fig. 4 and Fig. 13a). It is possible that these sequences mediated the 3- to 6-fold activation (Figs. 11b and 13c) of the *pea3* promoter lacking the RRE between sequences –256 and –217.

7. The Mouse *pea3* Promoter Is Responsive to c-Jun in McMA cells

The proteins that effect promoter activation through RREs are members of the AP-1 and Ets transcription factor families. Ets and Jun/Fos synergy was first discovered on the polyomavirus enhancer (Wasylyk *et al.*, 1989). Since then, co-operation between PEA3 and AP-1 elements have been demonstrated on a number of regulatory DNA elements, including the collagenase promoter (Gutman and Wasylyk, 1990), the urokinase enhancer (Nerlov, *et al.*, 1992), the gelatinase B promoter (Gum *et al.*, 1996) and the interleukin-8 promoter (Iguchi, *et al.*, 2000). In addition, many interactions between ETS and AP-1 proteins have been characterized (reviewed in Graves and Petersen, 1998). In particular, c-Jun has been shown to be able to interact with a variety of ETS proteins, including ETS-1 (Logan *et al.*, 1996), ERM (Nakae *et al.*, 1995), Elf-1, PU.1 and Fli-1 (Bassuk *et al.*, 1995). Such previous evidence of ETS/jun synergy led to the hypothesis that c-Jun

was a potential effector of Raf-1 activation on the *pea3* promoter. To test this hypothesis, the effect of c-Jun on the *pea3* promoter was assayed through co-transfection experiments.

Initially, the –356+21 luc promoter construct was co-transfected with increasing amounts of an expression plasmid containing c-Jun cDNA (Fig. 14a). The promoter was responsive to c-Jun transactivation in a dose-dependant manner over the wide range of concentrations tested. To identify the region(s) of the *pea3* promoter responsive to c-Jun, six different deletion mutants were assayed in the presence or absence of c-Jun (Fig. 14b and c). Interestingly, the effect of c-Jun on the *pea3* promoter was reduced by 3-fold when the sequences containing the RRE were deleted (Fig. 14c, compare -256+21 luc and –156+21 luc). In fact, all the constructs containing larger deletions than –156+21 were not affected by co-transfection of the c-jun expression plasmid. These results suggest that sequences between –256 and –156 are required for c-Jun transactivation.

In an attempt to confirm the role of the RRE in the responsiveness of the *pea3* promoter to c-Jun, the fine deletion mutants were assayed alongside the point mutants of the putative Ets and AP-1 binding sites in a similar co-transfection experiment. The effect of the c-Jun expression vector co-transfected with the different reporter constructs is illustrated in Figure 15. The –356+21 luc construct displayed an unusually low level of responsiveness to c-Jun transactivation as compared to –256+21 luc in this experiment.

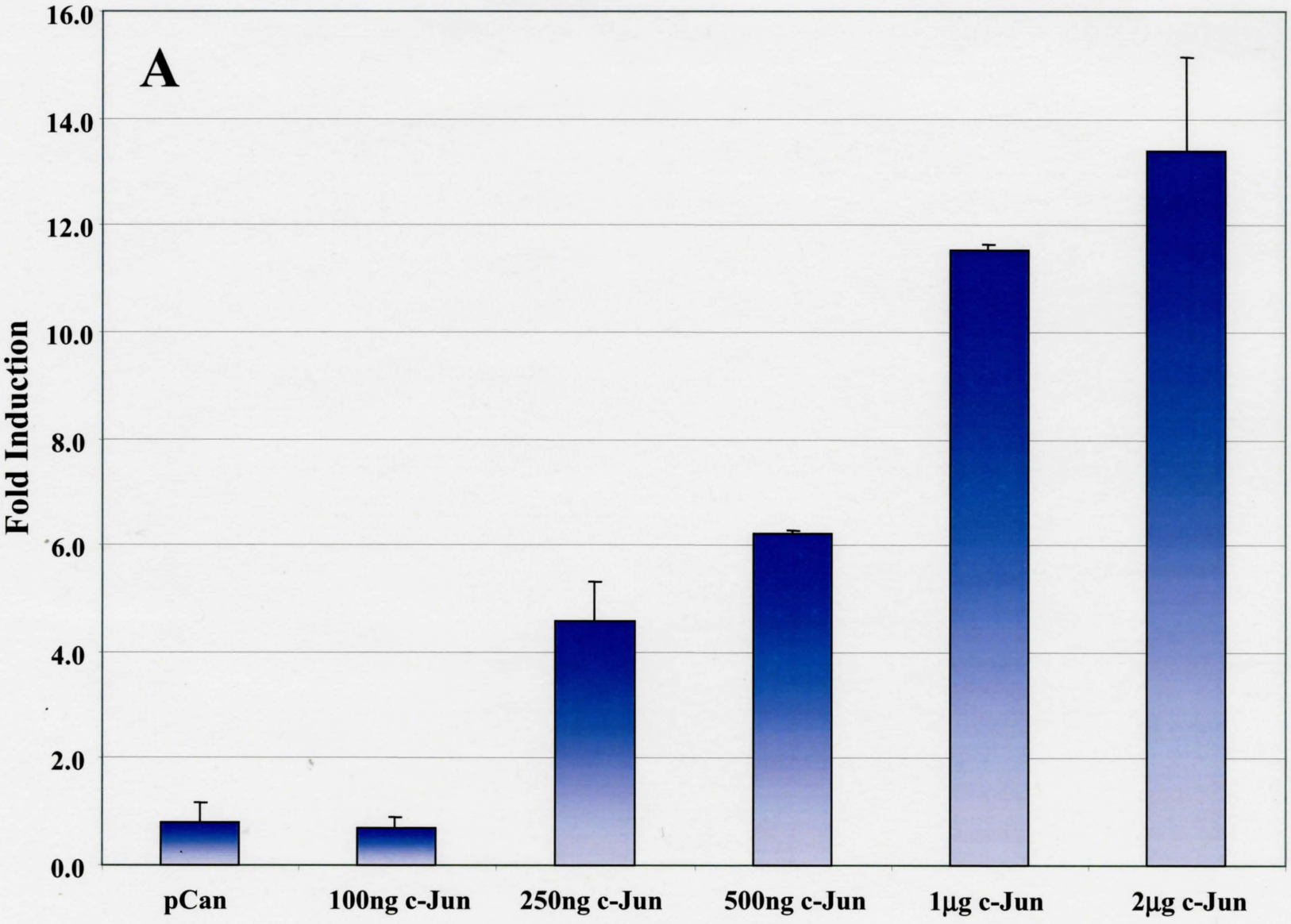
As shown previously in Fig. 14c, both constructs can achieve similar fold activation by c-Jun co-transfection. Progressive deletion or mutation of the Ets and AP-1 binding sites cause a small but consistent increase in basal promoter activity (Fig 15a, pCan only).

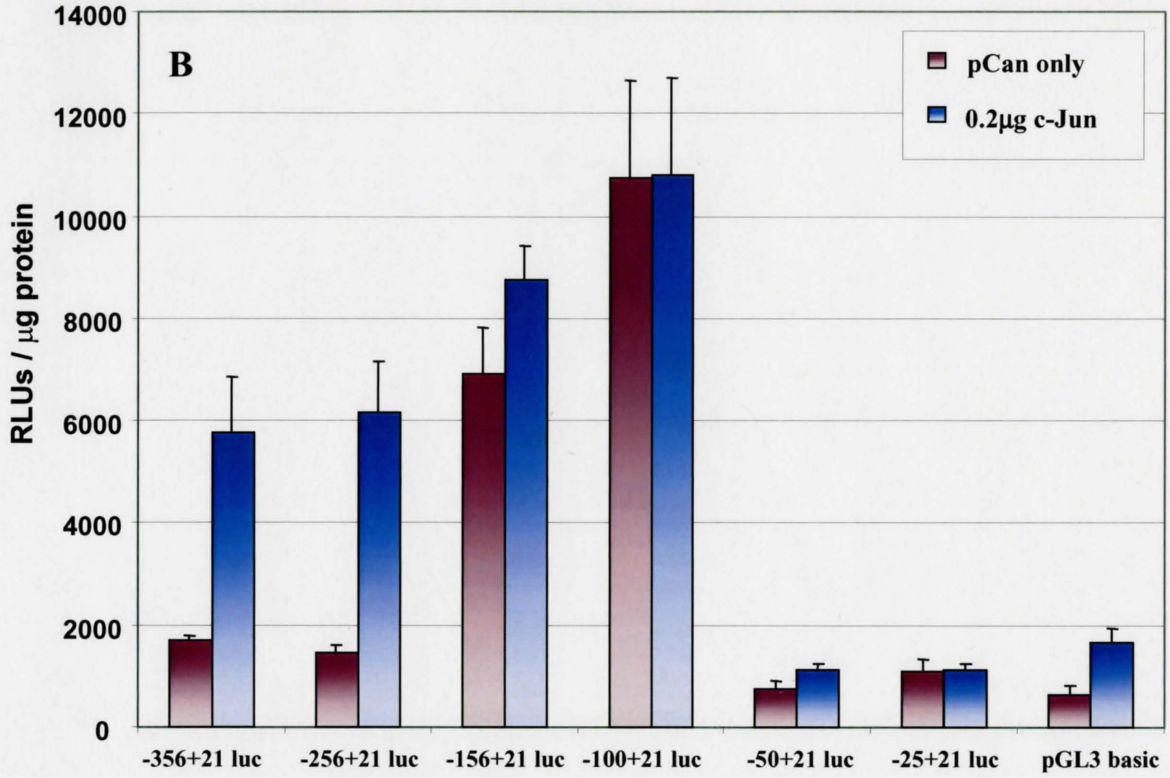
Figure 14. **The Mouse *pea3* Promoter is Responsive to c-Jun Transactivation in McMA Cells**

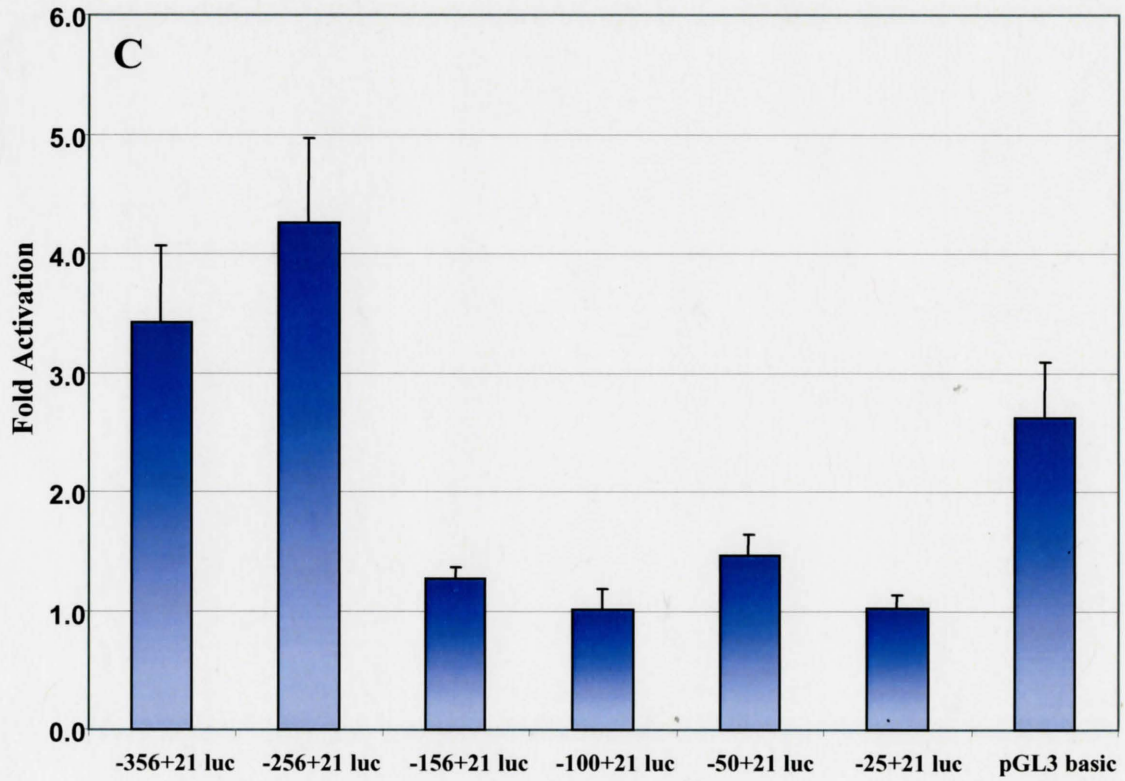
A. The –356+21 luciferase reporter construct was tested for its capacity to respond to increasing amounts of c-Jun in McMA cells. 0.5μg of the reporter was co-transfected with the indicated amounts of an expression vector encoding c-Jun. This experiment was repeated with similar results.

B. Different deletion mutants of the *pea3* promoter were tested for their ability to respond to c-Jun. 0.5μg of each reporter construct was transfected in McMA cells with (blue series) or without (red series) 0.2μg c-Jun expression plasmid. This experiment was repeated with similar results

C. The activity of each reporter construct transfected alone was set to 1 and the effect of c-Jun co-transfection illustrated as fold activation.


A
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Fold Induction
pCan
100ng c-Jun
250ng c-Jun
500ng c-Jun
1µg c-Jun
2µg c-Jun


B
pCan only
0.2µg c-Jun
RLUs / µg protein
14000
12000
10000
8000
6000
4000
2000
0
-356+21 luc
-256+21 luc
-156+21 luc
-100+21 luc
-50+21 luc
-25+21 luc
pGL3 basic

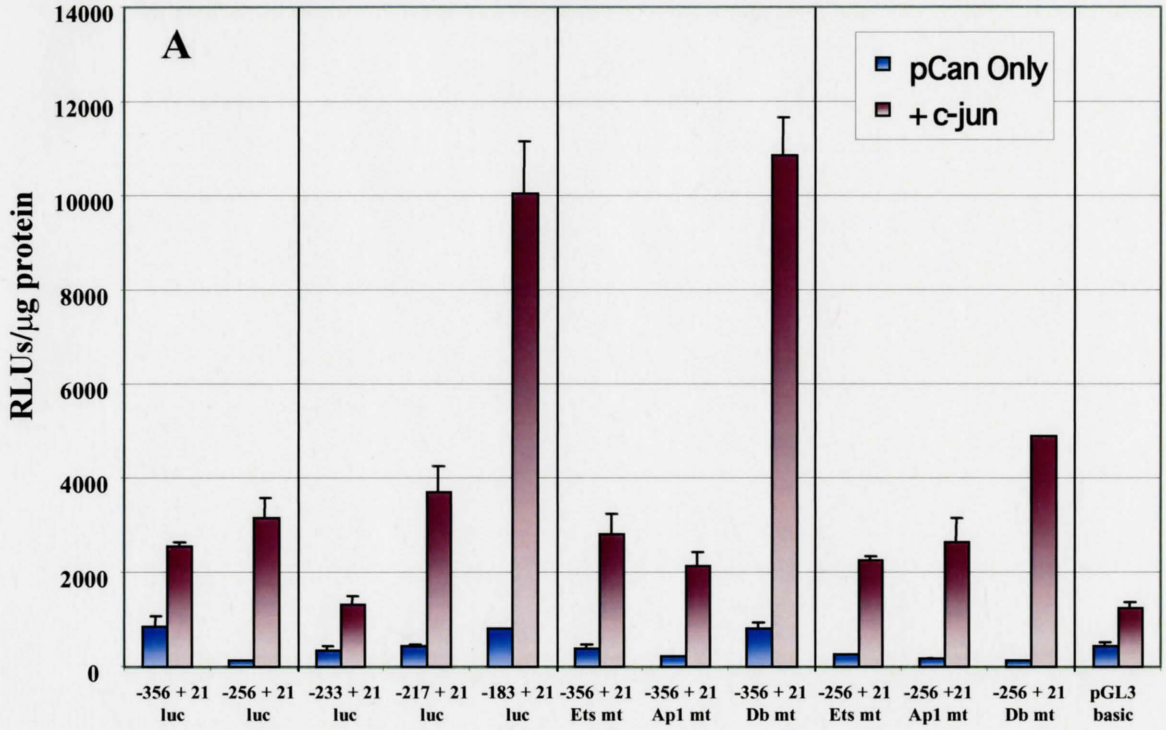

C
Fold Activation
6.0
5.0
4.0
3.0
2.0
1.0
0.0
-356+21 luc
-256+21 luc
-156+21 luc
-100+21 luc
-50+21 luc
-25+21 luc
pGL3 basic

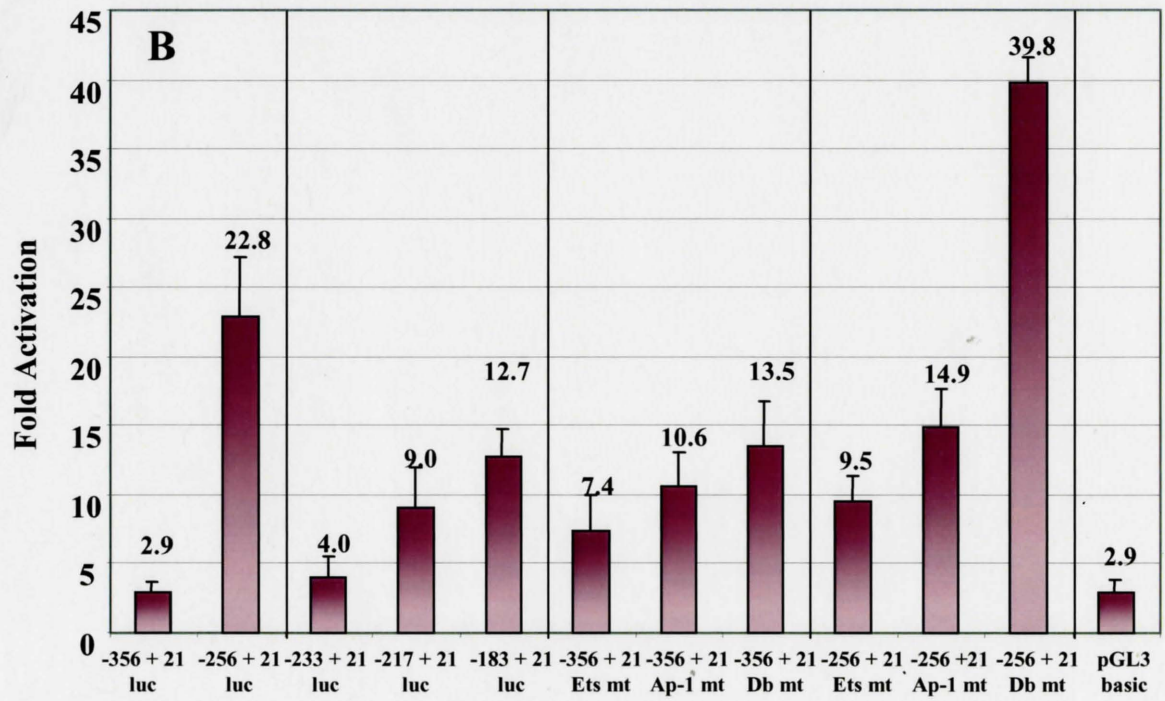
Deletion or mutation of the EBS at –247 caused a reduction in the level of promoter activity in the presence of c-Jun (Fig 15a, + c-Jun), mirrored by a decrease in fold activation by c-Jun (Fig 15b). This suggests that the EBS is important for responsiveness of the *pea3* promoter to c-Jun transactivation. Mutation of the AP-1 binding site by itself (-256+21 AP-1 mt and –356+21 AP-1 mt) affected the ability of c-Jun to induce *pea3* promoter activity to a lesser extent than the EBS mutation, as reflected by a higher fold activation by c-Jun (Fig. 15b) Deletion of the AP-1 binding site at –227, which efficiently deletes the entire RRE, increased c-Jun responsiveness by 5-fold, from the level achieved with the EBS deletion mutant. This suggests that, in this context, this AP-1 binding site may be playing an inhibitory role on the *pea3* promoter. This effect can also be observed by examining the results obtained with the double mutants (-256+21 Db mt and –356+21 Db mt). Each conferred the *pea3* promoter a greater responsiveness to c-jun than each individual mutation, and, in the case of –256+21 Db mt, a greater responsiveness to c-Jun than the wild type –256+21 luc (Fig 15b). Far from being expected, these results point to a complex role of c-Jun on the *pea3* promoter. The role of the RRE may not be limited to assisting in the transactivation of the *pea3* promoter. There is evidence through the results of this experiment that c-Jun may play a repressive role as well in regulation of *pea3* transcription. It is important to point out that these experiments were conducted in the absence of Raf-1 activation, and that the presence of Raf-1 kinase activity may have altered the results significantly.

Figure 15. Role of the Putative Ets and AP-1 Binding Sites in c-Jun Transactivation of the *pea3* promoter in McMA cells

A. Different deletion and site mutants of the *pea3* promoter were tested for their ability to respond to c-Jun. 0.5μg of each reporter construct was transfected in McMA cells with (blue series) or without (red series) 1.0μg c-Jun expression plasmid. This experiment was repeated with similar results

B. The activity of each reporter construct transfected alone was set to 1 and the effect of c-Jun co-transfection illustrated as fold activation.


A
pCan Only
+ c-jun
RLUs/μg protein
14000
12000
10000
8000
6000
4000
2000
0
-356 + 21 luc
-256 + 21 luc
-233 + 21 luc
-217 + 21 luc
-183 + 21 luc
-356 + 21 Ets mt
-356 + 21 Ap1 mt
-356 + 21 Db mt
-256 + 21 Ets mt
-256 +21 Ap1 mt
-256 + 21 Db mt
pGL3 basic


B
Fold Activation
45
40
35
30
25
20
15
10
5
0
2.9
22.8
4.0
9.0
12.7
7.4
10.6
13.5
9.5
14.9
39.8
2.9
-356 + 21 luc
-256 + 21 luc
-233 + 21 luc
-217 + 21 luc
-183 + 21 luc
-356 + 21 Ets mt
-356 + 21 Ap-1 mt
-356 + 21 Db mt
-256 + 21 Ets mt
-256 +21 Ap-1 mt
-256 + 21 Db mt
pGL3 basic

8. PEA3 and c-Jun Can Co-operate to Activate the *pea3* Promoter in the Presence of Raf-1 Activation

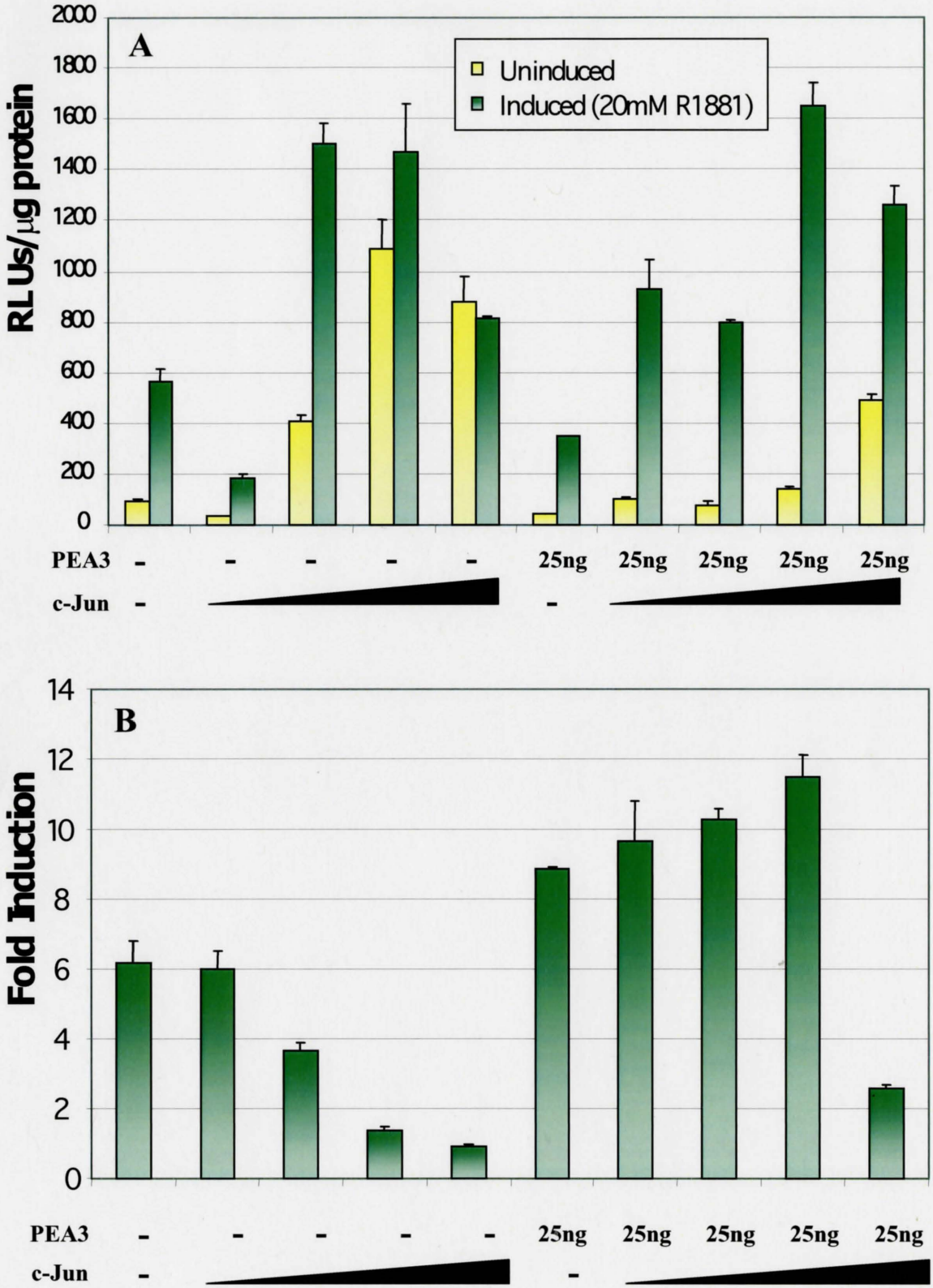
PEA3 was previously shown to regulate its own promoter up to 5-fold in Cos cells (Benz *et al.*, 1997). This observation was confirmed by reporter assays using the –356+21 luc construct in Cos-1 cells (not shown). Even though the EBS in the RRE is not a consensus PEA3 binding site, it was tempting to hypothesize that PEA3 is the ETS factor that synergizes with c-Jun to transactivate the *pea3* promoter. Co-transfection of increasing amounts of the PEA3-encoding vector did not succeed in increasing transactivation of the luciferase reporter gene from the *pea3* promoter (not shown). The deletion mutants of the *pea3* promoter were also tested for their responsiveness to PEA3 transactivation, with little effect from an amount of transfected PEA3 plasmid sufficient to achieve transactivation of the *pea3* promoter in Cos-1 cells (not shown).

In previous experiments, the importance of the RRE for the activation of the *pea3* promoter was assayed in the context of Raf-1 activation. It was therefore possible to believe that the effects observed with c-Jun co-transfection and the lack of effect with PEA3 co-transfection was due to the absence of Raf-1 activation in these experiments. To test this hypothesis, transfection/induction experiments were designed using c-Jun and/or PEA3 co-transfection, with the –356+21 luc reporter construct. The results of these experiments are illustrated in Figure 16. Co-transfection of increasing amounts of c-Jun gave a dose-dependent increase in *pea3* promoter activity, up to a plateau at 250ng (Fig. 16a, yellow series, left panel). As can be observed from Figure 16b, the addition of increasing amounts of c-Jun expression vector actually decreased the potential of Raf-1

Figure 16. **PEA3 and c-Jun can Co-operate to Activate the *pea3* Promoter in the Presence of Raf-1 Activation**

A. 0.5μg –356+21 mPEA3 luciferase reporter construct was co-transfected/induced, as before into McMA cells with expression vectors encoding PEA3, c-Jun, or both. The amounts of c-Jun expression plasmid used were 10ng, 50ng, 250ng and 750ng. The total amount of DNA transfected was kept constant using the empty expression vector (pCan). This graph represents a triplicate experiment that was repeated with similar results.

B. The ratio of the activity of the promoter in the induced samples to that of the same promoter in the mock-induced samples is illustrated here.


A
Uninduced
Induced (20mM R1881)
RLUs/μg protein
2000
1800
1600
1400
1200
1000
800
600
400
200
0
PEA3 - - - - - 25ng 25ng 25ng 25ng 25ng
c-Jun -
B
Fold Induction
14
12
10
8
6
4
2
0
PEA3 - - - - - 25ng 25ng 25ng 25ng 25ng
c-Jun -

induction of the *pea3* promoter (Fig. 16b, left panel). This is likely due to the large increase in activity of the promoter in the absence of Raf-1 activation. It is apparently impossible for activated Raf-1 to "super-activate" the *pea3* promoter in the presence of such a large excess of c-Jun. However, in the presence of PEA3, increasing amounts of c-Jun expression plasmid up to 250ng gave a small but gradual increase in Raf-1 induction of the *pea3* promoter, from 9-fold up to 11-fold (Fig. 16b, right panel). This suggests that PEA3 and c-Jun can co-operate to mediate induction of the *pea3* promoter by activated Raf-1. Co-transfection of 750ng of c-Jun, with or without PEA3, seemed to saturate the system and interfere with the ability of activated Raf-1 to induce transcription from the *pea3* promoter. It is possible that this effect is due to squelching, a phenomenon that is believed to occur when essential elements of the transcription initiation complex are sequestered away from the promoter of interest, when a large excess of a given protein is introduced into a cell (Gill and Ptashne, 1988).

CHAPTER 2: RATIONALE FOR STUDYING THE REGULATION OF *pea3* AND *CD1* PROMOTERS

Identification of target gene promoters for transcription factors can lead to a clearer understanding of the regulation of genes that are involved in control of normal cell growth, and differentiation, as well as provide insight into the development of cancer and its progression. Ets factors are overexpressed in a variety of cancers including prostate (Sementchenko *et al.*, 1998), colon (Ito *et al.*, 2002) and breast (Shepherd and Hassell, 2001; Barrett *et al.*, 2002) and ovary (Davidson *et al.*, 2003a; Davidson *et al.*, 2003b). In hopes to shed light on their contribution to tumor progression, investigation of their target genes has been intensive. DNA sequences corresponding to Ets binding sites were identified in a large variety of viral and cellular genes' promoters and enhancers, suggesting a role for Ets proteins in the regulation of cellular proliferation, differentiation, development, hematopoieis, apoptosis, metastasis, tissue remodelling, angiogenesis and transformation. A review of the literature up to the year 2000 identified over 200 Ets target genes (Sementchenko and Watson, 2000). With the increasing use of microarray technology, more potential Ets target genes are being identified at an incredible rate.

CYCLIN D1 is overexpressed in a large proportion of breast (Bartkova *et al.*, 1994a) and colorectal (Bartkova *et al.*, 1994b) cancers. In addition, studies in mouse models showed that overexpression of *CYCLIN D1* resulted in abnormal mammary cell proliferation and the development of mammary adenocarcinomas (Wang *et al.*, 1994).

CYCLIN D1 overexpression was also associated with intestinal adenomas and with increased cell proliferative activity in premalignant neoplastic cells in multiple intestinal neoplasia (Min) mice (Zhang *et al.*, 1997). It has been known for 10 years that *CYCLIN D1* accelerates and is required for progression through G1 to S phase in rodent (Quelle *et al.*, 1993) and human (Baldin *et al.*, 1993) fibroblasts. More recently, it was shown that *CYCLIN D1* is required to maintain the transformed phenotype in human gastric cancer cells (Chen *et al.*, 1999)

Accumulating evidence points to a direct correlation between *neu* mutation or overexpression and the levels of *CD1* in transformed cells such as MDA-MB-453, BT-483, which harbour amplification of *neu* (Lee *et al.*, 2000). MCF7 cells transfected with a transforming version of *neu* (*neuT*) also showed elevated levels of *CD1* compared to cells transfected with an empty expression vector (Lee *et al.*, 2000). Recent studies show that *CD1* is essential to transformation by *neu* and *ras* oncogenes in mice (Yu *et al.*, 2001) and in Rat-1 cells (Lee *et al.*, 2000). The involvement of the Ras/MAPK pathway in *CYCLIN D1* expression was shown to be essential to mediate Neu activation of the *cyclin d1* promoter in MCF7 cells (Lee *et al.*, 2000).

A large number of Ets proteins are overexpressed or otherwise up-regulated in different types of cancer (Dittmer and Nordheim, 1998, Oikawa and Yamada, 2003). A large number of these have been shown to be direct targets of the MAPK pathway (Sharrocks, 2001). *pea3* has been shown to be essential for tumor formation in MMTV-*neu* mice (Shepherd *et al.*, 2001). These facts led to the hypothesis that *CYCLIN D1* may be an ETS target gene, and more specifically a PEA3 subfamily target gene.

CHAPTER 3: THE *CYCLIN D1* PROMOTER

9. The *CYCLIN D1* Promoter Is Responsive to PEA3 Transactivation

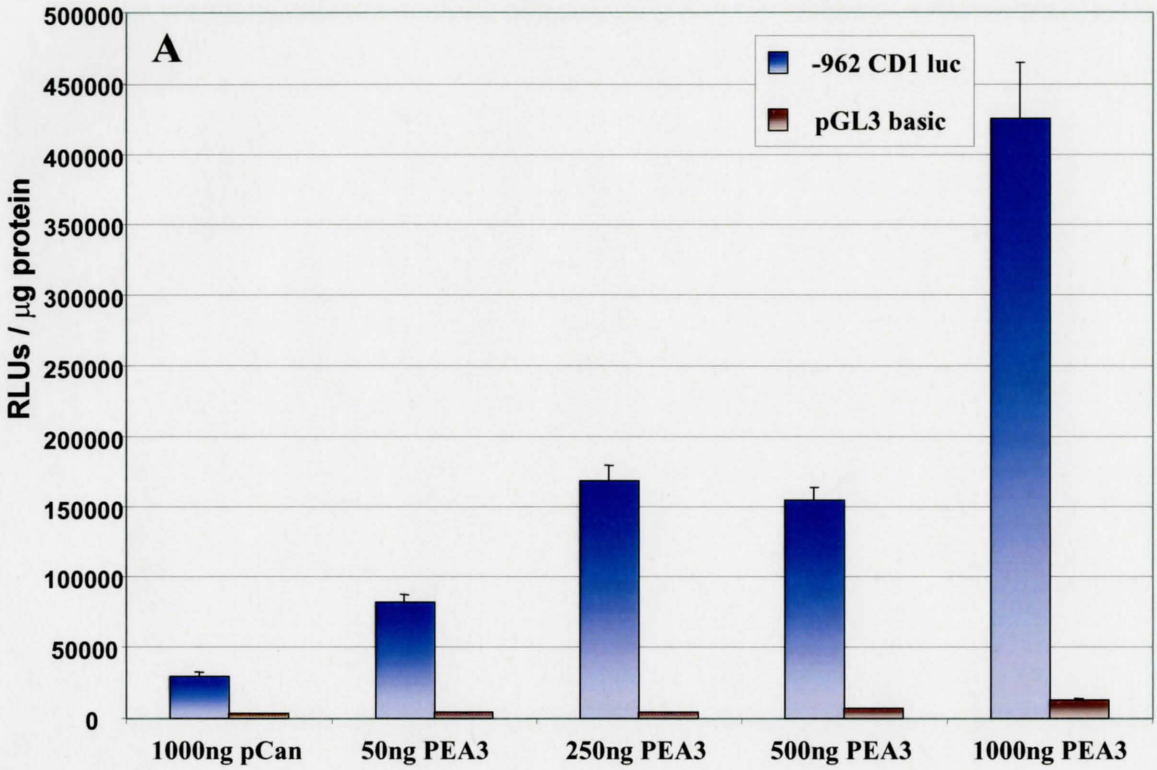
CYCLIN D1 (*CD1*) gene regulation is one of the most widely studied among the members of the cell cycle regulatory genes. Given that it is a direct player in cell cycle control, and that a large number of different cancers are associated with an increase in *CD1* mRNA levels, it is important to understand how its expression is regulated in normal cells to better understand what happens in the malignant cells. The *CD1* promoter contains at least four putative ETS binding sites, and has previously been shown to be regulated via a number of signaling pathways, including Wnt (Zhang *et al.*, 1997; Tetsu *et al.*, 1999; Rimerman *et al.*, 2000) and the Ras/MAPK pathway (Filmus *et al.*, 1994; Liu *et al.*, 1995; Lavoie *et al.*, 1996). Members of the Ets family of transcription factors such as Ets-2 have also been implicated in the regulation of the *CD1* promoter (Albanese *et al.*, 1995). Since the *CD1* gene is amplified in 20% of breast cancers (Dickson *et al.*, 1995) and the protein is overexpressed in 50% of human mammary carcinomas (Bartkova *et al.*, 1994a), and *PEA3* is also overexpressed in the majority of breast tumors, it is possible that *CD1* is a PEA3 target gene.

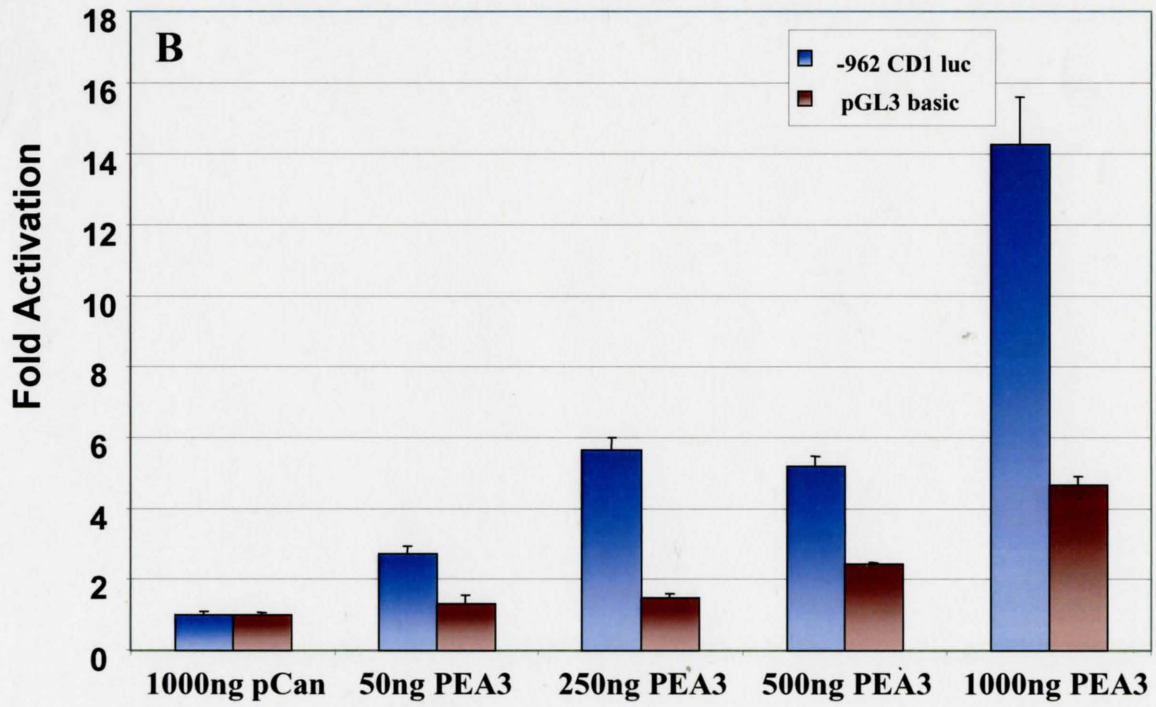
To test this hypothesis, increasing amounts of an expression plasmid encoding PEA3 was co-transfected with a reporter construct containing 1.1kb of the *CD1* promoter. The results of these experiments are illustrated in Figure 17. As can be easily observed, the *CD1* promoter is responsive to transactivation by PEA3 in a dose dependant manner,

Figure 17. **The *CYCLIN D1* Promoter is Responsive to PEA3 Transactivation in Cos-1 Cells**

A. 100ng of the –962CD1 promoter luciferase reporter construct was co-transfected in Cos-1 cells with the indicated amounts of an effector plasmid containing PEA3 cDNA. The total amount of DNA was kept constant by the addition of empty pCan effector plasmid. A dose-dependant increase in *CD1* promoter activity can be observed with increasing amount of PEA3. This effect is not mirrored when using the empty reporter plasmid as a control. This experiment was done in triplicate and is representative of three separate experiments.

B. The activity of each reporter construct transfected alone was set to 1 and the effect of PEA3 co-transfection illustrated as fold activation.


A
-962 CD1 luc
pGL3 basic
RLUs / μg protein
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
1000ng pCan
50ng PEA3
250ng PEA3
500ng PEA3
1000ng PEA3


B
-962 CD1 luc
pGL3 basic
Fold Activation
18
16
14
12
10
8
6
4
2
0
1000ng pCan
50ng PEA3
250ng PEA3
500ng PEA3
1000ng PEA3

up to 20-fold in certain experiments. Importantly, co-transfection of PEA3 with the promoterless pGL3 basic vector has little effect on the level of luciferase activity. This is solid evidence that *CD1* is a potential PEA3 target gene *in vivo*.

10. The *CYCLIN D1* Promoter Is Susceptible to Repression by DN-PEA3

To assess whether the effect of PEA3 was mediated by it binding directly to the *CD1* promoter, or through the activation of a different transcription factor which in turn would transactivate the *CD1* promoter, co-transfection experiments were performed using the *CD1* promoter luciferase reporter plasmid and an expression vector coding for a dominant negative form of PEA3 (DN-PEA3). This construct produces a hybrid protein with the PEA3 ETS domain fused to the Engrailed repression domain (Han *et al.*, 1993), and actively represses transcription of PEA3 regulated promoters (Shepherd *et al.*, 2001). DN-PEA3 was able to decrease the activity of the *CD1* promoter in a dose-dependant manner in Cos-1 cells (Fig. 18a, b) These results provide a strong argument to support the hypothesis that PEA3 acts directly on the *CD1* promoter.

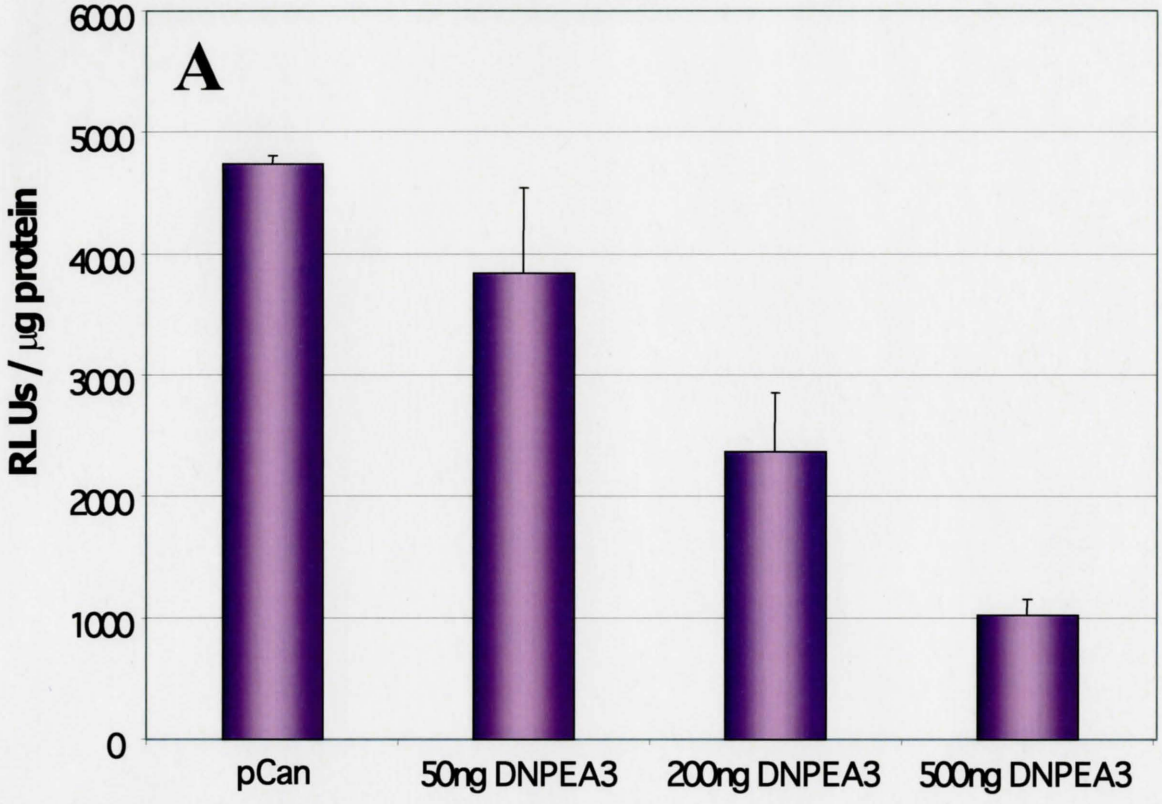
11. The *CYCLIN D1* Promoter Is Susceptible to Transactivation by Other Transcription Factors

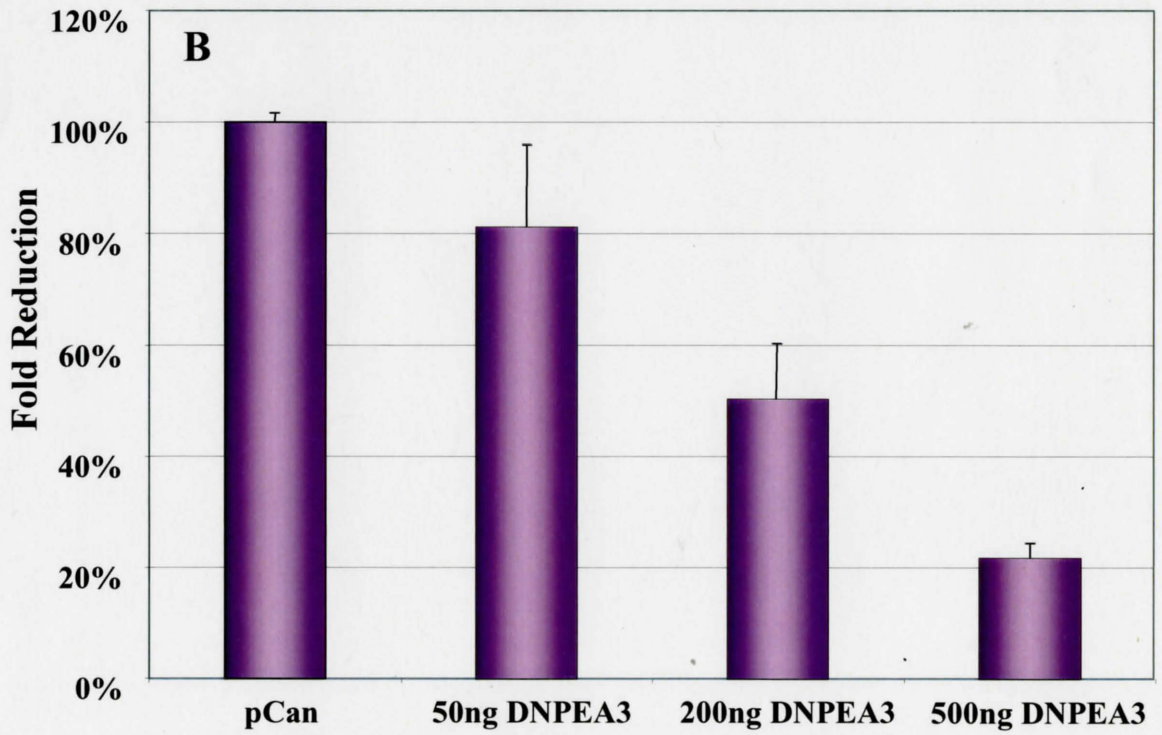
Promoters are highly complex regulatory DNA elements. Most promoters are regulated by a combination of transcription factors. The complexity of the promoters is what provides cell-specific and timely activation or silencing of each gene.

Figure 18. **The *CYCLIN D1* Promoter is Susceptible to Repression by DN-PEA3 in Cos-1 Cells**

A. 100ng of the –962CD1 promoter luciferase reporter construct was co-transfected into Cos-1 cells with increasing amounts of an expression plasmid encoding DN-PEA3. The total amount of DNA was kept constant using the empty expression vector (pCan). A dose-dependant decrease in promoter activity is observed proportional to the amount of repressor added. This experiment was done in triplicate and is representative of there separate experiments.

B. The activity of each reporter construct transfected alone was set to 100% and the effect of DN-PEA3 co-transfection illustrated as fold reduction.


A
RLUs / µg protein
6000
5000
4000
3000
2000
1000
0
pCan
50ng DNPEA3
200ng DNPEA3
500ng DNPEA3


B
Fold Reduction
120%
100%
80%
60%
40%
20%
0%
pCan
50ng DNPEA3
200ng DNPEA3
500ng DNPEA3

The *CD1* promoter has already been shown to be regulated by the Wnt-signaling pathway in HeLa cells (Tetsu and McCormick, 1999) and the Ras/MAPK pathway in normal rat intestinal epithelial cells (Filmus *et al.*, 1994), in mouse NIH 3T3 fibroblasts (Liu *et al.*, 1995) and in chinese hamster fibroblasts (Lavoie *et al.*, 1996). In order to establish a role for each of these independent pathways in Cos-1 cells, expression plasmids for some of their downstream effectors, PEA3, Lef-1, β-catenin and c-Jun, were co-transfected individually with the *CD1* reporter construct. The results of these experiments are depicted in Figure 19. Increasing amounts of each of the expression vectors for PEA3 and β-catenin gave a dose dependant increase in promoter activity up to 13-fold and 7-fold, respectively. By contrast, transfection of increasing amounts of the expression plasmids for Lef-1 and c-Jun had little effect on the activity of the *CD1* promoter (Fig. 19). Co-transfection of 0.6μg of all four transcription factors transactivated the promoter more than 30-fold (Fig. 19b, purple bar). This value is more than twice the sum of the effects observed from transfection of 0.6μg of each of the individual transcription factors: PEA3, 7.5-fold, β-catenin, 2-fold, Lef-1, 2.5-fold and c-Jun, 2-fold. This strongly suggests that these transcription factors act in synergy to transactivate the *CD1* promoter in Cos-1 cells.

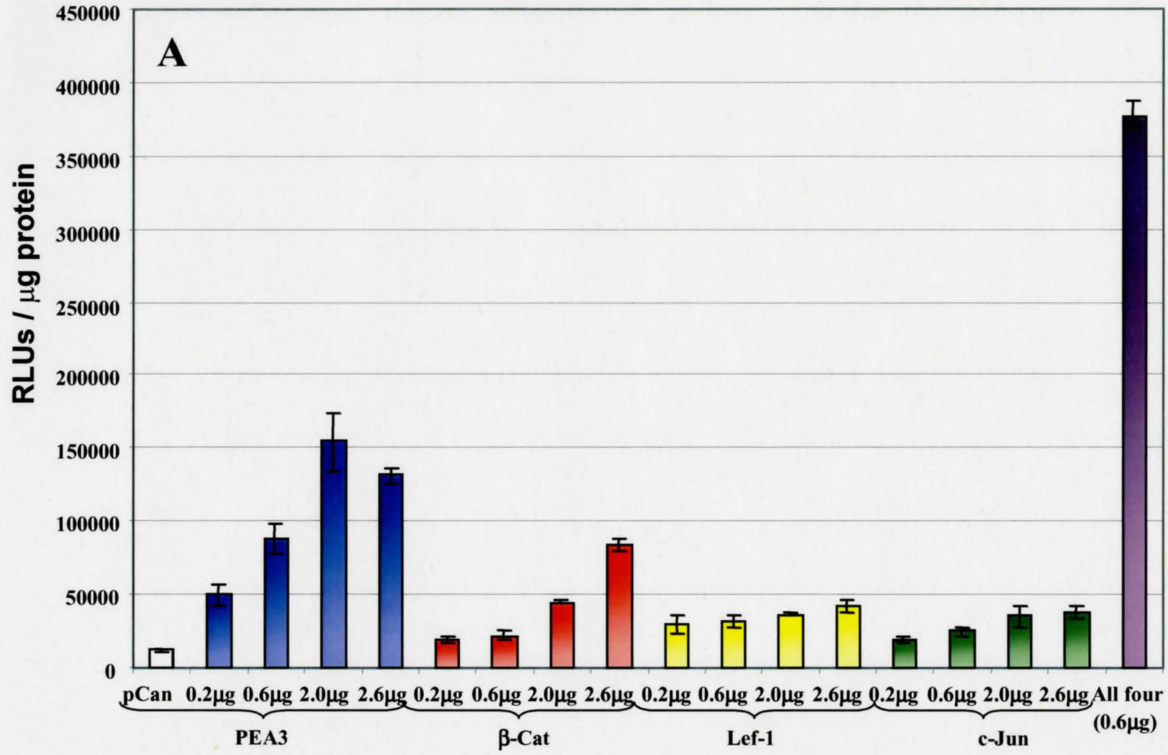
12. PEA3, β-catenin, c-Jun and Lef-1 Can Act Synergistically to Transactivate the *CYCLIN D1* Promoter

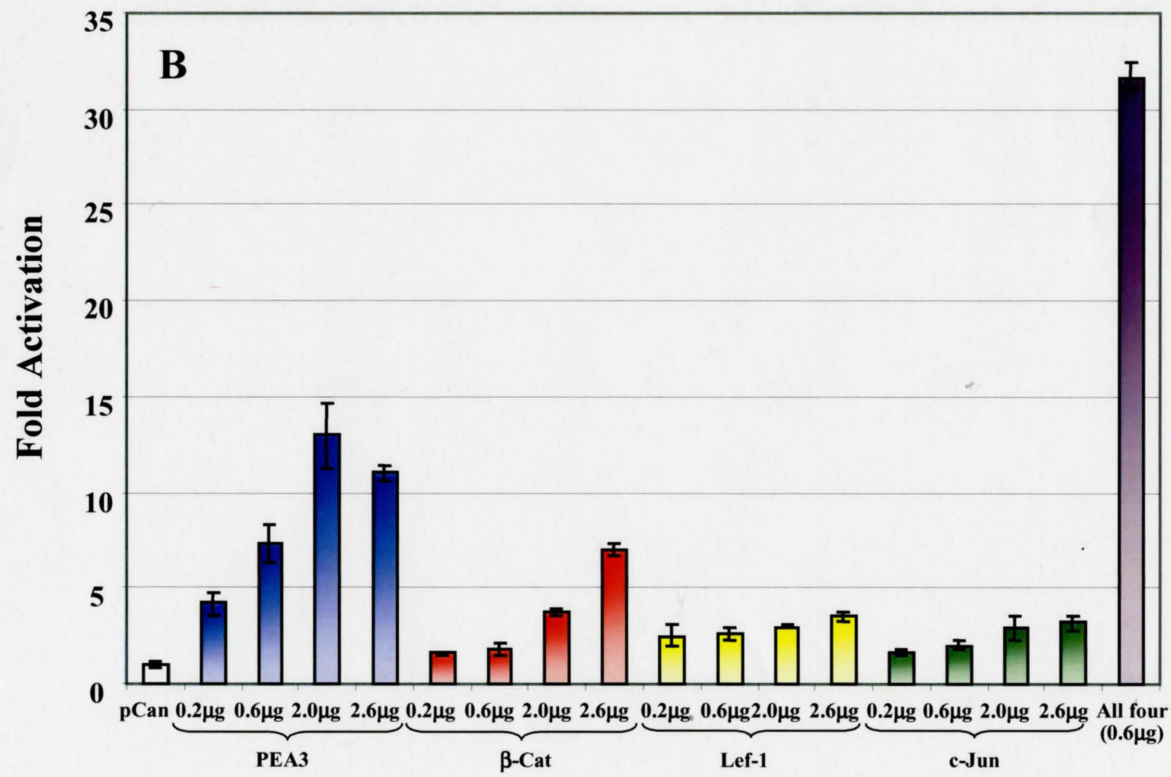
The finding that the transactivation potential of four transcription factors is greater than that of the sum of each of them individually raised an important question: Is the

Figure 19. **The *CYCLIN D1* Promoter is Susceptible to Transactivation by Other Transcription Factors**

A. 400ng of the –962CD1 promoter luciferase reporter construct was co-transfected into Cos-1 cells with increasing amounts of expression plasmids encoding PEA3, β-catenin, Lef-1 or c-Jun, as indicated. 0.6μg of all four effector plasmids were also co-transfected together. The total amount of DNA was kept constant using the empty expression vector (pCan). This experiment was performed in triplicate and is representative of three separate experiments.

B. The ratio of the activity of the reporter co-transfected with the indicated amount of expression plasmids over the reporter transfected alone is illustrated here.


A
RLUs / μg protein
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
pCan
0.2μg
0.6μg
2.0μg
2.6μg
0.2μg
0.6μg
2.0μg
2.6μg
0.2μg
0.6μg
2.0μg
2.6μg
0.2μg
0.6μg
2.0μg
2.6μg
All four (0.6μg)
PEA3
β-Cat
Lef-1
c-Jun


B
Fold Activation
35
30
25
20
15
10
5
0
pCan
0.2μg
0.6μg
2.0μg
2.6μg
0.2μg
0.6μg
2.0μg
2.6μg
0.2μg
0.6μg
2.0μg
2.6μg
0.2μg
0.6μg
2.0μg
2.6μg
All four (0.6μg)
PEA3
β-Cat
Lef-1
c-Jun

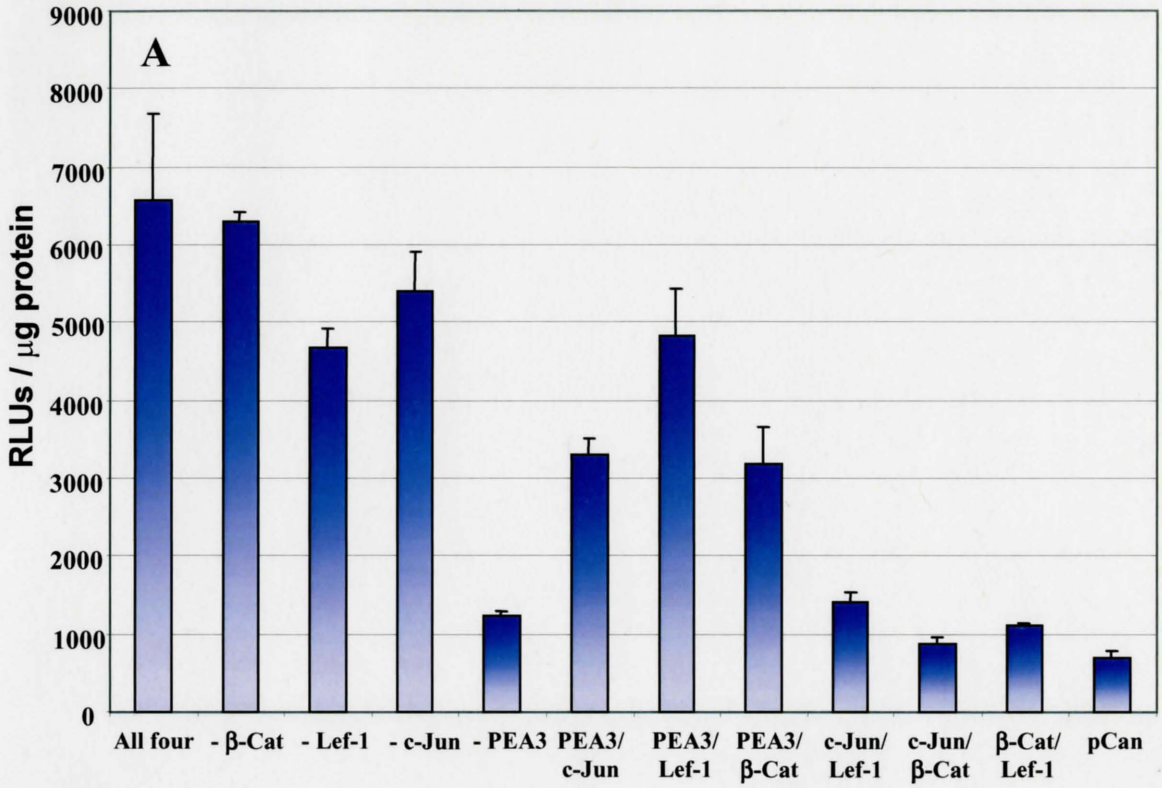
contribution of any of these transcription factors essential to this co-operation? In order to address this question, co-transfection experiments were performed in Cos-1 cells. PEA3, β-catenin, Lef-1 and c-Jun expression vectors were transfected together with the –962CD1 promoter reporter construct. In the same experiment, each of the transcription factors was withdrawn individually from the transfection mixture, to assess their effect on the co-operative transactivation of the promoter. Every possible pairwise combination of these factors was also assayed for the sake of thoroughness. The results of these experiments are illustrated in Figure 20.

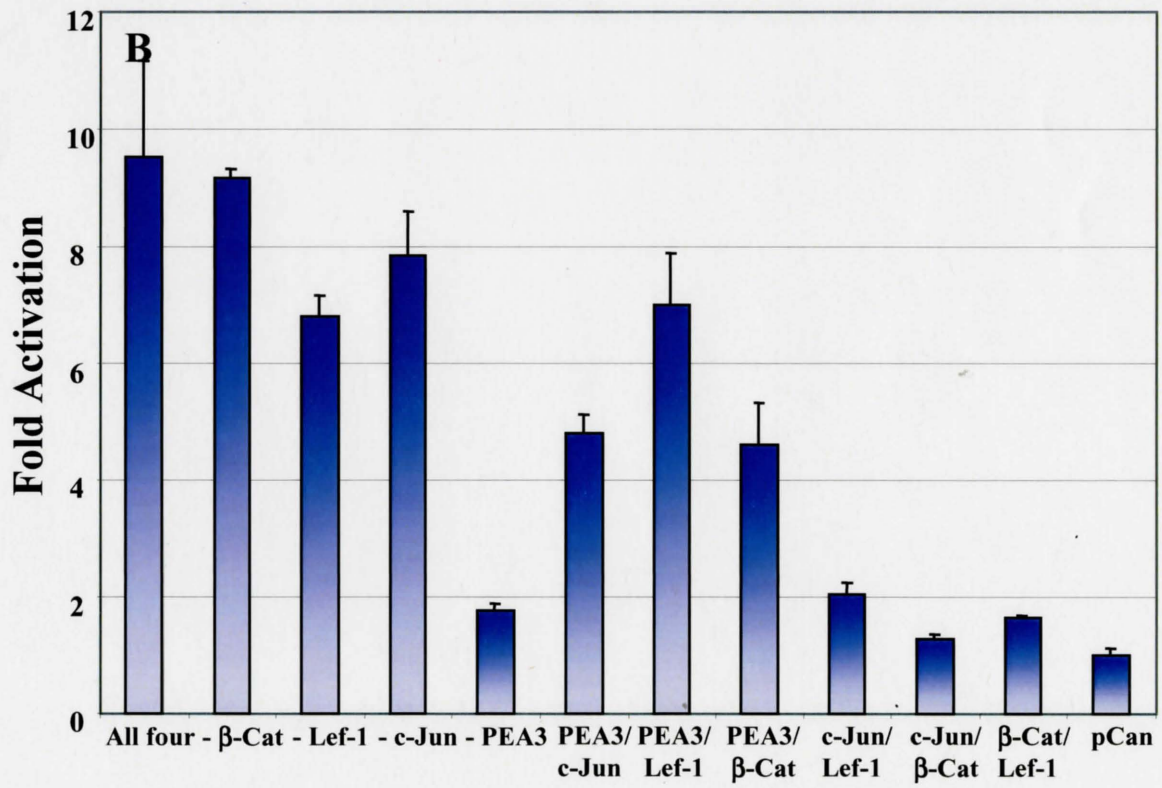
Interestingly, it seems that PEA3 is the essential element of the synergy observed between β-catenin, Lef-1, c-Jun and PEA3. Withdrawal of PEA3 from the transfection mixture causes the largest drop in transactivation of the *CD1* promoter, from 9.5-fold to less than 2-fold (Fig. 20b, compare All four with – PEA3). This strongly suggests that PEA3 is the limiting factor in the cooperative transactivation of the *CD1* promoter in this cell system. Also of interest is the fact that PEA3 and Lef-1 alone transactivated the promoter 7-fold. Introduction of β-catenin into the transfection mixture (Fig. 20b, -c-Jun) generated only a slight increase in transactivation of the *CD1* promoter, bringing it up to less than 8-fold. This suggests that β-catenin is present in sufficient amounts in Cos-1 cells to transactivate target promoters in the presence of Lef-1, and that introduction of additional β-catenin through the transfection of an expression vector is inconsequential. Another interesting observation arises by comparison of the 6 pairwise combinations that were assayed: PEA3/c-Jun, PEA3/Lef-1, PEA3/β-cat, c-Jun/Lef-1, c-Jun/β-cat, Lef-1/β-cat. All three combinations containing PEA3 transactivated the *CD1* promoter 5-fold or

Figure 20. **PEA3, β-Catenin, c-Jun and Lef-1 Can Act Synergistically to Transactivate the *CYCLIN D1* Promoter**

A. 400ng of the –962CD1 promoter luciferase reporter construct was co-transfected into Cos-1 cells with 0.6μg of expression plasmids encoding PEA3, β-catenin, Lef-1 and/or c-Jun, as indicated. 0.6μg of all four effector plasmids were also co-transfected together. The total amount of DNA was kept constant using the empty expression vector (pCan). This experiment was performed in triplicate and is representative of three separate experiments.

B. The ratio of the activity of the reporter co-transfected with the indicated expression plasmids over the reporter transfected alone is illustrated here.


A
RLUs / μg protein
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
All four
- β-Cat
- Lef-1
- c-Jun
- PEA3
PEA3/ c-Jun
PEA3/ Lef-1
PEA3/ β-Cat
c-Jun/ Lef-1
c-Jun/ β-Cat
β-Cat/ Lef-1
pCan


B
Fold Activation
12
10
8
6
4
2
0
All four
- β-Cat
- Lef-1
- c-Jun
- PEA3
PEA3/ c-Jun
PEA3/ Lef-1
PEA3/ β-Cat
c-Jun/ Lef-1
c-Jun/ β-Cat
β-Cat/ Lef-1
pCan

more, while the other three combinations gave a maximum transactivation of 2-fold. This provides further evidence to suggest that PEA3 is an essential element in the transactivation of the *CD1* promoter in Cos-1 cells.

13. The ETS B Site in the *CYCLIN D1* Promoter Is Essential for Promoter Function

The *CD1* promoter contains four putative ETS binding sites (Fig. 6 and Fig. 21a). In order to determine which binding site(s) were mainly responsible for PEA3 transactivation of the *CD1* promoter, site directed mutants of the ETS binding sites were used: ETS A mt, ETS B mt, ETS C mt and ETS D mt. The first three constructs were kindly provided by Dr. McCormick's laboratory (Tetsu *et al.*, 1999), while the fourth was isolated in our laboratory. Site-directed mutagenesis was used to mutate the EBS D as outlined in Methods.

Each of the reporter constructs was transfected in Cos-1 cells, either alone or with increasing amounts of the PEA3 expression plasmid (Fig. 21b and c). As observed from Figure 21b, the EBS B was the most important for basal activity of the promoter. The basal activity (1000ng pCan) of the ETS B mutant reporter construct was significantly lower than any of the other promoter luciferase constructs, and was in fact comparable to that of the promoter-less reporter plasmid, pGL3 basic. The ETS B site also seems to be important for transactivation by PEA3, as illustrated by the transactivation of this construct by increasing amounts of PEA3 in comparison to the other constructs (wild type and EBS mutants A, C and D). The wild type promoter was transactivated up to 14-fold with 1000ng of PEA3 expression plasmid (Fig. 21c). The EBS mutants A, C and D

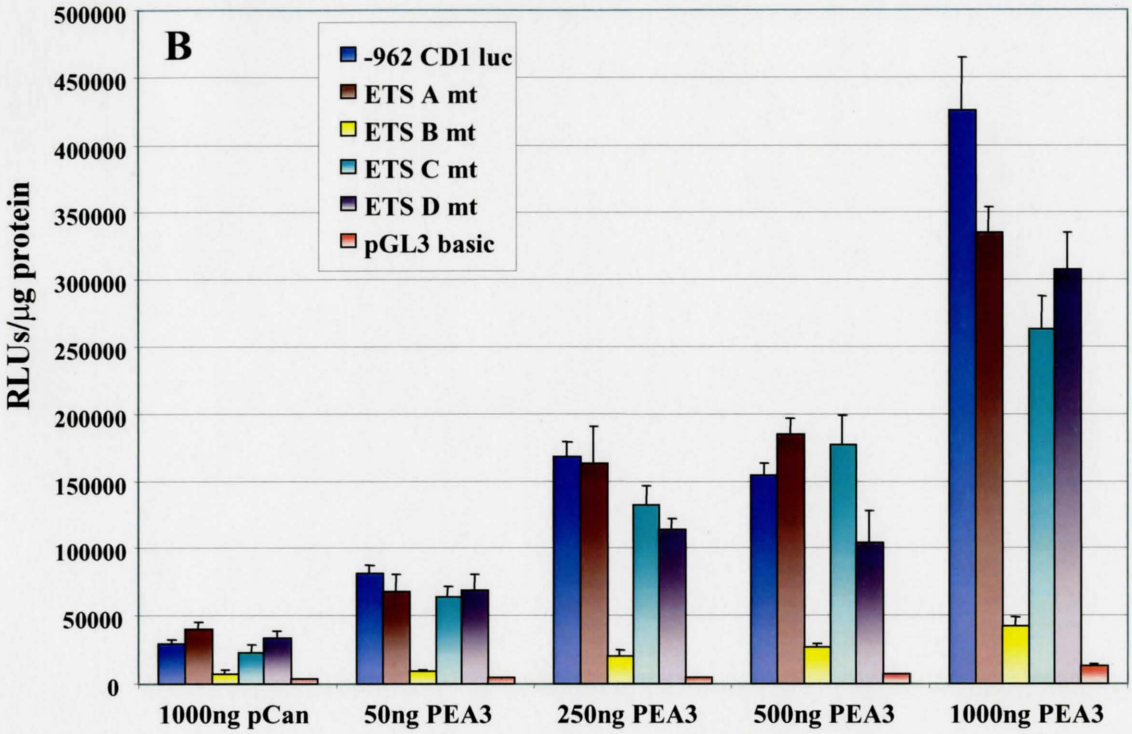
Figure 21. **The ETS B site in the *CD1* promoter is Essential for Promoter Function**

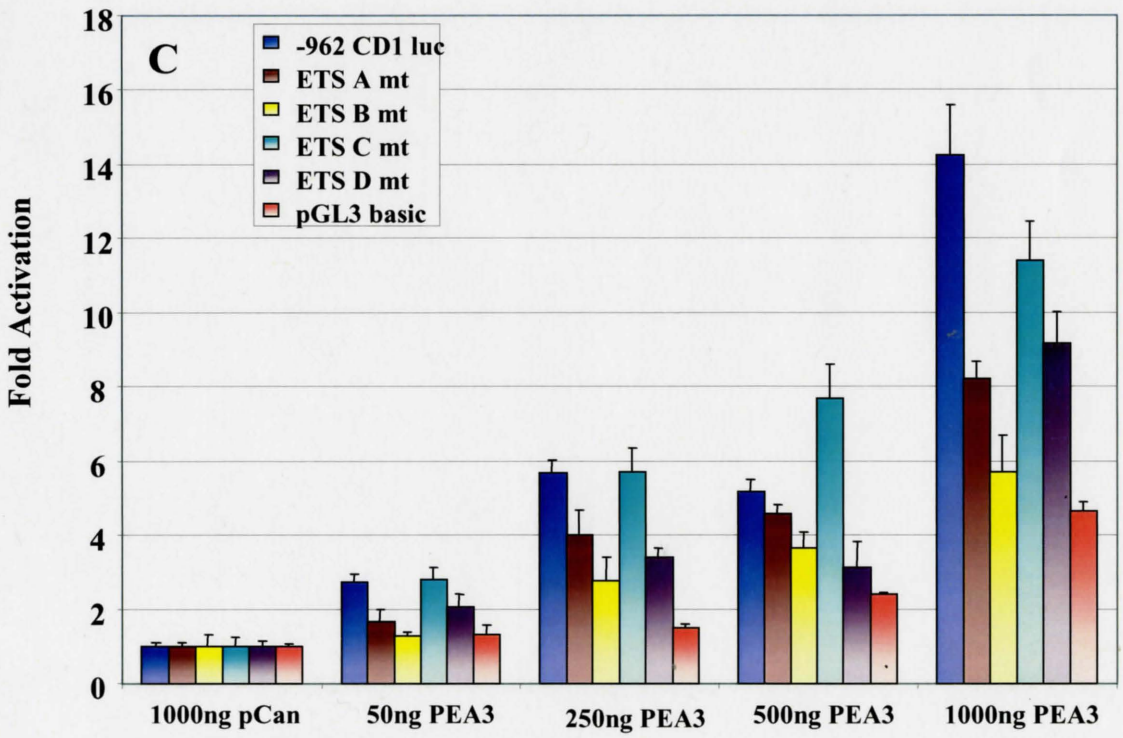
A. Sequence of the *CD1* promoter from –962 + 134 and illustration of the putative ETS binding sites, ETS A, ETS B, ETS C and ETS D. Numbers above the sequence refer to the position relative to the transcription start site (+1).

B. 100ng of the –962CD1 promoter luciferase reporter construct, or one of the four EBS mutants, was co-transfected into Cos-1 cells with increasing amounts of an expression plasmid encoding PEA3, as indicated. The total amount of DNA was kept constant by addition of empty expression vector (pCan). A dose-dependant increase can be observed in all cases except for the B mutant and the empty reporter control (pGL3 basic). This experiment was performed in triplicate and is representative of three separate experiments.

C. The activity of each reporter construct transfected alone was set to 1 and the effect of PEA3 co-transfection illustrated as fold activation.


A
-962
ETS A
ETS B
-750
ETS D
ETS C
-300
-70
+1
+134


B
-962 CD1 luc
ETS A mt
ETS B mt
ETS C mt
ETS D mt
pGL3 basic
RLUs/μg protein
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
1000ng pCan
50ng PEA3
250ng PEA3
500ng PEA3
1000ng PEA3


C
-962 CD1 luc
ETS A mt
ETS B mt
ETS C mt
ETS D mt
pGL3 basic
Fold Activation
18
16
14
12
10
8
6
4
2
0
1000ng pCan
50ng PEA3
250ng PEA3
500ng PEA3
1000ng PEA3

were slightly impaired in their capacity to be transactivated by PEA3, with maximum transactivation levels of 8- to 11-fold. However, the EBS B mutant was transactivated less than 6-fold, which is comparable to the transactivation levels of the pGL3 basic vector control (Fig 21c). This ability of the pGL3 basic vector to be transactivated by transcription factors is not uncommon and has been analysed in detail (Amanatullah *et al.*, 2001). The results in Figure 21 strongly suggest that even though the other three EBS in the *CD1* promoter likely contributed to the responsiveness of the promoter to PEA3, the ETS B site is the most important, both for basal promoter activity and for transactivation by PEA3. This observation corroborates the hypothesis stipulated by Dr. McCormick's laboratory that the ETS B site is important for transactivation by β-catenin in HeLa cells (Tetsu and McCormick, 1999). This particular DNA element may be central to the proper regulation of *CD1* transcription levels *in vivo*.

14. The ETS B Site in the *CYCLIN D1* Promoter is Essential for Co-operation of PEA3 with the Other Transcription Factors

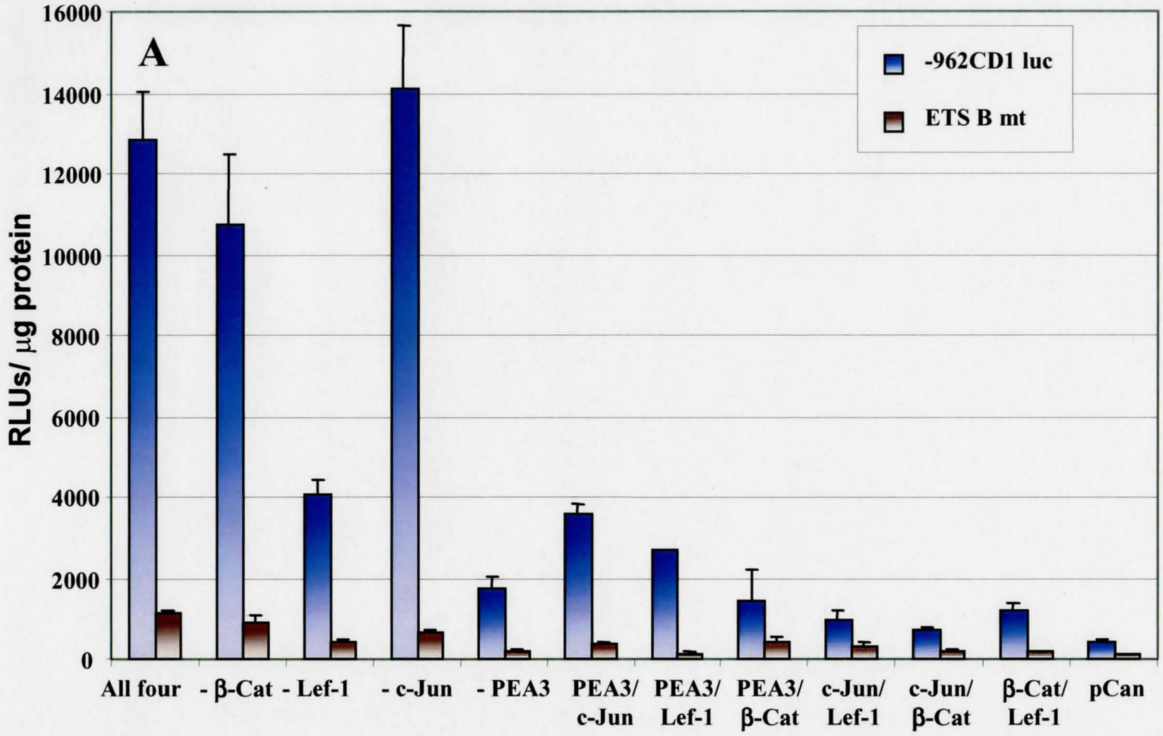
The discovery that the ETS B site in the *CD1* promoter was essential for PEA3 transactivation of the promoter raised yet another question: Is the ETS B site essential for co-operation between PEA3, β-catenin, Lef-1 and c-Jun? To assess this, co-transfection experiments were performed using the ETS B mt luciferase reporter in parallel with the wild type –962 CD1 luc. Expression plasmids for each of the four transcription factors, PEA3, β-catenin, Lef-1 and c-Jun, were co-transfected with the reporter costructs in Cos-1 cells. The same combinations of transcription factors were used as previously (Fig. 20),

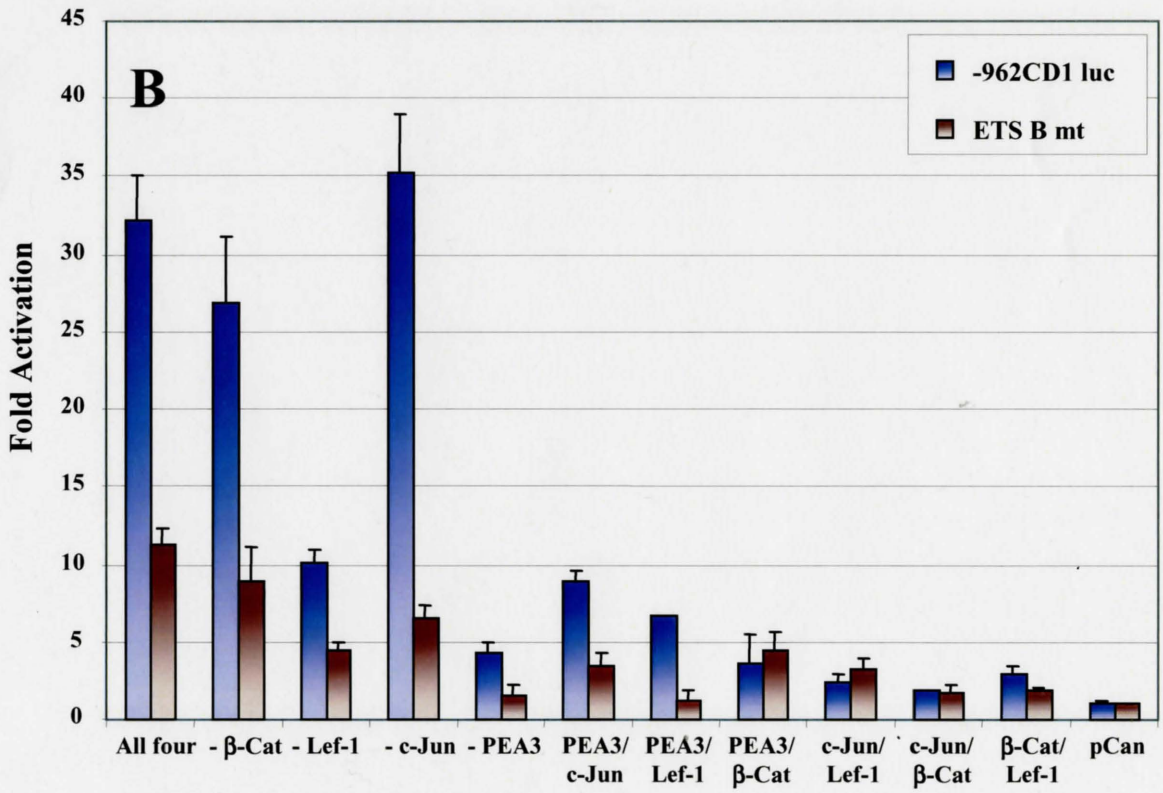
to allow for comparison. As can be observed from Figure 22, the B site mutant was severely impaired in its capacity to be transactivated by the combination of all four of these transcription factors. The wild type promoter was transactivated more than 30-fold, whereas the B mt was transactivated about 11-fold (Fig. 22b). Withdrawal of β-catenin or c-Jun from the transfection mixture had little effect on transactivation of the *CD1* promoter, as previously observed (Fig. 20). Withdrawal of Lef-1 from the transfection mixture had a more pronounced effect on transactivation of the promoter in this particular experiment. This kind of variation is common and can be attributed to small differences in experimental conditions between experiments, even though efforts were made to avoid such variations (see Methods). Withdrawal of PEA3 from the transfection mixture still generated the greatest decrease in transactivation of the *CD1* promoter: from 32-fold to 4-fold for the wild type promoter and from 11-fold to 1.6-fold for the ETS B site mutant promoter. This suggests that even in the context of the B site mutant, PEA3 is an important transcription factor for the transactivation of the *CD1* promoter in Cos-1 cells. This is also reflected by the capacity of PEA3 to co-operate with c-Jun and β-catenin to transactivate the ETS B site mutant promoter by 3.5- and 4.5-fold, respectively. These transactivation levels of the ETS B site mutant promoter were comparable to those achieved for the wild type promoter in the presence of PEA3 and c-Jun, and PEA3 and β-catenin (Fig. 22b) in certain experiments. These observations provide further evidence that PEA3 is an essential activator of the *CD1* promoter. Also, although the EBS B site may be the key DNA element in *CD1* promoter regulation, the other three EBS likely contributes to PEA3 transactivation and co-operation with β-catenin, Lef-1 and c-Jun.

Figure 22. **The ETS B site is Essential for Promoter Responsiveness to Different Transcription Factors**

A. 400ng of the –962CD1 promoter luciferase reporter construct (blue series), or the ETS B mt (red series), was co-transfected into Cos-1 cells with 0.6μg of all four effector plasmids. Each transcription factor was left out individually to assess its contribution to promoter activity. All the pair-wise combinations of transcription factors were also assayed. The total amount of DNA was kept constant by addition of empty expression plasmid (pCan). This experiment was performed in triplicate and was repeated with similar results.

B. The ratio of the activity of the reporter co-transfected with the indicated expression plasmids over the reporter transfected alone is illustrated here.


A
-962CD1 luc
ETS B mt
RLUs/ μg protein
16000
14000
12000
10000
8000
6000
4000
2000
0
All four
- β-Cat
- Lef-1
- c-Jun
- PEA3
PEA3/ c-Jun
PEA3/ Lef-1
PEA3/ β-Cat
c-Jun/ Lef-1
c-Jun/ β-Cat
β-Cat/ Lef-1
pCan


B
-962CD1 luc
ETS B mt
Fold Activation
45
40
35
30
25
20
15
10
5
0
All four
- β-Cat
- Lef-1
- c-Jun
- PEA3
PEA3/ c-Jun
PEA3/ Lef-1
PEA3/ β-Cat
c-Jun/ Lef-1
c-Jun/ β-Cat
β-Cat/ Lef-1
pCan

DISCUSSION

The objectives of this project were successfully completed. The sequences in the *pea3* promoter that are responsible for induction of promoter activity by the Ras/MAPK pathway will be discussed. The mechanisms of transactivation of the *CD1* promoter and the DNA sequences that are essential for it to occur will also be examined. Finally, the possible interactions between the different elements of the Ras/MAPK and Wnt-signaling pathways that are involved in regulation of *CD1*, and how they might co-operate in cases such as normal cell proliferation and oncogenesis will be outlined.

1. Analysis of the *pea3* Promoter

The influence of the Ras/MAPK signaling pathways on the *pea3* promoter, and the DNA sequences required for these effects to occur, were determined using co-transfections, transfection/induction experiments and luciferase assays. These methods were invaluable in that they allowed for relatively fast and efficient assay of a large number of different conditions simultaneously.

a. Influence of the Ras/MAPK Pathway on the *pea3* Promoter

The observation that *pea3* mRNA levels are increased following Raf-1 activation in NIH 3T3 cells led to two possible hypotheses: the stability of the mRNA was increased

or the rate of transcription of the *pea3* gene was accelerated. To test the first of these hypotheses would require run-on assays, which are both time-consuming and necessitate the use of radioactive isotopes. The second theory is a lot simpler to verify, since it involves reporter gene assays. These types of assays usually yield reliable results in a timely fashion.

pea3 promoter reporter gene constructs were used in transfection/induction assays to determine whether Raf-1 activation could increase transcription of the luciferase reporter gene. A series of deletion mutants of the *pea3* promoter were used in an attempt to identify DNA sequences responsible for the effect. All the reporter constructs tested that contained sequences up to –256 were reproducibly activated 6- to 10-fold by Raf-1 (Figures 12b and 13b). Interestingly, deletion of the putative RRE in the promoter led to a 2- to 3-fold decrease in the activation of the promoter by Raf-1. Confirmation of the involvement of these sequences in the responsiveness of the *pea3* promoter to Raf-1 was provided by point mutations of the two DNA sequences forming the RRE: AP-1 and ETS. The analysis of these mutant reporter constructs gave results similar to those obtained with the deletion mutants. Interestingly, there is a residual 2-fold induction by Raf-1 activation of the –156+21 construct and a residual 1.5-fold induction of the –100+21 luc construct. Both these promoter fragments lack the RRE. The presence of the AP-1 binding site at –107 (see Fig. 5) is a possible explanation for the effect of Raf-1 activation on the –156+21 luc construct. In addition, it is possible that some other poorly conserved ETS and/or AP-1 transcription factor binding sites, not depicted on Figure 5, are also responsible for the low level of induction observed with these shorter constructs.

Finally, the effect of Raf-1 activation could be indirect, inducing expression of another transcription activator, such as NFκB for example (Lee *et al.*, 1997) which in turn would bind to the *pea3* promoter and activate transcription of the luciferase reporter gene. This hypothesis is supported by the evidence of a need for protein synthesis to induce *pea3* expression, as demonstrated by the nothern blotting experiment using cycloheximide (Fig. 10). More work would be needed to discern between these hypotheses.

Given that AP-1 family members (Cooper, G.M., 1995) and ETS family members (Marais *et al.*, 1993; Yang *et al.*, 1996), are downstream targets of the Ras/MAPK pathway, further evidence of the involvement of this pathway in regulation of the *pea3* promoter was provided by the co-transfection experiments performed in McMA cells using PEA3 and c-Jun effector plasmids. Co-transfection of c-Jun effector plasmid with the *pea3* promoter luciferase construct transactivated the promoter up to 14-fold (Fig. 16b). However, an attempt to determine whether the RRE was the DNA element that mediated the effect of c-Jun on the *pea3* promoter yielded ambiguous results. The deletion analysis (Fig. 16b and 16c) results pointed to a crucial role of the RRE in the *pea3* promoter's capacity to respond to c-Jun transactivation. Deletion of 100bp around the RRE, from –256 to –156, abolished *pea3* promoter responsiveness to c-Jun (Fig. 16c). However, an attempt to confirm the role of the ETS and/or AP-1 binding sites in the ability of the *pea3* promoter to respond to c-Jun transactivation, through the use of the fine deletion and point mutations, yielded ambiguous results (Fig. 17). The observation that deletion or mutation of the AP-1 binding site in the RRE potentiated the ability of the *pea3* promoter to respond to c-Jun transactivation led to the hypothesis that c-Jun may act

in part as a repressor of *pea3* transcription. It is possible that, in the absence of MAPK signaling, the levels of *pea3* transcription are tightly regulated through a combination of inducive and repressive signals. Recently, the role of c-Jun as a transcriptional repressor has been unveiled. Several mechanisms by which c-Jun can act to suppress transcription have been described. c-Jun has been shown to physically interact with the corepressor Ski to stabilize the Smad2/Ski repressor complex, thereby actively participating in repression of Smad2 regulated genes in the absence of TGF-β signaling (Pessah *et al.*, 2002). c-Jun was also shown to interact with the corepressor TG-interacting factor (TGIF) to suppress Smad2 transcriptional activity, by preventing Smad2 association with p300 (Pessah *et al.*, 2001). In the case of the 5-aminolevulinate synthase (*ALAS*) promoter, it appears that induction of AP-1 by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) reduces transcription of the *ALAS* gene by impinging on the capacity to assemble the productive pre-initiation complex, including CBP, through sequestration of a limited amount of the coactivator (Guberman *et al.*, 2003). Thus, whether it would be through binding to a corepressor or through sequestration of CBP, it is possible that c-Jun acts as a repressor at the RRE in the absence of growth factor signaling, or Raf-1 induction. More work needs to be done to verify this hypothesis and to determine the mechanism by which c-Jun repression occurs. It also would be interesting to determine whether mutations in the endogenous mouse *pea3* promoter, at the ETS and/or AP-1 binding sites similar to those introduced in our laboratory, are responsible for increased expression of *pea3 in vivo*.

Members of the AP-1 family of transcription factors have previously been shown to act in synergy with Ets factors to transactivate promoters through an RRE (reviewed in Li *et al.*, 2000). Since both c-Jun (Fig. 16) and PEA3 (not shown) had previously been shown to up-regulate the *pea3* promoter, it was plausible that the two proteins could co-operate to transactivate the *pea3* promoter. This effect of synergy had been previously demonstrated in Cos-1 cells in our laboratory (not shown). Interestingly, c-Jun and PEA3 were able to co-operate to potentiate Raf-1 activation of the *pea3* promoter, from 6-fold with Raf-1 activation alone to almost 12-fold with optimal amounts of PEA3 and c-Jun (Fig. 18b). This strongly suggests that these two transcription activators are responsible for mediating Raf-1 induction of the *pea3* promoter. To determine whether the RRE is the DNA element responsible for the effect of c-Jun and PEA3 on induction of the *pea3* promoter by activated Raf-1, the deletion and site mutant reporter constructs would have to be tested in similar experiments. Also, comparison with other ETS factors should be made to determine if PEA3 is the favored ETS protein for this effect. *In vitro* DNA binding assays could be performed as well to confirm binding of the optimal ETS factor to the EBS in the RRE. Alternatively, chromatin immunoprecipitation experiments could be attempted, to identify the ETS protein(s) and other transcription factors that bind to the *pea3* promoter *in vivo* (Orlando, 2000).

b. Influence of Other Factors on the *pea3* Promoter

There is several other transcription factor binding sites in the *pea3* promoter sequence as it is depicted in Figure 5. The influence of most of these transcription factors on

transcription levels of the *pea3* gene has yet to be established. Preliminary evidence in our laboratory points to the involvement of the highly conserved Lef-1/TCF binding sites at –254 and –90 (Kann, G.S., 1999) in β-catenin regulation of the *pea3* promoter (not shown). It is possible to formulate hypotheses on how some of the other transcription factor binding sites could regulate the *pea3* promoter in certain cells. For example, the presence of an AP-2 putative binding site is of interest since the levels to AP-2 transcription factor is higher in all cell lines overexpressing c-*erb*B-2 (Bosher *et al.*, 1995). Since the c-*erbB*-2 promoter has also been shown to be upregulated by Ets factors (Scott *et al.*, 2000), it is possible that AP-2 and Ets proteins act together in regulating transcription from the *pea3* and c-*erb*B-2 promoters. Preliminary studies have shown that the AP-2 family of transcription factors were able to transactivate the *pea3* promoter in the HepG2 cell line, which expresses low levels of AP-2 family members endogenously (Barrett, 1997). Other assays such as DNase footprinting or electromobility shift assays (EMSAs) would need to be performed to confirm the putative AP-2 binding site as a *bona fide* regulatory element.

The presence of a tandem of putative NFAT/NFκB binding sites between –50 and –25 in the *pea3* promoter sequence suggests an interesting theory. Cooperation between nuclear factor of activated T cells (NFAT) and nuclear factor κB (NFκB) family members have been previously observed on the human immunodeficiency virus (HIV) enhancer (Bassuk *et al.*, 1997), the interferon-γ promoter (Sica *et al.*, 1997) and T cell receptor-CD3 (Badran *et al.*, 2002). Also, members of the NF-kB family have been shown to work synergistically with members of the AP-1 (Stein *et al.*, 1993) and Ets

(Thomas *et al.*, 1997) families of transcription factors to transactivate the 5' long terminal repeat of HIV type 1 and the human granulocyte-macrophage colony stimulating factor (GM-CSF), respectively. The presence of putative binding sites for each of these different transcription factors in the *pea3* promoter suggest that a similar cooperation could take place in the appropriate cellular environment. Co-transfection experiments using expression plasmids encoding members of the NFAT and NF-κB families along with the luciferase reporter gene linked to the *pea3* promoter could verify this hypothesis.

There are three putative myb binding sites in the *pea3* promoter sequence, between –100 and +21. The role of c-*myb* as an important regulator in hematopoiesis has been established for a few years (reviewed in Friedman, 2002). There are two other members of the Myb family, A-*myb* and B-*myb*, which share DNA binding sequence specificity with c-*myb*. While c-*myb* is the most extensively studied of the three family members, emerging evidence points to an important role for A-*myb* and B-*myb* in proliferation, differentiation and oncogenesis (reviewed in Oh and Reddy, 1999). A-*myb* has been shown to be essential for effective mammary tissue proliferation following pregnancy in mice (Toscani *et al.*, 1997). Given that A-*myb* is cell cycle regulated and phosphorylated by co-expression of cyclin A or cyclin E (Ziebold and Klempnauer, 1997), it ensues that A-*myb* is an attractive candidate for upregulation of *pea3* during mammary gland development. It would be interesting to determine the exact role of the Myb family members in regulation of the *pea3* promoter.

There are two putative binding sequences for hepatic nuclear factor 3β (HNF3β) in the –356 to –256 region of the *pea3* promoter. Since deletion of this region causes an

increase in basal promoter activity in NIH 3T3 cells, it is possible that it contains transcription repressor binding sites. Recent studies point to an inhibitory role for HNF3β in the regulation of the tyrosine aminotransferase promoter (Merkulova *et al.*, 2003) and nuclear hormone receptor-dependent hepatitis B virus replication (Tang *et al.*, 2002). Further analysis of the –356 to –256 region of the *pea3* promoter would be necessary to determine the exact influence of the two putative HNF3β sites in this region.

Since *pea3* is expressed in several stages of development (Chotteau-Lelievre *et al.*, 2001; Raible and Brand, 2001; Munchberg *et al.*, 1999; Chotteau-Lelievre *et al.*, 1997) and in several different tissues of the embryo and adult organisms (Shepherd and Hassell, 2001; Taylor *et al.*, 1997), it is likely that several different pathways and effectors have the potential to regulate *pea3* expression in a cell-specific and timely fashion. A role for the fibroblast growth factor (FGF) family of signaling molecules in regulating the expression of *pea3* family members is already established. FGF8 was shown to be required for normal *pea3* and *erm* expression in early zebrafish development (Raible and Brand, 2001) and in chick nasal mesenchyme (Firnberg and Neubuser, 2002). Recent studies point to a role for FGF7 in upregulating *pea3* and *erm* expression in early lung development, and contributes to maintaining the pool of endodermal progenitor cells (Liu *et al.*, 2003). A role for the Wnt-signaling pathway in regulation of *pea3* expression is also becoming clear (Howe *et al.*, 2001). Nonetheless, a lot more work needs to be done to fully understand the mechanisms that govern *pea3* expression *in vivo*.

2. Analysis of the *CYCLIN D1* Promoter

The influence of different transcription activators on the *CYCLIN D1* promoter was determined using co-transfection experiments and luciferase assays. These methods were invaluable in that they allowed for relatively fast and efficient assay of a large number of different conditions simultaneously.

a. Influence of PEA3 on the *CYCLIN D1* Promoter

There are four Ets binding sites (EBS) in the *CD1* promoter (Fig. 7). Since both *pea3* and *CD1* levels are increased in tumor compared to normal tissues, it was hypothesized that *CYCLIN D1* is a PEA3 target gene. Co-transfection experiments in Cos-1 and FM3A cells using the *CD1* promoter linked to a luciferase reporter gene showed that PEA3 can transactivate this promoter (Fig. 19) and that DN-PEA3 can suppress its activity (Fig. 20). Moreover, the repression effect of DN-PEA3 on the *CD1* promoter is even more pronounced in FM3A cells (not shown), which express relatively high levels of *pea3* mRNA (Xin *et al.*, 1992), suggesting that both proteins may compete directly for binding to the promoter DNA elements (Shepherd *et al.*, 2001). This observation introduces a novel way in which PEA3 can act as an oncogene, as well as an important developmental regulator, through the activated transcription of a powerful cell cycle regulator such as *CYCLIN D1.*

b. Influence of PEA3, β-Catenin, Lef-1 and c-Jun on the *CYCLIN D1* Promoter

CYCLIN D1 expression is regulated by a variety of signaling pathways. The *CYCLIN D1* promoter has been shown to respond to $p21^{ras}$ in transfection experiments in human trophoblasts (JEG-3 cells, Albanese *et al.*, 1995). Liu *et al.* showed that transformation

of mouse NIH 3T3 fibroblasts by Ras led to elevated levels of *CYCLIN D1* and acceleration of G1 progression (Liu *et al.*, 1995). *CYCLIN D1* was also shown to be required for transformation of rat-1 fibroblasts by *neu*T, and for tumorigenesis induced by MMTV-*neu*T NAFA mouse mammary epithelial tumor cells injected in nude mice (Lee *et al.*, 2000). The *CYCLIN D1* promoter was also shown to be responsive to co-transfected β-catenin and Lef-1 in 293T cells (Shtutman *et al.*, 1999). In addition, the Wnt and MAPK pathways have been shown to co-operate to promote transformation and *CYCLIN D1* mRNA accumulation in mouse NIH 3T3 fibroblast cells (Rimerman *et al.*, 2000) and in HeLa cells (Tetsu and McCormick, 1999). It was therefore tempting to ask whether the downstream effectors of the Wnt-signaling pathway, β-catenin and Lef-1, and of the MAPK pathway, c-Jun, could act in synergy with PEA3 to transactivate the *CD1* promoter linked to a luciferase reporter gene.

The results of co-transfection experiments presented here clearly show that cooperation between the four transcription factors exists, and that PEA3 is the base on which the activation of the promoter resides. Removing the PEA3 expression vector from the transfection mixture led to a large decrease in the activation potential of the luciferase gene, from 9-fold to less than 2-fold in one experiment (Fig. 22b) and from 32-fold to 4-fold in a separate experiment (Fig. 24b). Subtraction of any of the other three transcription activators had little effect on promoter activity in comparison. These results are similar to those obtained in similar experiments involving the matrilysin promoter (*MMP*-7, Crawford *et al.*, 2001) in which PEA3, or one of its subfamily members, ERM or ER81, was sufficient to induce high levels of transcription from the *MMP*-7 promoter,

and required for synergy with β-catenin, Lef-1 and c-Jun. More experiments are needed to determine the role of ERM and ER81 in the regulation of the *CD1* promoter. It is possible that the *CD1* and *MMP*-7 promoters are regulated in a similar fashion to insure the concerted expression of these two genes during certain phases of embryonic development or organogenesis, when both cellular proliferation and migration are required (Reviewed in Moscoso, 2002). It would be interesting to see how many more genes are transactivated synergistically by PEA3, β-catenin, Lef-1 and c-Jun. Potential targets would be other matrix remodelling enzymes and cell cycle regulators.

c. Mutational Analysis of the *CYCLIN D1* Promoter

Analysis of the 4 EBS mutants of the *CD1* promoter in transfection experiments in Cos-1 cells revealed that the EBS B is critical both for the basal activity of the promoter and for its activation by PEA3 (Fig. 28). Also, the same DNA sequence was shown to be important for co-operative activation of the *CD1* promoter by PEA3, β-catenin, Lef-1 and c-Jun. The importance of this site had previously been identified, both for transactivation by β-catenin and for RasV12 activation in HeLa cells (Tetsu and McCormick., 1999).

Even though binding of PEA3 protein to the B site is weak in electromobility shift assays (EMSA, not shown and Leidal, A., personal communications), it is detectable. This suggests that PEA3 is indeed capable of transactivating the *CD1* promoter through this site, albeit it may not be the ideal activator of this gene's expression. Also, several Ets-transcription factors often require the formation of multi-protein complexes to achieve higher specificity and affinity DNA binding (reviewed in Verger and Duterque-

Coquillaud, 2002). This process, called combinatorial control, is one of the ways by which the highly homologous Ets DNA-binding domains can regulate distinct promoters in a timely and efficient fashion. It is therefore possible that an appropriate co-activator for PEA3 is required, along with the additional DNA sequences, for efficient and readily detectable binding of PEA3 to *CD1* sequences *in vitro*.

Ets-2 has also been shown to transactivate the *CD1* promoter through a site in the proximal promoter region (Albanese *et al.*, 1995). In co-transfection experiments using a variety of Ets family members, Fli-1 was shown to transactivate the *CD1* promoter over 60-fold in Cos-1 cells (not shown). The same expression plasmid encoding Fli-1 had little effect on two other promoters (*stromelysin* and *pea3*), suggesting that the effect on the *CD1* promoter may be specific. More experiments would need to be designed to determine the comparative levels of expression of each Ets protein, and calculations of specific activity would allow a better assessment of the role of Fli-1, and other Ets proteins, in *CYCLIN D1* regulation.

3. Link Between Regulation of *pea3* and *CYCLIN D1*

Oncogenesis is usually the result of a number of different genetic mutations that contribute to uncontrolled cell growth and, eventually, cell invasion and metastasis. It is possible to imagine a scenario in which the results presented here would fit together like pieces in the large puzzle of tumor formation. For example, *neu* dysregulation could lead to *pea3* overexpression and trigger a feedback loop to generate more Neu protein. A second mutation could target the Wnt-signaling pathway or the Ras/MAPK signals to

induce more *pea3* expression. Eventually, a level of PEA3 sufficient to activate other genes, such as *CYCLIN D1,* would be achieved. Overexpression of *CD1* would result in cell cycle acceleration, leading to cellular proliferation. Obviously, this particular hypothesis is likely oversimplified and the actual events probably involve a multitude of other factors. Moreover, the situation is almost certainly unique for each type of tumors.

Other involvements of *cyclin d1* and *pea3* in carcinogenesis have been described. In a murine leukemic model, *cyclin d1* overexpression occurred at the initiation of tumoral development (Yerly-Motta *et al.*, 1999). In chondrosarcoma of the jaws, *CD1* was upregulated in 75% of cases (Si and Liu, 2001). Increased expression of *cyclin d1* was also detected in 34% of hepatocellular neoplasms in mice (Anna *et al.*, 2003). This strongly points to an important role for *CYCLIN D1* in a variety of malignancies and a better understanding of the mechanisms by which it becomes overexpressed could lead to the design of new treatment or preventative cancer therapies. Recently, involvement of PEA3 in ovarian carcinoma has been described (Davidson *et al.*, 2003a; Davidson *et al.*, 2003b). Expression of *PEA3* mRNA was detected in 92% of ovarian carcinomas and more often in carcinomas of short-term survivors. This suggests a poor prognosis associated with the overexpression of *PEA3* in these tumors (Davidson *et al.*, 2003a). Also, the expression of several matrix metalloproteinases was detected in a large number of primary and metastatic advance stage ovarian carcinomas that express *PEA3* (Davidson *et al.*, 2003b). These results point to an important role for *PEA3* in tumor progression and metastasis and warrant the continued study of *PEA3* expression in order to allow the design of preventive and curative therapies that target *PEA3* in cancer cells.

Some evidence of the involvement of PEA3 in regulation of *CYCLIN D1* expression also came from microarray experiments performed using RNA extracted from a HEK293 cell line engineered to inducibly express DN-PEA3 (DN3). In four separate experiments involving three different RNA isolates, *CD1* mRNA levels were decreased to an average of 0.69 ± 0.13, after 24 hours of DN-PEA3 induction (Peters J.R., personal communication). In infection experiments using an adenovirus coding for DN-PEA3, the levels of *CD1* mRNA in HEK 293 cells was significantly decreased by DN-PEA3, as ascertained by Light Cycler experiments (Vaz, D., personal communication). These results give some evidence to support the hypothesis that there is a direct link between regulation of *pea3* and *CD1*. A lot more work needs to be done before light is shed on the exact role of both *pea3* and *CYCLIN D1* in tumor formation.

CONCLUSIONS

The influence of the elements of the Ras/MAPK pathway on the *pea3* promoter was determined, using transfection/induction and co-transfection experiments in McMA cells. Sequences responsible for the effect of the Ras/MAPK pathway on the *pea3* promoter were identified as the RRE at –241/-227, both through deletion and site-directed mutagenesis. The *pea3* promoter also was shown to be responsive to c-Jun transactivation. Finally, PEA3 and c-Jun were shown to cooperate to potentiate Raf-1 activation of the *pea3* promoter.

The influence of PEA3 on the *CYCLIN D1* promoter was confirmed through the use of co-transfection experiments in Cos-1 cells using an expression plasmid coding for PEA3 and its dominant-negative form. The co-operation between PEA3, β-catenin, Lef-1 and c-Jun on the *CD1* promoter was also established. PEA3 was identified as the key factor responsible for this synergy to occur. The ETS B site was found to be essential both for basal promoter activity and for activated transcription of the reporter gene.

The possible link between the regulation mechanisms of these two proto-oncogenes is interesting and might provide insight for the development of new cancer treatments.

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