CONSTRUCTION AND CHARACTERIZATION OF A CHIMERIC TRANSCRIPTION FACTOR: EWS-PEA3

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ABSTRACT

The activity of PEA3, a member of the Ets proto-oncogene family of transcription factors, is associated with various normal and aberrant developmental processes. PEA3 is believed to function *in vivo* as a transcription factor which regulates the gene expression of its cellular targets. In particular, PEA3 motifs are found in the regulatory regions of several genes implicated in tumor progression and metastasis. Aberrant PEA3 activity has been linked to the development and progression of several types of cancer, particularly, breast cancer and Ewing's sarcoma. To assist in the understanding of the role that PEA3 plays in these processes, constitutively-activated alleles of PEA3 were created by fusing a segment of the EWS gene to murine PEA3; to essentially mimic the EWS-Ets fusion proteins isolated from the human cancer syndrome, Ewing's sarcoma. Five EWS-PEA3 chimeric genes were constructed and one (EWSΔN268PEA3), is an exact murine version of an EWS-human PEA3 fusion gene isolated from Ewing's sarcoma. Transcriptional analysis of the EWS-PEA3 chimeras revealed that with the exception of one mutant, all the chimeras possessed increased transcriptional activity in comparison to normal PEA3. Furthermore, it was suggested that the transcriptional activity of the chimeras may be regulated by Ras, although this observation requires additional validation. The chimeras were not found to possess any transforming activity, however, this finding does not suggest that the chimeras are not oncogenic in nature. In conclusion, these findings suggest that the EWS-PEA3 chimeras may indeed function as activated versions of the PEA3 transcription factor.

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1.0 Introduction

Transcription factors are regulatory proteins which modulate cellular phenotype by controlling gene expression. By binding to short sequences within the promoter regions of genes, transcription factors govern the transcription of genes and also coordinate the stimulated gene expression induced in response to extracellular stimuli. Consequently, the proper functioning of these proteins is critical for normal cell growth. Cellular transformation, the initial event in the development of cancer, is often mediated by aberrant transcription factor activity and subsequent alterations in gene expression. In lower vertebrates, some retroviruses are known to induce oncogenesis by mechanisms such as the deregulation of normal cellular transcription factor expression or by the alteration of transcription factor activity via the expression of mutated viral forms. In humans, chromosomal translocations involving the loss, disruption or alteration of transcription factors is a prevalent cause of many types of leukemias and soft tissue tumors. Thus, the study of transcription factors is relevant to the understanding of normal cellular function and provides insight into the molecular mechanisms responsible for the development of cancer.

1.1 The Ets Family of Transcription Factors

The Ets proto-oncogene family of transcription factors is composed of a diverse group of proteins whose members are found in a wide variety of vertebrate species (for reviews see Wasylyk *et al.*, 1993; Macleod *et al.*, 1992; Janknecht and Nordheim, 1993). The first member of the Ets family to be identified, *v-ets*, was discovered as a product of the avian E26 transforming retrovirus (ETS, E26 transformation-specific or E-twenty six) (LePrince *et al.*, 1983; Nunn *et al.*, 1983). The development of erythroid and myeloid leukemias in chickens following infection with E26 is attributed to the expression of a viral chimeric gene which encodes the $p135^{gag\text{-}myb\text{-}ets}$ fusion protein (LePrince *et al.*, 1983; Nunn *et al.*, 1983). Analysis of E26 revealed that the fusion protein is necessary and required for both erythroblast and myeloid transformation (Nunn and Hunter, 1989; Golay *et al.*, 1988). The *v-ets* oncogene was later found to be derived from a cellular progenitor, chicken Ets-1 (LePrince *et al.*, 1983, Nunn *et al.*, 1983).

Currently, over 30 genes have been classified as belonging to the Ets family based on sequence similarity within a highly conserved 85 amino acid region. This region, termed the ETS domain, is generally sufficient and necessary for binding to DNA sequences containing a 10-12 base pair purine-rich motif centered around a C/A GGA A/T core (Karim *et al.*, 1990; Janknecht *et al.*, 1992; Nye *et al.*, 1992; Klemsz *et al.*, 1990; Urness *et al.*, 1990; Woods *et al.*, 1992; Bowman *et al.*, unpublished). The binding specificity of the various Ets proteins is determined by the sequences flanking the core motif and in some cases, by adjacent transcription factor binding sites which may facilitate ETS specificity through protein-protein interactions. Ets proteins bind to DNA as

monomers (Thompson *et al.*, 1991; Nye *et al.*, 1992; Wasylyk *et al.*, 1992). However, many Ets proteins collaborate with additional transcription factors to mediate gene transcription. Structurally, the ETS domain is unique among DNA binding proteins. Although it possesses a basic region and three evenly-spaced conserved tryptophan repeats (Karim *et al.*, 1990) which slightly resemble the DNA binding domains of transcription factors such as *myb* (Anton and Frampton, 1988), it assumes a winged helix-turn-helix motif to mediate DNA interactions (Liang *et al.*, 1994, Werner *et al.*, 1995; Donaldson *et al.*, 1996).

Members of the Ets family are classified into distinct subgroups based on the location of the ETS domain and sequence similarity both within and outside of the ETS domain (Wasylyk *et al.*, 1993). PEA3 is the founding member of the PEA3 subfamily whose other members include ERM (Monte *et al.*, 1994) and ER81 (Brown and McKnight, 1992). Together they possess 95% amino acid identity within their ETS domains which are divergent from the ETS domains found in other Ets proteins. In particular, the PEA3 ETS domain is found to possess only 60% amino acid similarity to the ETS domains belonging to Ets-1 subfamily members (Xin *et al.*, 1992). The PEA3 subfamily members also display high degrees of similarity outside their DNA binding domains. All possess an acidic domain amino terminal to the ETS domain that is 85% conserved at the amino acid level and overall, are almost 50% identical throughout the entire coding sequences of PEA3, ERM and ER81 (Monte *et al.*, 1995). Although the PEA3 subfamily members are highly similar in their primary structure, differences in their function may be partially attributed to variations in their expression patterns. Human

ERM is ubiquitously expressed (Monte *et al.*, 1994, 1995), but PEA3 (Xin *et al.*, 1992; Monte *et al.*, 1994) and ER81 (Brown and McKnight, 1992; Monte *et al.*, 1994) have more restricted expression patterns.

1.2 PEA3

PEA3 (polyomavirus enhancer activator 3) is a member of the ETS transcription factor family. PEA3 was originally described as a cellular factor able to interact with a region of the polyomavirus (Py) enhancer and is required for early and late viral gene expression and Py DNA replication (Martin *et al.*, 1988; Mueller *et al.*, 1988; Muller *et al.*, 1988; Murakami *et al.*, 1990). To date, human (hPEA3 or E1A-F) and murine (mPEA3) cDNAs of PEA3 have been cloned from HeLa and FM3A cDNA expression libraries, respectively (Xin *et al.*, 1992; Higashino *et al.*, 1993). They are encoded by 2.5 kb and 2.4 kb mRNA transcripts in their respective cell lines and have estimated molecular weights of 60 kDa (human) and 61 kDa (murine). At the amino acid level, human and murine PEA3 are completely identical within their ETS domains and possess 94% identity overall (Higashino *et al.*, 1993). Analysis of the genomic organization of mPEA3 has revealed that it is encoded by 13 exons that span across 17.6 kb of chromosomal DNA (Liang *et al.*, unpublished). Human PEA3 maps to chromosome 17q21.3 (Barrett *et al.*, unpublished) while murine PEA3 localizes to chromosome 11, band D (Laing *et al.*, unpublished).

1.2.1 PEA3 Expression

The expression of PEA3 is developmentally regulated during mouse embryogenesis. Using *in situ* hybridization, PEA3 mRNA expression is detected in the brain, somites and developing limb buds of mouse embryos (Laing *et al.*, unpublished). PEA3 expression is not critical for mouse development since transgenic mice bearing a PEA3-null genotype are viable and do not exhibit any overt physical abnormalities. The single phenotypic observation from PEA3-null mice is a male fertility defect, the cause of which is currently under investigation (Laing *et al.*, unpublished). In the normal adult mouse, PEA3 expression is highly restricted. PEA3 is detected most abundantly in the epididymis and brain; however, lesser amounts of PEA3 mRNA expression is also observed in colon, kidney, and intestinal (small) tissue (Xin *et al.*, 1992; Laing *et al.*, unpublished). Female mice also express PEA3 mRNA specifically within their mammary gland, ovarian, and uterine tissues (Laing *et al.*, unpublished; MacNeil *et al.*, unpublished). The critical factors which govern the temporal and tissue specific expression of PEA3 are currently unknown.

1.2.2 PEA3 Transcription Factor Activity

PEA3 is a sequence specific DNA binding transcriptional activator which is capable of directing gene transcription from promoters containing a 5'-A/C,GGAAGT-3' element (Xin *et al.*, 1992). Although definitive PEA3 target are not known, several cellular and viral genes contain Ets binding site (EBS) motifs within their regulatory regions. Within the Py enhancer, the PEA3/AP1 motif is required for the transcription of

viral genes and replication of the viral genome (Wasylyk *et al.*, 1989). Although several Ets proteins have been shown to bind to this site, including Ets-1 (Wasylyk *et al.*, 1990), both recombinant and endogenous mPEA3 protein bind to this region of the Py enhancer (Xin *et al.*, 1992; Martin *et al.*, 1988). Another viral gene whose transcription is governed from an element containing Ets binding sites is the adenovirus (Ad5) E1A gene (Hearing *et al.*, 1983). Human PEA3 isolated from HeLa cells is capable of binding to this motif and hPEA3 mRNA is coordinately upregulated in Ad5-infected HeLa cells (Higashino *et al.*, 1993). PEA3 motifs are also found in the human T-cell leukemia virus long terminal repeat (HTLV-1 LTR), however, specific PEA3 binding to this motif has never been demonstrated (Bosselut *et al.*, 1990).

Putative Ets-regulated cellular genes generally possess ETS motifs in conjunction with AP-1 transcription factor sites within their regulatory domains. They encode for a broad spectrum of proteins involved in processes relevant to cellular proliferation, migration and metastasis (Wasylyk *et al.*, 1989; Gutman and Wasylyk, 1990a; Liotta *et al.*, 1991; Macleod *et al.*, 1992; Wasylyk *et al.*, 1993). The increasing evidence on the critical regulatory factors governing expression of several of these putative target genes supports a role for PEA3 in their transcriptional regulation.

PEA3 and Matrix Metalloprotease Gene Expression

Matrix metalloproteases (MMPs) comprise a related group of degradative enzymes involved in tissue remodelling and cell migration that are believed to play a critical role in tumor progression and metastasis (Liotta *et al.*, 1991). The transcriptional regulation of

the type I collagenase (MMP-1), stromelysin (MMP-3), and 92 kDa type IV collagenase (gelatinase B or MMP-9) genes are possibly modulated *in vivo* through PEA3/AP1 motifs situated within their promoter regions. In transient assays, hPEA3 directs high levels of transcription from all three promoters (Higashino *et al.*, 1995); and acts synergistically with jun/fos homo- or heterodimers (AP-1) on the type 1 collagenase promoter (Gutman *et al.*, 1990; Higashino *et al.*, 1995).

Another gene whose expression may be regulated *in vivo* through PEA3 is the urokinase-type plasminogen activator (uPA). The uPA gene encodes for a serine protease that modulates extracellular matrix proteolysis cascades and is believed to play a critical role in tumor metastasis (Carmeliet *et al.*, 1994; Blasi *et al.*, 1990). An enhancer located 2 kilobases upstream of the uPA gene transcription start site contains conserved PEA3/AP-1 and AP-1 elements which together are necessary for both constitutive and phorbol-stimulated uPA gene expression (Rorth *et al.*, 1990; Nerlov *et al.*, 1991).

PEA3 and PEA3 Gene Expression

Recently, evidence has been presented to suggest that PEA3 is involved in its own regulation. The promoter region of mPEA3 contains several putative Ets binding sites (Barrett *et al.*,unpublished) and DNase I footprinting of the -355 to +1 region reveals the induction of hypersensitive sites in response to PEA3 binding. Furthermore, PEA3 is able to transactivate a segment of the PEA3 promoter in a dose-dependent manner in transient reporter gene assays (O'Hagan *et al.*, unpublished).

1.2.3 Regulation of PEA3 Transcriptional Activity

The DNA binding and transcriptional activation properties of PEA3 are regulated at the post-translational level; likely involving transient protein phosphorylation in response to mitogenic and proliferative signals. *In vivo*, the activity of Ets proteins which bind to the PEA3 element (PEA3, by extension) is enhanced by serum factors, the expression of non-nuclear oncogenes including Py middle T antigen, *v-src*, Ha-ras, *v-mos*, and *v-raf*, and agents such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (Wasylyk *et al.*, 1989; Asano *et al.*, 1990). Furthermore, the signals elicited by these factors are transduced to their effectors via known protein kinase-mediated phosphorylation cascades. PEA3 transcriptional activity has recently been demonstrated to be regulated by two signal transduction cascades mediated through Ras (O'Hagan *et al.*, 1996).

Activation of PEA3 by Ha-Ras

Ha-Ras, belongs to a superfamily of low molecular weight proteins (p21) which mediate the transduction of signals from the cell surface to the nucleus (reviewed in Marshall, 1993). Activation of Ras in response to a specific stimulus directly results in the activation of distinct protein kinase networks which transduce the signal through substrate phosphorylation. Two signalling cascades which have been shown to be downstream of Ras are the ERK (extracellular regulated kinases; also known as MAP kinases, mitogen activated kinases) and the SAPK (stress activated protein kinases; also known as JNK, Jun regulated kinases) modules. Several ETS proteins are among the downstream targets of Ras-mediated signalling cascades. Elk-1 phosphorylation by ERK1 and ERK2 leads to

increased transcriptional activity by Elk-1/SRF complexes at the serum response element (SRE) of the c-fos promoter (Hill *et al.*, 1995). Phosphorylation of a conserved threonine residue in Ets-1 and Ets-2 by activation of the Ras-Raf1-ERK pathway is required for the transactivation of promoter fragments containing Ets-responsive elements (Yang *et al.*, 1996). Finally, the transcriptional activity of the PEA3 subfamily members, ERM and ER81 are both increased in response to Ras-Raf1-ERK activation and *in vitro* are directly phosphorylated by ERK1 and ERK2 respectively (Janknecht *et al.*, 1996; Janknecht *et al.*, 1996).

Evidence exists to suggest that PEA3 regulation is also mediated, in part, by effectors downstream of Ras. In transient assays, the ability of mPEA3 to transactivate a PEA3-responsive reporter construct is enhanced at least 10 fold in the presence of a constitutively-activated Ras construct, Ha-RasV12 (O'Hagan *et al.*, 1996). Furthermore, Ras activation is conferred through the Ras-Raf1-ERK and Ras-Rac1-SAPK cascades (O'Hagan *et al.*, 1996). Thus, PEA3 activation by Ras is attributed to the independent but synergistic actions of these two pathways.

PEA3 contains eight putative ERK/SAPK consensus phosphorylation sites and *in vitro* is readily phosphorylated by recombinant ERK and SAPK proteins (Cox *et al.*, unpublished). *In vivo*, PEA3 is phosphorylated exclusively on serine residues (O'Hagan *et al.*, 1996). Phosphopeptide analysis of *in vivo* and *in vitro* phosphorylated PEA3 show identical patterns, indicating phosphorylation on the same serine residues (Tozer and Cox, unpublished). Of the eight putative sites, phosphorylation at serines 90, 101 and 134 contribute the majority of stimulation-induced phosphorylation. Whether PEA3 is a direct

in vivo target of phosphorylation by the ERK or SAPK protein kinases and whether phosphorylation is the modification responsible for PEA3 activation still requires to be determined.

Activation of PEA3 by the HER2/neu Receptor

The HER2/neu gene encodes a 185 kDa protein which belongs to the epidermal growth factor (EGF) family of receptor tyrosine kinases (Coussens *et al.*, 1985; Bargmann *et al.*, 1986; Hynes *et al.*, 1994). Upon binding of an unidentified ligand, the HER2/neu receptor is activated by dimerization and subsequent autophosphorylation of specific tyrosine residues located in its cytoplasmic domain. The phosphotyrosine residues then act as docking sites for effector molecules such as GRB2/SOS, phosphatidylinositol 3' kinase (PI3'K), phospholipase Cγ, src, and Shc (reviewed in Hynes *et al.*, 1994) which initiate signal transduction cascades to the nucleus. The HER2/neu receptor is linked to the Ras pathway by GRB2/SOS, a nucleotide exchange factor which has been shown to activate Ras by facilitating GTP binding. Thus, through the activation of Ras and Ras-modulated protein phosphorylation cascades, HER2/neu is ultimately able to regulate PEA3 activity. In transient assays, both normal and a constitutively activated neu receptor (NeuNT(V664E); Bargmann *et al.*, 1986) increase the ability of exogenous PEA3 to direct the transactivation of a PEA3-dependent reporter gene construct. Furthermore, this activation is significantly abbrogated if Ras signalling is blocked at Ras itself, or downstream within either the Raf1-ERK or Rac1-SAPK pathways (O'Hagan and Hassell,

submitted). Together, these results begin to present a clearer understanding of the mechanisms which regulate the transcriptional activity of PEA3.

1.2.4 The Role of Ets proteins in Oncogenesis

Several cellular ETS proteins are implicated in oncogenic processes. In mice, the increased expression of Fli-1 and Spi-1(PU.1) following proviral insertion of the Friend Murine Leukemia and Spleen Focus-forming viruses, respectively, is sufficient to induce erythroleukemias (Ben-David *et al.*, 1991; Moreau-Gachelin *et al.*, 1988). Simple overexpression of other ETS proteins is sufficient to render them oncogenic. c-Ets-1 (Seth and Papas, 1990), c-Ets-2 (Seth *et al.*, 1989) and human ERG (Hart *et al.*, 1995) overexpression in NIH 3T3 cells results in cellular transformation as determined by morphological changes and the acquisition of properties necessary for growth under low serum and anchorage-independent conditions. Furthermore, subcutaneous injection of c-Ets-1, c-Ets-2 or ERG transformed NIH 3T3 clones into nude mice is sufficient to induce solid tumor growth.

In humans, chromosomal translocations involving the Ets protein, Tel, are associated with the acquisition of several types of leukemias. Chronic myelomonocytic leukemia (CMML) is a syndrome characterized by myeloid cell proliferation which eventually progresses to acute myelogenous leukemia (AML). Development of CMML is associated with a t(5;12)(q33;p13) translocation (Keen *et al.*, 1987; Srivastava *et al.*, 1988; Berkowicz *et al.*, 1991; Lerza *et al.*, 1992; Wessels *et al.*, 1993) which results in the fusion of the amino terminus of Tel to the transmembrane and tyrosine kinase domains of

the platelet-derived growth factor receptor β (PDGFR-β) (Golub *et al.*, 1994). Rearrangement of the Tel genomic locus following translocation also results in Tel-ABL (Papadopoulos *et al.*, 1994) and Tel-AML1 (t(12;21)(p13;q22)) in acute lymphoblastic leukemia (ALL) (Golub *et al.*, 1995).

In addition to leukemias, chromosomal translocations involving Ets proteins which result in solid tumors have also been reported. In particular, the Ewing's sarcoma family of extraosseous bone tumors are caused by translocations which result in the fusion of the Ets proteins Fli-1 (Delattre *et al.*, 1992), ERG (Sorensen *et al.*, 1994), ETV1/hER81 (Joen *et al.*, 1995), FEV (Peter *et al.*, 1997) and E1A-F/hPEA3 (Kaneko *et al.*, 1996; Urano *et al.*, 1996) to the RNA binding protein, EWS. Additional information on this syndrome will be presented in the following sections.

1.2.5 Role of PEA3 in Oncogenesis

Several lines of evidence implicate a putative role for PEA3 in the development and progression of several types of cancer both in humans and in mice.

PEA3 and Breast Cancer

As discussed previously, HER2/neu (also known as c-erbB-2) belongs to the epidermal growth factor family (EGF) of receptor tyrosine kinases. Members of this family have been implicated in the development and progression of several types of cancer. In particular, HER2/neu mRNA is present at elevated levels in an estimated 20-30% of all human breast carcinomas and is considered a poor prognostic factor for survival due to

increased tendency to metastasize and lesser response to hormonal and chemotherapeutic agents (Slamon *et al.*,1987; Singleton *et al.*, 1992). Overexpression is attributed both to amplification of the HER2/neu gene (Liu *et al.*, 1992) and to increased expression of the gene at the transcriptional level. Comparative analysis of normal and mammary tumor cell lines exhibit an increase of 6-8 fold more HER2/neu mRNA per gene copy in mammary tumor cells (King *et al.*, 1989; Iglehart *et al.*, 1990; Parkes *et al.*, 1990)

The clinical significance of HER2/neu in human breast cancer has lead to the development of several transgenic mouse models. Transgenic mice engineered to overexpress the normal rat neu gene under the control of the mouse mammary tumor virus (MMTV) promoter/enhancer, develop focal mammary adenocarcinomas which are highly metastatic to the lung (Guy *et al.*, 1992b). These primary mammary tumors and lung metastases exhibit elevated levels of PEA3 mRNA in the transformed tissue as compared to the surrounding normal mammary epithelium and lung tissue (Trimble *et al.*, 1993). The correlation between HER2/neu and PEA3 expression is further substantiated by analysis of human breast tumors. In the analysis of 74 primary breast tumors (both ductal carcinoma *in situ*, and invasive tumor samples), PEA3 and HER2/neu overexpression is correlated in 90% of the tumor samples. In contrast, less than half of human breast tumors that do not express elevated HER2/neu levels overexpress PEA3 (Benz *et al.*, in press).

The similar expression patterns of HER2/neu and PEA3 in breast epithelial tissue suggests a functional relationship between the two proteins in mediating breast tumorigenesis. As discussed previously, activation of the HER2/neu receptor ellicits an

intracellular signalling cascade which functions through Ras to activate PEA3 and subsequent PEA3-dependent transcription. It is possible that in HER2/neu overexpressing cells, the transduced signal results in activation of PEA3 and overaccumulation of the protein products of PEA3 target genes. Given the evidence suggesting that PEA3 may regulate the expression of several genes implicated in metastatic processes, it is interesting and perhaps significant that the murine mammary adenocarcinomas which express elevated levels of PEA3 mRNA are also extremely metastatic (Guy *et al.*, 1992; Trimble *et al.*, 1993). Futhermore, the lung metastases derived from the primary transformed mammary epithelium also express significantly higher levels of PEA3 mRNA than observed in the surrounding normal tissue (Trimble *et al.*, 1993). The suggestion that overexpression of PEA3 may contribute to the development of a metastatic phenotype is further supported by experiments performed with human breast cancer cell lines. The ectopic expression of hPEA3 in MCF-7 cells, a non-metastatic breast cancer cell line, is sufficient to induce cell motility and invasiveness (Kaya *et al.*, 1996); a phenotype which is abbrogated if PEA3 activity is blocked through the use of a dominant-negative PEA3 allele (O'Hagan, Ashraf, and Shepherd, unpublished).

Another gene which may be upregulated in response to PEA3 activity in mammary tumors is the HER2/neu gene, which possesses conserved PEA3 binding sites within its upstream regulatory region (Scott *et al.*, 1994). In transient transcriptional activation assays, PEA3 transactivates the neu promoter in a dose-dependent manner which is contingent on DNA binding (Benz *et al.*, in press). The suggestion that PEA3 may regulate HER2/neu gene expression provides insight into a mechanism by which both the

PEA3 and HER2/neu proteins may be simultaneously overexpressed in breast tumors. Given the fact that HER2/neu activation stimulates PEA3 transcriptional activity, it is possible that once initiated, PEA3 could upregulate both its own and HER2/neu gene expression simultaneously. The resultant increases in both proteins could potentially support a stimulatory loop which would effectively result in more distortions of protein expression.

The role of PEA3 and its correlation with HER2/neu activity in mammary tumorigenesis and metastasis requires further investigation. Based on the proposed model, PEA3 is a critical factor in the maintainance of transformation and possibly, directly initiates metastasis through the upregulation of required degradative enzymes. Currently, there is no evidence to suggest that the stimulation of PEA3 activity in the absence of other events is sufficient to induce tumorigenesis in breast tissue.

PEA3 in Ewing's Sarcoma

The first direct evidence to illustrate that aberrant PEA3 activity could result in human malignancy was obtained from the analysis of a Ewing's sarcoma tumor excised from the cheek of a newborn (Kaneko *et al.*, 1996). Molecular characterization of the tumor revealed a chromosomal translocation, t(17;22)(q12;q12), which directly involved the genomic locus of hPEA3 (Kaneko *et al.*, 1996). This finding was substantiated by more extensive analysis which demonstrated that a small fraction of diagnosed Ewing's sarcoma tumors directly express EWS-PEA3 fusion transcripts (Urano *et al.*, 1996).

1.3 Ewing's Sarcoma

The Ewing's family of tumors (Table 1.1-A) comprise a subset of human solid tumor malignancies which generally manifest in childhood and early adulthood as osteosarcomas. Characteristics of the disease include small round cell tumors of non-osseous origin which occur primarily in bone, and to a lesser degree, in soft tissues (Dahlin *et al.*, 1961). Histologically, Ewing's sarcoma consists of uniform sheets of undifferentiated small round cells (mainly of neuroectodermal origin) whose only distinguishing marker is the cell surface antigen $p30/32^{MIC2}$ (Fellinger *et al.*, 1991). Karyotypic analysis of Ewing's sarcoma reveals specific chromosomal translocations involving chromosome 22.

All the Ewing's sarcoma translocations characterized to date, juxtapose various ETS genes to the EWS gene on chromosome 22 (Table 1.1-B). The ensuing novel genes, under the control of the EWS promoter, encode chimeric proteins with amino terminal EWS sequences fused to the Ets DNA binding domain. Consequently, these chimeras are thought to function as aberrant or deregulated ETS transcription factors whose expression and activities are oncogenic.

1.3.1 The EWS-Fli-1 Chimeric Transcription Factor

The EWS-Fli-1 products are the best characterized since the t(11;22) translocation accounts for 85% of all Ewing's sarcoma cases (Zucman *et al.*, 1992). The breakpoint regions, which are highly recurrent, tend to occur specifically within the 7-10 intron region of the EWS gene, whereas, Fli-1 gene disruption occurs within the region containing

Table 1.1-A Summary of the Ewing's Sarcoma Family Members

Ewing's Family Member	Reference
Ewing's sarcoma of bone	Aurius *et al.*, 1983; Turc-Curel *et al.*, 1983
Extraskeletal Ewing's sarcoma	Becroft *et al.*, 1984
Peripheral neuroepithelioma	Whang Peng *et al.*, 1986
Askin tumor	Whang Peng *et al.*, 1986

Table 1.1-B Summary of all the Ewing's sarcoma chromosomal translocations

Translocation	Fusion Product	Reference
t(11;22)(q24;q12)	EWS-Fli-1	Delattre *et al.*, 1992
t(21;22)(q22;q12)	EWS-ERG	Sorensen *et al.*, 1994
t(07;22)(p22;q12)	EWS-ETV-1	Joen *et al.*, 1995
t(17;22)(q12;q12)	EWS-E1A-F (hPEA3)	Kaneko *et al.*, 1996; Urano *et al.*, 1996
t(2;21;22)(q33;q22;q12)	EWS-FEV	Peter *et al.*, 1997

introns 3-9 (Zucman *et al.*, 1993). Variation in breakpoint regions and alternate splicing results in a heterogeneous population of EWS-Fli-1 transcripts (Zucman *et al.*, 1993; May *et al.*, 1993). Invariant features of all EWS-Fli-1 chimeras are a minimal amino terminus consisting of the first 264 amino acids of EWS and the retention of the DNA binding domain of Fli-1 (Zucman *et al.*, 1992;1993).

Expression of EWS-Fli-1 is restricted exclusively to Ewing's sarcoma tumor tissue. Stable transfection of EWS/Fli-1 in NIH 3T3 cells is sufficient to confer growth in liquid culture and soft agar under low serum conditions, producing macroscopic colonies within 10 days (May *et al.*, 1993). Furthermore, the ability of EWS-Fli-1 to transform NIH 3T3 cells requires both the EWS and Fli-1 domains present in the chimera (Lessnick et al., 1995; May et al., 1993, 1994). Under similar conditions, wild type Fli-1 expression does not transform NIH 3T3 cells (May *et al.*, 1993). The rapid and efficient transformation of NIH 3T3 cells suggests that expression of EWS-Fli-1 is directly oncogenic in these cells. Cell lines established from Ewing's sarcomas express physiological levels of EWS-Fli-1 from the endogenous EWS promoter. Transfection of these cell lines with plasmids encoding antisense EWS-Fli-1 results in decreased colony formation and growth in soft agar likely owing to marked decreases in chimera expression (Ouchida *et al.*, 1995). Furthermore, the antisense-transfected cell lines lose the ability to induce solid tumor formation following subcutaneous injection in nude mice (Ouchida *et al.*, 1995).

The putative physiological role of EWS-Fli-1 is as a transcription factor with altered activity from the wild type Fli-1 protein. EWS-Fli-1 binds to DNA with sequence

specificity and its expression is localized to the nucleus (May *et al.*, 1993). Analysis of binding specificity by electrophoretic mobility shift assays (EMSAs) reveals that EWS-Fli-1 exhibits identical sequence recognition and binding to the ETS core motif as Fli-1 protein (Bailley *et al.*, 1994). Furthermore, EWS-Fli-1 transactivates with the same sequence specificity as wild type Fli-1, but with increased potency (Bailley *et al.*, 1994). In transient assays with reporter constructs containing the ets-responsive region (ERR1) of the human T-cell leukemia virus type I long terminal repeat (HTLV LTR), EWS-Fli-1 activates transcription to levels 10 fold greater than observed with Fli-1 (Bailley *et al.*, 1994). The increase in transactivation ability evident in EWS-Fli-1 is attributed to a stronger transcriptional activation domain present in the amino terminal EWS sequences which replaces the analogous Fli-1 region. When fused to the DNA binding domain of the yeast transcriptional activator, GAL4, the region of EWS found in the chimeras stimulated transcription at least 30 fold over the amino terminal region of Fli-1 (May *et al.*, 1993; Bailley *et al.*, 1994). Taken together, it is possible that one mechanism by which EWS-Fli-1 mediates oncogenesis is the simple upregulation of Fli-1 target genes.

Although EWS-Fli-1 binds with the same sequence specificity as Fli-1, there is also evidence to suggest that the chimera can transactivate a unique subset of target genes. Ewing's sarcoma tumor cells possess elevated expression levels of the transcription factor, c-myc (McKeon *et al.*, 1988). Transient assays in HeLa cells show that EWS-Fli-1, but not Fli-1, is able to stimulate transcription from the c-myc promoter (Bailley *et al.*, 1994); suggesting that *in vivo* EWS-Fli-1 may recognize and transactivate from a more diverse range of ETS motifs than Fli-1. The serum response element (SRE) of the c-fos promoter

is a cis-regulatory element which the ETS proteins ELK-1 and SAP-1 transactivate following ternary complex formation with serum response factor, SRF (Dalton *et al.*, 1992; Hipskind *et al.*, 1991). *In vitro*, EWS/Fli-1 associates with SRF and forms a ternary complex on the c-fos SRE; Fli-1 does not possess this binding ability (Magnaghi-Jauhlin *et al.*, 1996). EWS-Fli-1 target genes have also been identified by Representational Difference Analysis (RDA). In EWS-Fli-1 overexpressing NIH 3T3 cells, upregulated genes include stromelysin 1 (MMP-3), murine cytochrome P-450, cytokeratin 15 (Braun *et al.*, 1995) and several novel genes such as EAT-2 (EWS-Fli-1 activated transcript-2) (Thompson *et al.*, 1996). The expression of stromelysin and EAT-2 are evident within 4-8 hours following the induction of EWS-Fli-1, implying that both may be direct targets of EWS-Fli-1 upregulation (Braun *et al.*, 1995; Thompson *et al.*, 1996). EWS-Fli-1 expression also results in the down-regulation of several gene transcripts in NIH 3T3, however, none have been identified to date (Braun *et al.*, 1995).

Characterization of EWS-Fli-1 and its expression in Ewing's sarcoma tumors is at present more comprehensive than for EWS-ERG, EWS-ETV-1, EWS-hPEA3 or EWS-FEV. Thus, it is plausible that the other EWS-ETS chimeras act by similar mechanisms as those elucidated for EWS-Fli-1. Consequently, the oncogenic effects of the EWS-ETS chimeras are likely governed by increased transactivation properties, altered transcriptional targets and/or deregulation of normal ETS activity.

1.3.2 The EWS Gene

The EWS gene was discovered by virtue of its involvement in the translocations responsible for Ewing's sarcoma (Delattre *et al.*, 1992). Located at the chromosome 22q12 locus, the EWS gene contains 17 exons which code for a ubiquitously expressed 656 amino acid protein (Delattre *et al.*, 1992). The physiological function of EWS is currently unknown, however, protein sequence analysis reveals several distinct functional domains.

The first 285 amino acids of EWS constitute the amino terminal domain of EWS (EWS NTD) and includes the region involved in Ewing's sarcoma translocations. The EWS NTD is rich in glutamine, threonine, and proline residues and encodes a strong transcription activation domain (Bailley *et al.*, 1994; May *et al.*, 1993). Replacement of the amino terminus of Fli-1 with EWS NTD results in an oncogenic transcription factor which transactivates with higher potency than wild type Fli-1. Deletion studies of the EWS-Fli-1 protein functionally distinguish the EWS sequences required for transformation to include the first 82 amino acids while transactivation is attributed to the region contained between amino acids 82 and 264 (Lessnick *et al.*, 1995). Removal of the transactivation domain from EWS-Fli-1 (amino acids 82-264) results in retention of transformation ability and suggests that important protein-protein interactions may be mediated within the first 82 amino acids of EWS to effect oncogenicity (Lessnick *et al.*, 1995).

Also contained within the amino terminus of EWS is a region between amino acids 157-262 which is 40% homologous to the carboxy terminal domain located on the largest

subunit of eukaryotic RNA polymerase II (CTD pol II) (Delattre *et al.*, 1992). The CTD possesses multiple Tyr-Ser-Pro-Thr-Ser-Pro-Ser repeats (Dahmus *et al.*, 1994) that are direct phosphorylation targets. The phosphorylation state of CTD pol II is implicated in the regulation of RNA pol II activity and is thought to mediate processes such as promoter selection, preinitiation complex formation, promoter clearance, and trancriptional elongation of mRNA transcripts (reviewed in Dahmus, 1996). The functional significance of the homology between EWS and the CTD pol II heptapeptide repeats is unknown.

The carboxy terminus of EWS which is absent in EWS-Fli-1 chimeras, contains several putative RNA binding regions. The RNA recognition motif (RRM or RNP motif) is a conserved 85 amino acid glycine-rich region which is found in several proteins, such as human hnRNP A1, known to possess RNA binding activity (Dreyfuss *et al.*, 1988; Kenan *et al.*, 1991; Haynes *et al*, 1992). Although the RNA annealing activity of hnRNP A1 is mediated *in vitro* through the RNP motif, no RNA binding activity mediated through this motif has been identified for EWS. The carboxy terminus of EWS also contains two RGG boxes homologous to RNA binding regions found in other proteins (Kiledjian and Dreyfuss, 1992). Through the C-terminal RGG box (last 86 amino acids), EWS binds specifically to poly G and poly U tracts (Ohno *et al.*, 1994). The presence of these RNA binding regions and *in vitro* binding studies suggests an analogous *in vivo* function for EWS. The significance of the loss of the EWS RNA binding motifs in the Ewing's sarcoma translocations are unknown.

EWS, TLS/FUS and Human Malignancy

EWS, as well as the related protein TLS/FUS, are members of a novel family of RNA binding proteins whose involvement in human malignancy is being discovered in an increasing number of solid tumors and leukemias. TLS/FUS (translocated in liposarcoma) was isolated by virtue of its fusion to the transcription factor, CHOP, in a myxoid liposarcoma tumor caused by a t(12;16) chromosomal translocation (Crozat *et al.*, 1993). Together, EWS and TLS/FUS are fused to a variety of transcription factors to produce a large spectrum of soft tissue tumor syndromes (Table 1.2). Similar to the Ewing's sarcoma translocation products, these chimeric transcription factors all retain the DNA binding activity of the parent factor and the development of each tumor syndrome is attributed to aberrant transcription factor activity in affected cells.

Table 1.2 Summary of the chromosomal translocations involving EWS or TLS/FUS and various DNA Binding Proteins

Chromosomal Translocation	RNA Binding Protein	DNA Binding Protein	Tumor	Reference
t(12;16)(q13;p11)	EWS	CHOP	Myxoid Liposarcoma	Panagopoulos *et al.*, 1995
t(12;22)(q13;q12)	EWS	ATF-1	Malignant melanoma of soft parts	Zucman *et al.*, 1993
t(9;22)(q22-31;q11-12)	EWS	CHN	Myxoid chondrosarcoma	Clark *et al.*, 1996
t(11;22)(p13;q12)	EWS	WT1	Desmoplastic small round cell tumor	Gerald *et al.*, 1995
t(12;16)(q13;p11)	TLS/FUS	CHOP	Myxoid liposarcoma	Crozat *et al.*, 1993
t(16;21)(p11;q22)	TLS/FUS	ERG	Acute myeloid leukemia	Ichikawa *et al.*, 1994

1.4 Project Objective

PEA3 is a highly regulated transcription factor whose expression and activity is correlated with various normal and aberrant developmental processes. In particular, PEA3 has been implicated both in tumor progression and metastasis (discussed previously); and the current aim of much investigation is the precise role(s) PEA3 plays in their manifestation. Given that PEA3 expression is highly restricted and its activity subject to regulatory mechanisms that have not been fully characterized, the study of PEA3 function is difficult.

The primary objective of this project was to engineer a constitutively-activated and/or non-regulated PEA3 allele for use in future studies aimed at assessing the physiological role of PEA3 in processes such as tumorigenesis, metastasis and development. Given the evidence suggesting the involvement of PEA3 in breast tumorigenesis, it seemed particularily relevant to construct an activated PEA3 gene which could be constitutively expressed in the mammary epithelium of transgenic mice and used to determine the precise role of PEA3 in this process. Furthermore, an activated PEA3 construct would also be invaluable to the study of other potential PEA3-regulated processes, including metastasis.

To achieve this end, several EWS-murine PEA3 chimeric genes were artificially constructed to resemble the mutant genes isolated from Ewing's sarcoma tumors. The decision to use the EWS-Ets chimeric genes as a model for the construction of an activated PEA3 allele was based on the fact that characterization of the EWS-Ets fusion proteins revealed them to be potent Ets-specific transcription factors and more

transcriptionally active than their normal counterparts. The relevance of the EWS-PEA3 fusion was later substantiated by the isolation of a true fusion of the EWS and PEA3 genes from a Ewing's sarcoma tumor (Urano *et al.*, 1997).

Following their construction, the characteristics of the EWS-PEA3 chimeras were assessed by studying their transcriptional activation and transformation properties. To determine what effect the addition of EWS conferred on the activity of the murine PEA3 protein, the fusion proteins were tested for the ability to transactivate PEA3-responsive reporter genes in transient transcriptional activation assays. To assess the relative oncogenicity of the EWS-PEA3 chimeras, their ability to affect morphologically transformation of NIH 3T3 and Rat-1 cells in focus and soft agar assays was determined. Finally, the EWS-PEA3 chimeras were subcloned into an expression vector which would allow for their use in mouse transgenics.

MATERIALS AND METHODS

The sources of all reagents, commercial or otherwise, are as described.

2.0 Construction and Subcloning of EWS-PEA3 Fusion Genes

The EWS cDNA sequences utilized were obtained from the plasmid pKS$^+$-EWS-Fli-1, kindly donated by Dr. O. Delattre. The source of PEA3 cDNA sequence was a clone isolated from a murine FM3A cDNA expression library (Xin *et al.*, 1992) and subcloned into the plasmid vector pGEM®-7 (Stratagene) by U. Ashraf, Hassell laboratory. The construction of the five EWS-PEA3 fusion genes (Table 2.1) required the amplification of relevant DNA sequences by the polymerase chain reaction (PCR) method. The E244P, E304P and E334P constructs were created by the independent PCR amplification of EWS and PEA3 amino terminal deletions, followed by restriction endonuclease digestion and subsequent ligation. The construction of EWSΔN268PEA3, E268P, involved a modified PCR protocol called overlap extension PCR, which utilized E244P as a template. The primer sequences utilized for PCR amplification are outlined in Table 2.2 and were synthesized in the Mobix Central Facility. Following is an outline of the cloning methods used to create the EWS-PEA3 chimeras. All were directly cloned into the eukaryotic expression vector, pCanMyc1, obtained from Onyx Pharmaceuticals

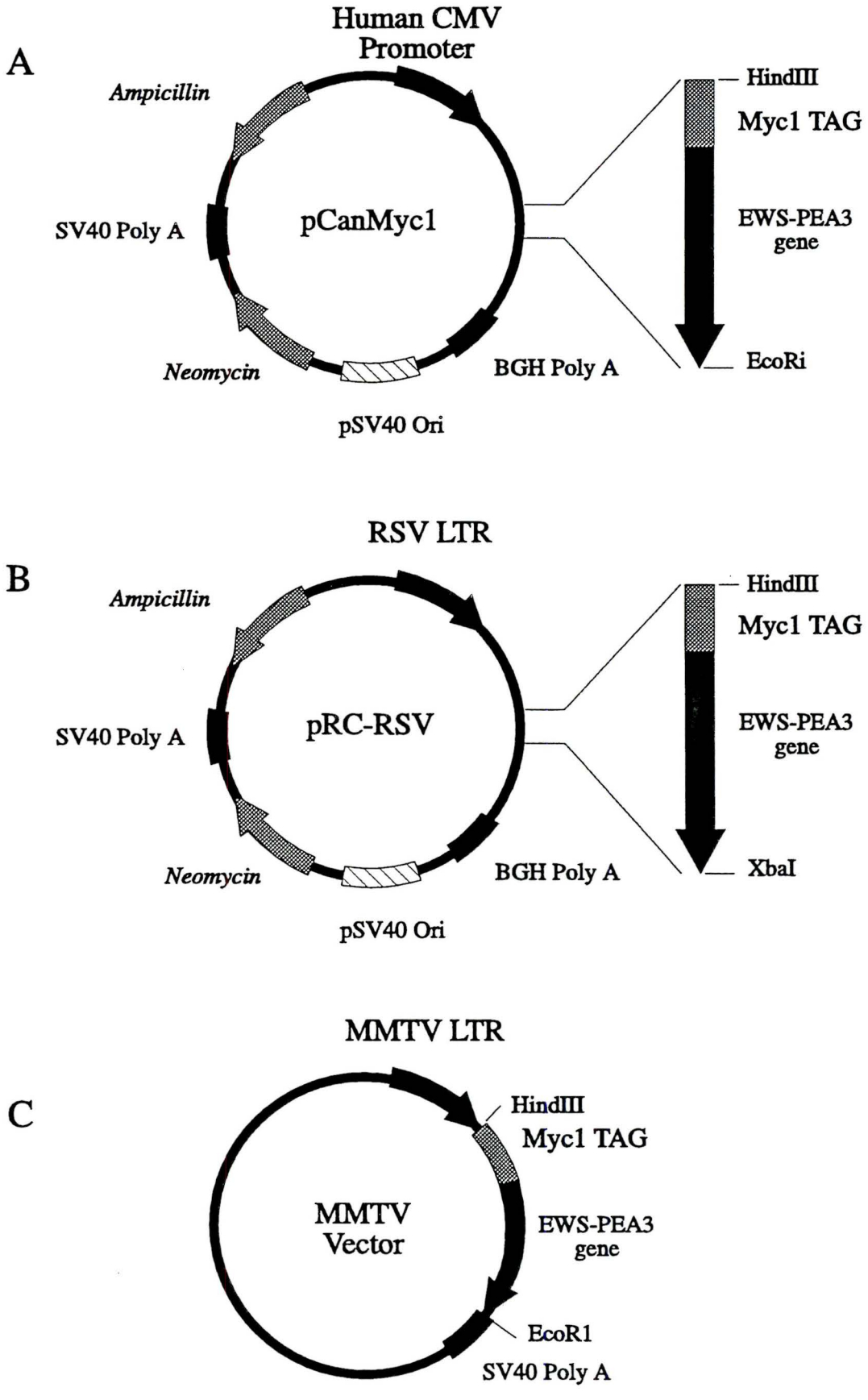
Figure 2.1 Structure of the recombinant plasmids encoding the EWS-PEA3 chimeric genes

A. Structure of the pCanMyc1 eukaryotic expression vector. This plasmid, which was obtained from Onyx Pharmaceuticals (CA), is modified from the pcDNA3 (Invitrogen) backbone by the addition of a myc epitope sequence directly upstream of the multiple cloning site. Gene transcription in eukaryotic cells is directed from the human cytomegalovirus (hCMV) promoter.

B. Structure of the pRc-RSV eukaryotic expression plasmid (Invitrogen). Transcription of the EWS-PEA3 chimeric genes in eukaryotic cells is directed from the Rous sarcoma virus long terminal repeat (RSV LTR).

Both the pCanMyc1 and pRc/RSV plasmids carry ampicillin and neomycin resistance genes. In addition, both also possess an SV40 origin of replication which enables plasmid replication in cell lines expressing SV40 large tumor (T) antigen.

C. Structure of the MMTV expression plasmid. All the myc-tagged EWS-PEA3 chimeric genes were subcloned into this plasmid to allow for their future use in transgenic mouse experiments.


A
Human CMV
Promoter
Ampicillin
SV40 Poly A
pCanMyc1
Neomycin
BGH Poly A
pSV40 Ori
HindIII
Myc1 TAG
EWS-PEA3
gene
EcoRi
B
RSV LTR
Ampicillin
SV40 Poly A
pRC-RSV
Neomycin
BGH Poly A
pSV40 Ori
HindIII
Myc1 TAG
EWS-PEA3
gene
XbaI
C
MMTV LTR
MMTV
Vector
HindIII
Myc1 TAG
EWS-PEA3
gene
EcoR1
SV40 Poly A

(Figure 2.1-A) and subsequently shuttled into the pRc-RSV (Figure 2.1-B) and MMTV plasmids (Figure 2.1-C).

Table 2.1 List of EWS-PEA3 Fusion Constructs

Full Name	Abbreviated Name
EWSΔN344PEA3	E334P
EWSΔN304PEA3	E304P
EWSΔN268PEA3	E268P
EWSΔN244PEA3	E244P
EWS/PEA3	EP

Construction of pCanMyc1-EWS. The pCanMyc1-EWS vector constitutes the backbone into which all the EWS-PEA3 constructs were subcloned. The EWS sequences encoding amino acids 1-264 were PCR amplified from the plasmid pKS$^+$- EWS-Fli-1 using the primers AB 6877 and AB 7112. The amplified EWS product (808 base pair) contained BglII restriction endonuclease cleavage sites at both the 5' and 3' termini, enabling insertion at the BamH1 site within the pCanMyc1 multiple cloning site. The final product was sequenced (Mobix Central Facility) through the EWS insert and found to contain no mutations.

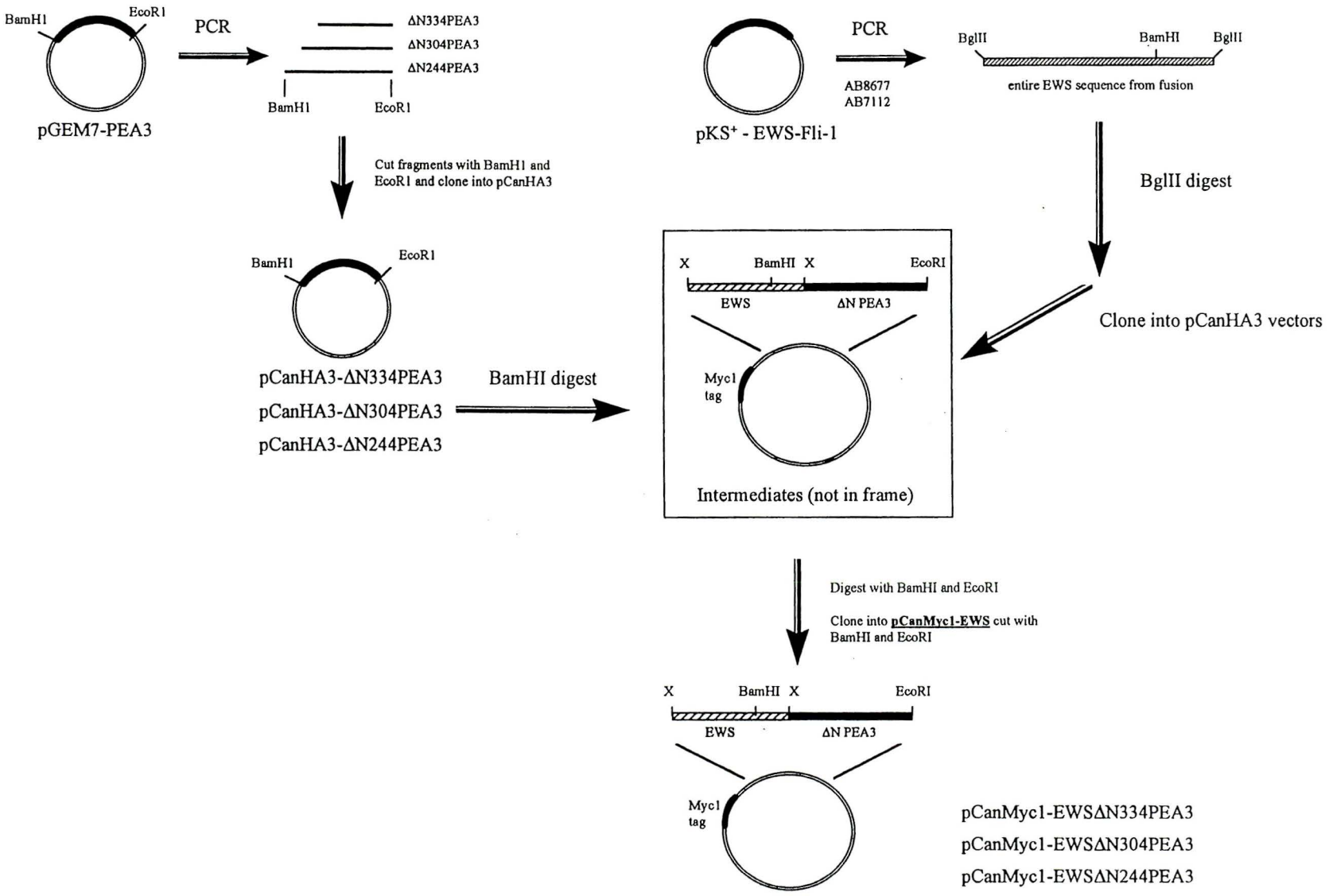
Table 2.2 List of Single Stranded Oligonucluotide Primers Utilized for Polymerase Chain Reactions

PRIMER NAME	PRIMER SEQUENCE	USAGE
AB 5744	5' - CG GGA TCC CGG GGT GCC TTA CAA - 3'	5' primer for ΔN334PEA3
AB 5745	5' - CG GGA TCC CAG AGG TAC CCA GGG - 3'	5' primer for ΔN2444PEA3
AB 5745	5' - CG GGA TCC GAT GTC TGC ATT GTC - 3'	5' primer for ΔN304PEA3
AB 5747	5' - G GAA TTC CTA GTA AGA ATA TCC ACC - 3'	3' primer for ΔN334PEA3, ΔN304PEA3, ΔN244PEA3
AB 6877	5' - GA AGA TCT ATG GCG TCC ACG GAT - 3'	5' primer for EWS
AB 7112	5' - GA AGA TCT CTG CTG CCC GTA GCT GC - 3'	3' primer for EWS
AB 8608	5' - AGC TAT TCC TCT ACA CAG - 3'	3' primer for EWS (overlap extension Primer A)
AB 8609	5' - GCA GAA TTC CTA GTA AGA ATA TCC - 3'	3' primer for PEA3 (overlap extension Primer D)
AB 8610	5' - TCC AGG GAC ATT CTG CTG CCC GTA GCT GCT - 3'	3' primer for EWS (overlap extension Primer B)
AB 8611	5' - GGG CAG CAG AAT GTC CCT GGA TGT GCA TCA ATG - 3'	5' primer for PEA3 (overlap extension Primer C)
AB 8180	5' - GA AGA TCT ATG GAC GGG ACT ATT - 3'	5' primer for mouse Fli-1
AB 8181	5' - GC TCT AGA CTA GTA GTA GCT GCC - 3'	3' primer for mouse Fli-1

Construction of E244P, E304P and E334P. Amino terminal deletions of PEA3 (ΔN244PEA3, ΔN304PEA3 and ΔN334PEA3) were constructed by PCR amplification of the pGEM7-PEA3 template. To facilitate cloning, a BamH1 restriction site was added at the 5' end of all the PEA3 deletions by the forward primers AB 5745 (ΔN244PEA3), AB 5746 (ΔN304PEA3) and AB 5744 (ΔN334PEA3). The common reverse primer, AB 5747, added an EcoR1 restriction site at the 3' end of all the genes. The amplified PEA3 fragments were then subcloned into the BamH1 and EcoR1 restriction sites of the pCanHA3 vector. To create intermediate EWS-PEA3 fusions, EWS sequences were PCR amplified (AB 6877, AB 7112) and inserted upstream of the PEA3 deletions at the BamH1 restriction site. The ligation of EWS to PEA3 utilizing the cohesive ends generated following BglII and BamH1 restriction digest resulted in permanent fusions at the EWS-PEA3 junction site. These intermediate vectors, pCanHA3-E244P, pCanHA3-E304P and pCanHA3-E334P, contained in-frame EWS-PEA3 junctions, however, were not in frame with the upstream sequences encoding the hemagglutinin (HA) epitope tag. To construct expressible EWS-PEA3 chimeric genes, the intermediate vectors pCanHA3-E244P, pCanHA3-E304P and pCanHA3-E334P were cut with BamH1 and EcoR1 to excise subfragments containing a portion of EWS fused to the PEA3 deletions. These partial fragments were then subcloned into the pCanMyc1-EWS vector (described previously) within the BamHI and EcoRI sites of the multiple cloning region. A schematic representation of the construction of pCanMyc1-E244P, pCanMyc1-E304P and pCanMyc1-E334P is depicted in Figure 2.2.

Figure 2.2 Schematic representation of the Cloning Strategy used to construct EWSΔN334PEA3, EWSΔN304PEA3 and EWSΔN244PEA3

PEA3 deletion mutants (ΔN334PEA3, ΔN304PEA3 and ΔN244PEA3) were created by PCR amplification and inserted into the pCanHA3 vector. The entire region of EWS contained within the EWS/Fli-1 fusion gene was PCR amplified and subsequently inserted upstream of the PEA3 genes within the pCanHA3 vectors. Since the EWS-PEA3 fusion genes are not in frame with the HA epitope of the pCanHA3 vectors, partial fragments of each chimeric gene were excised with BamH1 and EcoR1 and subcloned into the pCanMyc1-EWS vector backbone.


BamH1
EcoR1
pGEM7-PEA3
PCR
ΔN334PEA3
ΔN304PEA3
ΔN244PEA3
BamH1
EcoR1
Cut fragments with BamH1 and EcoR1 and clone into pCanHA3
BamH1
EcoR1
pCanHA3-ΔN334PEA3
pCanHA3-ΔN304PEA3
pCanHA3-ΔN244PEA3
BamHI digest
pKS+ - EWS-Fli-1
PCR
AB8677
AB7112
BglII
BamHI
BglII
entire EWS sequence from fusion
BglII digest
Clone into pCanHA3 vectors
X
BamHI X
EcoRI
EWS
ΔN PEA3
Myc1 tag
Intermediates (not in frame)
Digest with BamHI and EcoRI
Clone into pCanMyc1-EWS cut with BamHI and EcoRI
X
BamHI X
EcoRI
EWS
ΔN PEA3
Myc1 tag
pCanMyc1-EWSΔN334PEA3
pCanMyc1-EWSΔN304PEA3
pCanMyc1-EWSΔN244PEA3

Construction of EWS-PEA3. To construct a fusion of EWS to the full length PEA3 cDNA, EWS was amplified by PCR (AB 6877, AB 7112) from the pKS^{+}-EWS-Fli-1 template, digested with BglII and inserted at the BamH1 site of pGEM7-PEA3. A portion of EWS/PEA3 (last 60 base pairs of EWS permanently fused to full length PEA3 cDNA sequence) was excised from pGEM7-EWS/PEA3 with BamH1 and EcoR1 and inserted into the pCanMyc1-EWS expression vector.

Construction of E268P. To construct the murine analog, E268P, of the characterized EWS-hPEA3 fusion found in a Ewing's sarcoma tumor (Urano *et al.*, 1996), a PCR based method for creating mutations called overlap extension was used. The E268P chimera was constructed utilizing the pCanMyc1-E244P expression plasmid as a template for overlap extension, which resulted in a 69 base pair deletion within PEA3 encoding the amino acids 244 to 267. In addition, a novel asparagine residue was added at the junction of EWS and ΔN268PEA3. The 650 base pair overlap extension product was then digested with BamHI and EcoRI and placed downstream of EWS in the pCanMyc1-EWS vector. The overlap extension protocol used to create pCanMyc1-E268P is schematically represented in Figure 2.3 and discussed in more detail in Section 2.1.1.

Subcloning of PEA3 into pCanMyc1 Expression Vector. PEA3 was subcloned into the pCanMyc1 eukaryotic expression vector to enable gene expression from the same promoter as the EWS-PEA3 chimeric genes. The PEA3 cDNA sequence was excised from pGEM7-PEA3 by sequential digestion with SacI, treatment with Klenow to blunt the

3' SacI overhang and followed by cleavage with BamH1 restriction endonuclease. The pCanMyc1 plasmid was digested with BamH1 and EcoRV to produce an insert site flanked by one cohesive and one blunt end, which would enable insertion of the PEA3 cDNA fragment.

Subcloning of EWS-Fli-1 and Fli-1 into pCanMyc1. pKS^{+}-EWS-Fli-1 was digested with the restriction endonucleases, BamHI and XhoI to release a 720 base pair fragment representing the Fli-1 portion of the fusion gene. The Fli-1 cDNA fragment was then inserted downstream of EWS at the BamHI and XhoI restriction sites of pCanMyc1-EWS. The final construct was named pCanMyc1-EWS-Fli-1.

To construct pCanMyc1-Fli-1, the entire Fli-1 cDNA sequence was PCR amplified from pECE-Fli-1 (obtained from A. Bernstein) using the primers AB 8180 and AB 8181. The amplified gene containing BglII and XbaI restriction sites at its 5' and 3' termini, respectively, was then digested and inserted into the pCanMyc1 vector.

Shuttle of Myc-tagged constructs into the pRc-RSV Expression Vector. To direct the transcriptional expression of the constructs from an alternate promoter, the Rous sarcoma virus long terminal repeat (RSV LTR), all the constructs including the myc tag sequences were subcloned into the eukaryotic expression vector, pRc-RSV (Invitrogen; Figure 2.1-B). Myc1-EP, Myc1-E244P, Myc1-E268P, Myc1-E304P, Myc1-E334P and Myc1-Fli-1 sequences were excised from their respective pCan vectors with HindIII / XbaI digestion and subcloned directly into pRc-RSV. Myc1-EWS-Fli-1 sequence was released from the

pCanMyc1-EWS-Fli-1 vector by HindIII digestion and inserted into the HindIII cloning site of pRc-RSV.

Shuttle of Myc-tagged constructs in the MMTV Expression Vector. To enable the use of the EWS-PEA3 chimeras in future transgenic mouse experiments, the myc-tagged constructs were subcloned into the MMTV eukaryotic expression vector (obtained from W. Muller; Figure 2.1-C). The MMTV-Myc1-E244P, -E268P, -E304P and -E334P plasmids were constructed by the excision of the Myc1-E244P, Myc1-E268P, Myc1-E304P and Myc1-E334P sequences with HindIII and EcoRI from their respective pCan vectors and insertion into the HindIII / EcoR1 cloning site of MMTV vector.

2.1 Nucleic Acid Techniques

The section outlines the DNA manipulation techniques utilized in the construction and cloning of the previously described plasmids.

2.1.1 Polymerase Chain Reaction (PCR) Protocols

Amplification of DNA segments by PCR. In a reaction volume of 100 µL, 20 ng of template DNA was mixed with 1 µM of each single stranded oligonucleotide primer, 1.5 mM magnesium chloride, 100 µM total of deoxyribonucleotides (dATP, dCTP, dGTP, dTTP) (Pharmacia) and buffering system (20 mM Tris-HCl, pH 8.4, and 50 mM KCl)

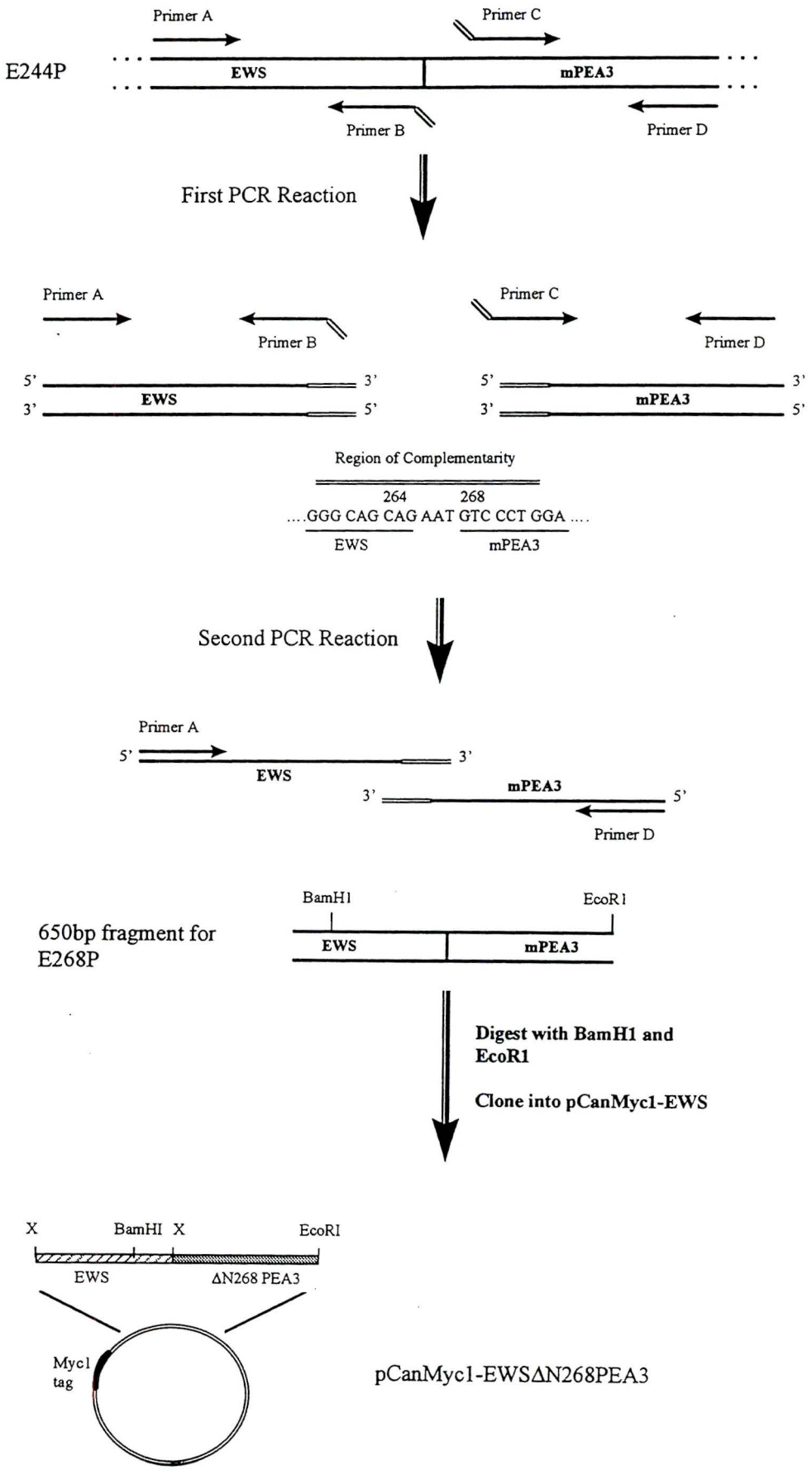
(GibcoBRL). Prior to thermocycling, 5 units of *Taq* DNA polymerase I (Gibco BRL) was added. The reaction was conducted in the Perkin Elmer GeneAmp PCR system 9600 which was programmed to perform 25 cycles of denaturation (95°C, 35 seconds), annealing (60°C, 35 seconds), and primer extension (72°C, 35 seconds). Product validation was achieved by visualization under ultraviolet light following electrophoresis of 10 μL of each reaction mixture in an 1% agarose/TAE gel containing 0.5 μg/mL of ethidium bromide. Products obtained were stored at 4°C.

Overlap Extension PCR The overlap extension polymerase chain reaction protocol was first described as a novel method of site-directed mutagenesis (Ho *et al.*, 1989). The first round of PCR involves the amplification of the two DNA fragments with two independent sets of primers. The two internal primers contain complementary regions of overlap and may include novel sequence to generate point mutations. The two generated products from the first reaction are then purified and utilized in a second PCR reaction, in which the amplification is initiated from the two external primers of the first reaction. This method was utilized specifically in the construction of E268P.

First round of PCR Amplification: pCanMyc1-E244P template was used in two independent PCR reactions. Reaction 1 amplified a 200 base pair (bp) fragment of EWS using 1 μM of AB 8608 and AB 8610 and Reaction 2 amplified a 650 bp fragment of PEA3, using 1 μM of AB 8611 and AB 8609. Both Reaction 1 and 2 were performed

Figure 2.3 Diagram of Overlap Extension Protocol Used to Construct EWSΔN268PEA3

(refer to Section 2.1.1)


Primer A
Primer C
E244P
EWS
mPEA3
Primer B
Primer D
First PCR Reaction
Primer A
Primer B
Primer C
Primer D
5'
3'
EWS
mPEA3
Region of Complementarity
264
268
....GGG CAG CAG AAT GTC CCT GGA ....
EWS
mPEA3
Second PCR Reaction
Primer A
EWS
mPEA3
Primer D
650bp fragment for E268P
BamH1
EcoR1
EWS
mPEA3
Digest with BamH1 and EcoR1
Clone into pCanMyc1-EWS
X
BamHI X
EcoRI
EWS
ΔN268 PEA3
Myc1 tag
pCanMyc1-EWSΔN268PEA3

using identical thermocycling conditions. 20 ng of pCanMyc1-E244P DNA was mixed with the required pair of single stranded oligonucleotide primers, 1.5 mM magnesium chloride, 100 μM total of deoxyribonucleotides (dATP, dCTP, dGTP, dTTP) (Pharmacia) and buffering system (20 mM Tris-HCl, pH 8.4, and 50 mM KCl) (GibcoBRL) in a 100 μL reaction volume. Prior to thermocycling, 5 units of *Taq* DNA polymerase I (Gibco BRL) was added. The reaction was conducted in the Perkin Elmer GeneAmp PCR system 9600 which was programmed to perform 25 cycles of denaturation (95°C, 35 seconds), annealing (55°C, 35 seconds), and primer extension (72°C, 35 seconds). One-fifth of the PCR products (20 μL) were electrophoresed on a 1.2% agarose/TAE gel containing 0.5 μg/mL of ethidium bromide. Fragments corresponding to the expected products for EWS and PEA3 amplification were excised and extracted using a gel extraction kit (QIAGEN) in a 50 μL elution volume.

Second round of PCR amplification: 2 μL each of purified EWS and PEA3 template from first round amplification were combined with 2 μL of the primers AB 8608 and AB 8609 in a 100 μL reaction volume also containing 1.5 mM $MgCl_2$, 5 mM each deoxyribonucleotide (dATP, dCTP, dGTP, dUTP) (Pharmacia) and 5 units of Taq polymerase (GibcoBRL). The thermocycling conditions were identical to first round amplification. The expected 650 base pair amplified fragment was confirmed by electrophoresis on a 1.0 % agarose/TAE gel and visualization under UV light.

PCR Product Extraction. PCR products were purified from contaminating primers and nucleotides by utilizing the QIAquick PCR Purification Kit (QIAGEN). The protocol used was as per manufacturer's directions and resulted in the concentration of the PCR products in 30-50 μL of 10 mM Tris-HCl, pH 8.0.

2.1.2 Restriction Endonuclease Digestion of Nucleic Acids

1-10 μg of supercoiled DNA plasmid and/or purified linear PCR products were generally digested at 37°C (or specified permissive enzyme temperature) for one to several hours. Simple digests were performed in 20-30 μL reaction volumes, in which, the DNA was diluted in the appropriate restriction buffer supplied by the enzyme manufacturer (GibcoBRL; Pharmacia; Boehringer Mannheim) and incubated with 1-2 units of each restriction endonuclease. The use of two restriction enzymes possessing different buffer requirements for optimal activity necessitated sequential restriction digests separated by the precipitation of the DNA with ethanol. Ethanol precipitations were performed by the dilution of digest volume to 100 μL, addition of 10 μL of 3M sodium acetate and the addition of 2 volumes of cold ethanol. The DNA was allowed to precipitate at -20°C for 30 minutes prior to centrifugation at 13,000 rpm for 30 minutes. The dry DNA pellets were resuspended in 17 μL of TE buffer (10 mM Tris-HCL, pH 8.0; 1 mM EDTA) and the second digest performed.

To generate blunt ends following restriction endonuclease digestion, Klenow, the large fragment of *Escherichia coli* DNA polmerase I (GibcoBRL), was used. 1-5 units of

Klenow was added to the reaction mixture with 2 μM of deoxyribonucleotides (0.5 μM each dATP, dTTP, dGTP and dCTP; Pharmacia) and allowed to incubate for 30 minutes at 30°C. Klenow was deactivated by incubation at 70°C for 15 minutes and removed by extraction with TE-buffered phenol:chloroform:isoamyl alcohol (25:24:1). The DNA was ethanol precipitated and resuspended in TE buffer.

Plasmid vectors digested with only one restriction endonuclease prior to cloning were dephosphorylated to hinder re-circularization and increase cloning efficiency. Phosphatase buffer was added to the digested plasmid DNA with 0.5 units of calf alkaline intestinal phosphatase (CIAP; various sources) and the reaction was allowed to incubate for 30 minutes at 37°C. The CIAP was inactivated at 70°C for 15 minutes and phenol extracted as described previously.

To separate cleaved DNA fragments, the digestion reactions were electrophoresed at 80-100 volts on a 0.8-1.2 % agarose (GibcoBRL) gel in TAE buffer (40 mM Tris-HCl, 20 mM acetate, 1 mM EDTA, pH 8.0), containing 0.5 μg/mL of ethidium bromide. Agarose gels were visualized under ultraviolet light, the desired DNA fragments excised and eluted from agarose gel slices using the QIAquick Gel Extraction Kit (QIAGEN).

2.1.3 Cloning of Chimeras

Cloning of PCR amplified or restriction endonuclease cleaved DNA fragments into linearized plasmid vector was achieved by incubation of 10:1 ratio of insert to linearized plasmid with T4 DNA ligase (10,000 units/reaction, New England Biolabs) in ligation buffer containing 10 mM ATP overnight at 4°C. The next day, the entire ligation

reaction (15 μL) was transformed into 100 μL of Epicurian Coli® JM109 competent cells (Stratagene) [e14$^-$(McrA$^-$)recA1 endA1 gyrA96 thi-1 hsdR17 ($r_k^- m_k^+$) supE44 relA1 Δ(lac-proAB) {F' traD36 proAB lacI^{q}ZΔM15]] which had been thawed on ice and allowed to incubate with 0.8 μL of β-mercaptoethanol (1.42 M) for 10 minutes on ice. The competent bacteria and ligation reaction were then allowed to incubate for 30 minutes on ice prior to heat shock at 42°C for 45 seconds and then an additional two minutes on ice. Transformed bacteria were then grown for 40 minutes at 37°C with shaking in 1 mL of SOC media (per litre: 20.0 g tryptone, 5.0g of yeast extract; 0.5 g NaCl; 10 mL of 1 M $MgCl_2$ and 10 mL of 1 M $MgSO_4$; to 100 mL add 2 mL of 20% (w/v) glucose). The entire transformed bacterial culture was then spun down and the pellet resuspended in 100 μL of SOC media and plated onto LB (Luria-Bertolli) agar plates containing 80 μg/mL of ampicillin. The plates were incubated in an inverted position overnight at 37°C.

2.1.4 Verification of Clones

To isolate the recombinant plasmid clones containing the desired ligation product, independent bacterial colonies were picked using sterile technique to inoculate 3 mL of LB media containing ampicillin (80 μg/mL). The cultures were grown for 15-18 hours at 37°C with shaking. The extraction of plasmid DNA was performed by the alkaline lysis method (Sambrook *et al.*, 1989) or by use of QIAGEN plasmid Mini purification kit.

The isolated plasmid DNA was characterized by single or multiple restriction endonuclease digestion. Bacterial clones expressing the correct plasmid were frozen down in 15% glycerol stocks and stored at -80°C.

2.1.5 Large Scale Plasmid Preparation

Larger quantities of plasmid DNA was isolated using either a commercial plasmid preparation kit (QIAGEN, Plasmid Maxi Kit) or by the traditional alkaline lysis of bacteria followed by high speed centrifugation (Sorvall ultracentrifuge) in a cesium chloride gradient (Sambrook *et al.*, 1989). The isolated plasmid DNA was resuspended in TE buffer, pH 8.0. The preparation concentration was determined by measurement of absorbance at a wavelength of 260 nm and purity by the ratio of absorbance at 260 and 280 nm.

2.2 Techniques of Recombinant Protein Analysis

2.2.1 Transfection Method for Transactivation Assays

One day prior to transfection, COS cells were seeded at 1.0×10^5 cells per 35 mm plate in Dulbecco's Modified Eagles Medium (DMEM) containing 10% fetal bovine serum and maintained at 37°C in a humidified incubator. Cells were transiently transfected utilizing a commercial reagent called LipofectAMINE™ (GibcoBRL); a 3:1 (w/w) ratio of 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) to dioleoylphosphatidyl ethanolamine (DOPE) in water.

DOSPA is a polycationic lipid shown to enhance DNA uptake into eukaryotic cells. To create DNA-liposome complexes, the quantity of effector and reporter plasmid DNA was diluted in a 100 μL volume of serum/antibiotic-free DMEM. The total DNA concentration was maintained at 2 μg per transfection by the addition of sheared salmon sperm DNA carrier. Sufficient lipofectAMINE reagent for all transfection reactions was diluted in DMEM to ensure that 6 μL of lipid reagent was delivered per transfection. Following the addition of 100 μL of diluted LipofectAMINE reagent, DNA-liposome complexes were allowed to form for 30 minutes at room temperature and the reaction volume increased to 1 mL by the addition of 800 μL of DMEM. Prior to overlaying the transfection mixture, the cells were washed twice with 2 mL of DMEM to remove residual traces of serum and antibiotics which interfere with DNA uptake during liposome-mediated transfection. Duplicate plates were then overlayed with 1 mL of the liposome-DNA complexes and allowed to incubate at 37°C, 5% CO_2 for 4.5 hours. Each transfection set was performed in quadruplicate to allow for duplicate processing of samples for reporter assay or Western blot analysis.

Following the 4.5 hour transfection period, the DNA-liposome mixtures were then removed from the plates and the cells washed twice with 5 mL of DMEM containing 10% fetal bovine serum to facilitate residual liposome-complex removal and once with phosphate buffered saline (1xPBS). The cells were supplemented with 2 mL of DMEM (with or without serum) and maintained for an additional 24 hours at 37°C, 5% CO_2.

2.2.2 Cell Lysate Preparation

Twenty four hours post transfection, cells were harvested for luciferase assay and Western blot analysis. To prepare samples for luciferase assays, the plates were removed from the incubator and media aspirated. Cells were washed twice with 5 mL of room temperature 1x PBS and lysed directly on the plate using 200 μL of Reporter Lysis Buffer (Promega) for 15 minutes at room temperature. Cells were then dislodged from the plates using a Costar cell scraper and the entire contents transferred to a microcentrifuge tube. Cellular debris was pelleted by centrifugation at 13,000 rpm for 30 seconds and the supernatants transferred to a new tube. Samples were assayed for luciferase activity immediately or stored at -80°C.

To correlate transactivation ability with protein abundance, the duplicate plate from each transfection set was lysed for Western analysis. To harvest, cells were washed twice with cold phosphate buffered saline and incubated with 200 μL of NP-40 lysis buffer (1.0% Nonident P-40 detergent, 400 mM NaCl, 50 mM Tris pH 7.5 and 5 mM EDTA; containing the protease inhibitors aprotinin (20 μg/mL), pepstatin (20 μg/mL), leupeptin (10 μg/mL) and PMSF (200 μg/mL)) for 20 minutes on ice. The cellular lysates were collected from the plates and cellular debris was pelleted by cold centrifugation for 5 minutes at 13,000 rpm. Supernatants were transferred to new microcentrifuge tubes and stored on ice for immediate use, or at -80°C for subsequent processing.

2.2.3 Luciferase Assays

To assay cellular lysates for luciferase activity, 20 µL of each extract was aliquoted in duplicate into luminometer tubes (Sarstedt) and allowed to equilibrate to room temperature. A Berthold Lumat LB 2401 luminometer was used to measure the relative light intensity (or RLU, relative light units) emitted from the samples over a 10 second period following the injection of 100 µL of luciferase assay reagent (Promega). The components of the luciferase assay reagent include 20mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2{\cdot}5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM dithiothreitol (DTT), 270 µM Coenzyme A, 470 µM luciferin and 530 µM adenosine triphosphate (ATP), pH 7.8.

2.2.4 Protein Quantitation

The protein concentration in cellular extracts processed for luciferase assay or Western analysis was determined by the Bradford method for protein quantitation (1976). The Bradford assay is a quantitive colorimetric method which utilizes the protein-binding compound, Coomassie® Brilliant Blue G-250, and a spectrophotometer to measure the changes in absorbance at 595nm. Each sample was assayed in duplicate with 5 µL of lysate in 1 mL of diluted Bradford reagent (BioRad). The absorbances of each sample were measured in a spectrophotometer set at 595 nm. Standard curves were constructed using serial dilutions of a 1 mg/mL bovine serum albumin (BSA) stock solution.

2.2.5 Data Analysis

To determine the relative luciferase activity (RLA) in a particular sample, the following calculation was used:

$$RLA = \frac{\textit{(Relative Light Units (RLU))}}{\textit{(Protein Concentration, } \mu g/\mu L\textit{) x 20 } \mu L \textit{ assay volume}}$$

(where both RLU and protein concentration (Bradford assay) were measured in duplicate)

Within a given experiment, data measuring the activity of each sample construct was obtained in duplicate from two independent transfection reactions. Results shown represent the average (or mean) RLA obtained across the given number of experiments. Variation in the measured relative luciferase activities of each sample construct both within and between experiments was determined by calculating the standard error associated with the average RLA. Standard error (SE) is defined as:

$$SE = \frac{\textit{Standard deviation}}{\sqrt{n}\ \textit{(n= total number of data measurements)}}$$

Data is presented graphically as the average RLA (indicated as the average RLU/ μg protein) with error bars representing the standard error associated with the mean.

2.2.6 Western Analysis

Proteins were resolved by electrophoresis on sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE) as described in Current Protocols (1987). The resolving gel was composed of 10% polymerized acrylamide (29:1 ratio of acrylamide to N,N'-methylene-bis-acrylamide) in buffer containing 375 mM Tris, pH 8.8 and 0.1% SDS. The concentration of the stacking gel was 5% acrylamide in 125 mM Tris, pH 7.6 and 0.1% SDS. Polymerization was catalysed in the presence of 0.10% ammonium persulphate (Sigma) and N,N,N',N'-tetramethylethylenediamine (TEMED, GibcoBRL). Prior to gel loading, the desired protein quantity (10-20 μg of COS cell lysate) was boiled in the presence of a 4x Boiling Buffer (12 mM Tris, 6 % (w/v) SDS, 6% (w/v) β-mercaptoethanol, 3% (w/v) glycerol and bromophenol blue, pH 6.9) for 5 minutes. Samples were loaded alongside 20 μL of pre-stained low molecular weight marker (BioRad) and electrophoresed for 15 hours at 30-70 volts or for shorter periods of time at 120 volts. The electrophoresis was performed in running buffer containing 10 mM Tris-HCL, 5 mM glycine and 0.7% SDS.

Following electrophoresis, the separated proteins were transferred to polyvinylidene difluoride (PDVF) or nitrocellulose membranes by electroblotting. Prior to use, Immobilon-P membranes (PDVF, Millipore) were pre-wetted in methanol for 1 minute, rinsed in water and then equilibrated in transfer buffer (20 mM Tris-HCL, 150 mM glycine, 18.2% (v/v) methanol, pH 8.0) for 20 minutes. Nitrocellulose membranes were wetted in transfer buffer just prior to transfer apparatus assembly. The transfer

sandwich was assembled as follows. Two 15 x 17 cm filter papers (Whatman 3MM) were soaked briefly in transfer buffer and placed on top of a Scotch-Brite pad supported by a plastic transfer case. The 10% SDS-PAGE gel was removed from the electrophoresis appartus, the stacking gel removed, and placed onto the filter paper. The Immobilon-P or nitrocellulose membrane was then positioned on the polyacrylamide gel, ensuring the absence of air pockets. This sandwich was succeeded by an additional two filter papers, Scotch-Brite pad and plastic case. The apparatus was positioned in a BioRad tank containing transfer buffer to enable transfer of proteins from the polyacrylamide gel to the membrane. The electroblot was performed at 60 volts for 4.5 hours and 4°C.

Following transfer, the membrane was incubated in blocking buffer (BB) overnight at 4°C or two hours at room temperature. BB is a 5% solution of skim milk powder (Carnation) in TBS-T containing 10 mM Tris pH 7.3, 150 mM NaCl and 0.05% Tween-20 (Sigma). To detect protein, the blocked membrane was incubated with 10 mL of the appropriate primary antibody in BB (Table 2.2.1) for 2 hours with rotation. The unbound or excess primary antibody was removed with three successive washes in 100 mL of TBS-T for 10 minutes each at room temperature.

Table 2.2.1 Primary Antibody Description and Source

Primary Antibody	Epitope Recognition	Dilution	Reference/ Source
Mycl- 9E10 (Primary monoclonal)	- Residues 408-439 of human c-myc - MEEQKLISEEDLL epitope in pCanMyc vector series	1:1000 or 3.0-5.0 μg/mL	Evans *et al.*, 1985
MP13 (Primary monoclonal)	- Recognition mapped to residues 157-256 of murine PEA3 - Exact sequence recognition unknown	1:1000	Hassell *et al.*, unpublished
MP16 (Primary monoclonal)	-Recognition mapped to residues 256-337 of murine PEA3 -Exact sequence recognition unknown	1:1000	Hassell *et al.*, unpublished
MP113 (Primary monoclonal)	-Recognition mapped to residues 337-380 of murine PEA3 -Exact sequence recognition unknown	1:100	Hassell *et al.*, unpublished

Detection of Proteins by Chemiluminescence. To detect proteins using the BM chemiluminescence (ECL) system (Boehringer Manneheim), the membrane was incubated in BB containing a secondary goat α-mouse polyclonal antibody conjugated to horse radish peroxidase (HRP) enzyme for one hour at room temperature with rotation. The membrane was then washed several times with 100 mL volumes of TBS-T for 30 minutes. During membrane washing, 10 mL (25-30 μL/cm ^{2}) of the chemiluminescence blotting substrate containing hydrogen peroxide and the diacylhydrazide, luminol, was prepared as per manufacturer's protocols and allowed to equilibrate to room temperature. The chemiluminscent substrate was incubated with the membrane for 1 minute, excess

removed and the coated membrane transferred to an X-ray film cassette. BioMax® MR film (Kodak) was exposed to the membrane for time periods of 1 second to 1 hour and developed.

Detection of Proteins by ^{125}I-conjugated Secondary Antibody. To enable protein detection and quantitation, a secondary goat α-mouse antibody conjugated with ^{125}I was used to probe Western blots. Following the initial primary incubation and wash, the membrane was incubated with 5 μCi of radiolabelled secondary antibody in 10 mL of BB for 1 hour at room temperature and with rotation. The membrane was washed several times in large volumes of TBS-T for 30-40 minutes and then sealed between two acetate sheets. The membrane was exposed to Kodak X-Omat AR (XAR) film in cassettes containing intensifying screens for 24 hours to several days. To quantify proteins, the membrane was placed on a phospho-detector screen and exposed for several hours. The screen was scanned and analysed using a PhosphoImager (Molecular Dynamics) which quantifies by volume integration.

Membrane Stripping and Re-Probing. To reprobe a membrane with an alternate monoclonal antibody or to quantify protein levels using the ^{125} I-conjugated secondary antibody, a method outlined in Kaufmann *et al.*, 1987 (Amersham TechTips) was used to strip membranes following the chemiluminescence reaction. The dried membrane was initially washed three times in 100 mL of TBS-T for 10 minutes each and subsequently

incubated with stripping buffer for 30 minutes at 70°C. The stripping buffer was comprised of 62.5 mM Tris-HCl, pH 6.8, 2% SDS and 100 mM β-mercaptoethanol in a 15 mL volume. Following removal of the stripping buffer, the membrane was re-washed with TBS-T as described previously and checked for signal removal by incubation with chemiluminescence substrate and exposure to film. The stripped membrane was then rinsed in TBS-T, re-blocked and processed as desired.

2.3 Cell Culture Techniques

2.3.1 Maintenance of Cell Lines

All cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL) containing the antibiotics penicillin G (sodium salt) (100 units/mL), streptomycin sulphate (100 μg/mL) and the antifungal compound fungizone® (Gibco BRL) (0.25 μg/mL amphotericin B). Media for COS and Rat-1 cells was supplemented with 10% fetal bovine serum (FBS) while NIH 3T3 cells were maintained in DMEM containing 10% calf or donor calf serum (CS). Cells were propagated at 37°C in a humidified CO_2 incubator (5%) to maintain the pH range of the media.

Passage of Cells. The maximum confluence any cell line was allowed to reach was 50 to 60% coverage of plate surface area. To passage, cells were washed twice with 5 mL of phosphate buffered saline solution (1x PBS; 137 mM NaCl, 3.0 mM KCl, 4.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4; pH 7.3) and incubated with a diluted trypsin solution (625

μg/mL in versene: PBS containing 0.53 mM ethylenediaminetetraacetic acid, EDTA) for 2-5 min at 37°C. The dislodged cells were resuspended in DMEM containing the appropriate serum and aliquoted to fresh 100 mm plates (Nunc™) at various dilutions (generally 1/10). Rat-1 and NIH 3T3 cells were maintained only for a limited number of passages to maintain their characterized features. Fresh stocks of both cell lines were thawed for focus assay, G418-selection assay or soft agar assay experiments and used prior to their tenth passage.

Freezing and Storage. To freeze cell lines for storage at -80°C, cells were grown to 85% confluence on 100 mm plates. Cells were washed, trypsinized and resuspended in 5 mL to a final concentration of 6.0×10^6 cells/mL. Cells were pelleted by centrifugation at 500 rpm for 5 minutes, resuspended in 4 mL of calf serum and 1 mL of Tris-buffered dimethylsulphoxide (50% DMSO, pH 6.7) was added. Aliquots of 1 mL per freezing vial and vials stored in the -80°C freezer (Revco).

2.3.2 Focus Assays

To assay the ability of the EWS-PEA3 fusion proteins to mediate the transformation, populations of NIH 3T3 and Rat-1 cells were transfected, allowed to grow to confluent monolayer and maintained for 14-21 days. Transforming ability was scored as the induction of focal growth on the cell population monolayer, indicative of a loss of contact inhibition, a phenotype of transformed cells. Introduction of DNA into Rat-1 and

NIH 3T3 cells were performed by two transfection methods: calcium phosphate mediated transfection and liposome mediated transfection.

Calcium Phosphate Transfection. The calcium phosphate mediated transfection of DNA into mammalian cells is outlined in Graham and van der Eb, 1973. NIH 3T3 or Rat-1 cells were seeded at 2.5 x 10^5 cells per 100 mm plate in DMEM supplemented with 10% calf serum or fetal bovine serum, respectively, one day prior to transfection. Calcium phosphate precipitates were prepared by incubating the given quantities of each plasmid (Table 2.4) with sufficient sheared calf thymus DNA carrier to bring the total DNA concentration to 20 μg per 100 mm plate. Five plates were transfected for each given plasmid and concentration.

Table 2.4 Plasmid DNA and Quantities Transfected into NIH 3T3 and Rat-1 cells for Focus Assays

Plasmid	Transfected Quantity per 100 plate
pCanMyc1-PEA3	0.1, 1.0 or 5.0 μg
pCanMyc1-E244P	5.0 μg
pCanMyc1-E268P	0.1, 1.0 or 5.0 μg
pCanMyc1-E304P	5.0 μg
pCanMyc1-E334P	5.0 μg
pCanMyc1-EWS/PEA3	5.0 μg
pCanMyc1-EWS/Fli-1	5.0 μg
pCanMyc1-Fli-1	5.0 μg
pCanMyc1 (empty vector)	0.1, 1.0 or 5.0 μg
pPyMT-1	0.1 μg

Precipitates were overlayed onto cells and incubated for 4.5 hours at 37°C and 5% CO_2. Following transfection, the media was aspirated from the plates and cells were washed once with PBS containing 1mM EGTA (ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid), twice with PBS, and finally supplemented with DMEM containing either 10% fetal bovine serum (Rat-1 cells) or 10% calf serum (NIH 3T3 cells). Cells were allowed to recover for an additional 24 hours in a 37°C humidified incubator. One day post transfection and every three days thereafter, the media on the plates were changed to DMEM supplemented with only 1 or 2 % of the appropriate serum and maintained for 14-21 days.

Liposome-mediated Transfection. Liposome-mediated stable transfection of NIH 3T3 and Rat-1 cells was performed using a commercial reagent, LipofectAMINE™ (GibcoBRL). Cells were seeded at 1.0×10^5 cells per 35 mm plate one day prior to transfection and incubated with DMEM containing 10% serum at 37°C and 5%CO_2. Prior to transfection, the media was removed and cells were washed twice with 2 mL of DMEM to remove residual traces of serum and antibiotics which interfere with DNA uptake. Cells were then overlayed with 1 mL of the liposome-DNA complex reaction containing plasmid (Table 2.5) and 6μL of lipofectAMINE™ reagent diluted in serum free DMEM for one hour at 37°C, 5%CO_2.

Table 2.5 List of Plasmids and Quantities Transfected into NIH 3T3 and Rat-1 cells for Focus Assay (LipofectAMINE™)

Encoded Protein	Vector	Transfected Quantity per 100 plate
PEA3	pCan, RSV	1.0 or 2.0 μg
E244P	pCan,RSV	1.0 μg
E268P	pCan,RSV	1.0 or 2.0 μg
E304P	pCan	1.0 μg
E334P	pCan	1.0 μg
EWS/PEA3	pCan, RSV	1.0 μg
EWS/Fli-1	pCan, RSV	1.0 μg
Fli-1	pCan, RSV	1.0 μg
Empty vector	pCan, RSV	1.0 or 2.0 μg
pPyMT-1	N/A	0.04 μg

Following transfection, the media was aspirated and cells were washed twice with 2 mL of DMEM containing 10% serum, once with PBS and supplemented again with 2 mL of DMEM containing 10% serum. The following day, each 35 mm plate was trypsinized and split onto two 100 mm tissue culture plates in DMEM containing 10% serum, initially, but reduced to 1% prior to the cells reaching confluence. The cells were refed at three day intervals and maintained in this manner for 14-21 days.

Fixing and Staining of Cells. 21 days post transfection, cells were fixed and stained to visualize and enumerate foci. The cells were washed twice with 5 mL of PBS and fixed in 5-8 mL of 10% buffered formalin phosphate (Fisher Scientific) for 30 minutes at room temperature. The fixing solution was then aspirated and the cells stained overnight with

10 mL of 5% Giesma (Fisher Scientific) / PBS solution. The next day, the stain was removed and plates were rinsed in water and dried in an inverted position.

2.3.3 Selection and Maintenance of Subcloned Cell Lines

To create stable cell lines, NIH 3T3 cells were transfected with 1 μg of the plasmids RSV-E268P, RSV-PEA3, RSV-EWS-Fli-1, RSV-Fli-1 or RSV (empty vector) using lipofectAMINE™ (GibcoBRL) reagent. The transfection protocol is similar to that outlined in Section 2.3.2 for focus assays. Following transfection, the NIH 3T3 cells were allowed to recover for 24 hours at 37°C, 5%CO_2 in 2 mL DMEM containing 10% calf serum. The next day, cells were split onto two 100 mm plates and allowed to recover for an additional 24 hours, after which the media (DMEM + 10% calf serum) was supplemented with 40 μg/mL of G418 sulfate (Geneticin®, GibcoBRL). The RSV plasmid encodes the neomycin resistance marker, thus addition of G418 selects only those cell populations which have integrated and stably express the neomycin resistance gene, and by extension, the encoded genes of interest. Selection was maintained for 5-7 days, or until individual clonal cell populations were obtained. Approximately, 10-12 colonies were picked for each cell line and transferred onto 60 mm plates. Those cell lines which survived further G418 selection, were propagated and frozen down.

2.3.4 Soft Agar Assays

To assay whether any of the isolated cell lines were capable of anchorage independent growth, an indicator of transformation, the NIH 3T3 cell lines were seeded in

soft agar. Cells were trypsinized, counted and resuspended in a 0.3% agarose/ DMEM/ 10% calf serum mixture maintained at 45°C. Final overlay concentrations of 10^2, 10^3, 10^4 or 10^5 cells per plate were plated over 5 mL of 0.6% agarose/DMEM solid base on 60 mm plates and incubated at 37°C, 5% CO_2. Cells were re-fed every three to four days with 2 mL of 0.3% agarose/DMEM mixture containing 10% calf serum. The plates were maintained for 14-21 days and scored for evidence of macroscopic clonal growth in the semi-solid agarose.

2.3.5 Detection of Stable Cell Line Expression by Immunoprecipitation

To detect the stable or constitutive expression of E268P in the isolated NIH 3T3 cell lines, cellular lysates were immunoprecipitated with the Myc1-9E10 monoclonal antibody conjugated to Sepharose®4B. To harvest lysate, media was aspirated off the plates and the cells were washed twice with cold phosphate buffered saline (PBS). The cells were collected in 5 mL of cold PBS and the contents of four 100 mm plates from each cell line were pooled. Cells were pelleted by centrifugation at 1000 rpm/4°C for 5 minutes and resuspended in 250 µL of a hypotonic NP-40 lysis buffer (1.0% Nonident P-40 detergent, 50 mM Tris pH 7.5 and 5 mM EDTA; containing 20 µg/mL aprotinin, 20 µg/mL pepstatin, 10 µg/mL leupeptin and 200 µg/mL PMSF). Following incubation for 5 minutes on ice, the salt concentration of the lysis buffer was increased to 400 mM by the addition of 22 µL of 5 M NaCl. The lysates were maintained on ice for 15 minutes and

then centrifuged at 4°C and 13,000 rpm for 5 minutes to pellet cellular debris. The supernatants were transferred to new microcentrifuge tubes and stored on ice until immunoprecipitation.

Coupling of Myc1-9E10 to Sepharose. The Myc1-9E10 monoclonal antibody was coupled directly to Sepharose since it has been shown to have a low affinity for Protein A and consequently works poorly in immunoprecipitation (Evans *et al.*, 1985). To produce 1.7 mL of Myc1-9E10- Sepharose slurry, 17 mg of the antibody was required. The antibody was dialyzed in coupling buffer (0.1 M $NaHCO_3$, pH 8.3; 0.5 M NaCl) for 48 hours at 4°C with frequent addition of fresh buffer. The cyanogen bromide-activated Sepharose® 4B (0.5 g; Pharmacia) was swollen in 1 mM HCl and washed with an additional 200 mL of 1 mM HCl on a sintered glass funnel. The dialyzed Myc1-9E10 and washed CNBr-Sepharose® 4B were mixed and rotated for one hour at room temperature. Following removal and storage of the antibody-depleted supernatant, the Sepharose beads were washed with 5 mL of coupling buffer to removed excess ligand. The remaining active groups on the Sepharose were blocked with 1.0 M ethanolamine, pH 8.0 for 2 hours. The Myc1-9E10-coupled Sepharose was then washed on a sintered glass funnel with three alternating cycles of 0.1 M acetate buffer, pH 4.0 with 0.5 M NaCl and 0.1 M Tris-HCl buffer, pH 8.0 with 0.5 M NaCl. The dried product was stored in PBS containing 0.5 % sodium azide at 4°C.

Immunoprecipitation. The NIH 3T3 cell line lysates (300 μL volume) were immunoprecipitated with 40 μL of the Myc1-9E10-Sepharose 4B complex for 5 hours at 4°C with rotation. The supernatants discarded, the sepharose beads were washed three times with 500 μL of modified NP-40 lysis buffer (1.0% Nonident P-40, 150 mM NaCl, 50 mM Tris, pH 7.5 and 5 mM EDTA). To prepare samples for SDS-PAGE, the sepharose beads were resuspended in 35 μL of boiling mix and incubated at 100°C for 5 minutes. The electrophoresis and electroblotting of the samples to Immobilon-P membrane was performed as described in 2.2.6. To visualize the protein spectrum pulled down in the immunoprecipitation reaction, the membrane was probed initially with the Myc1-9E10 monoclonal antibody and subsequently with goat α-mouse HRP-conjugated antibody (also as described in 2.2.5). The membrane was incubated with chemiluminescence substrate (Boehringer Mannheim) and exposed to BioMax MR (Kodak) film.

RESULTS

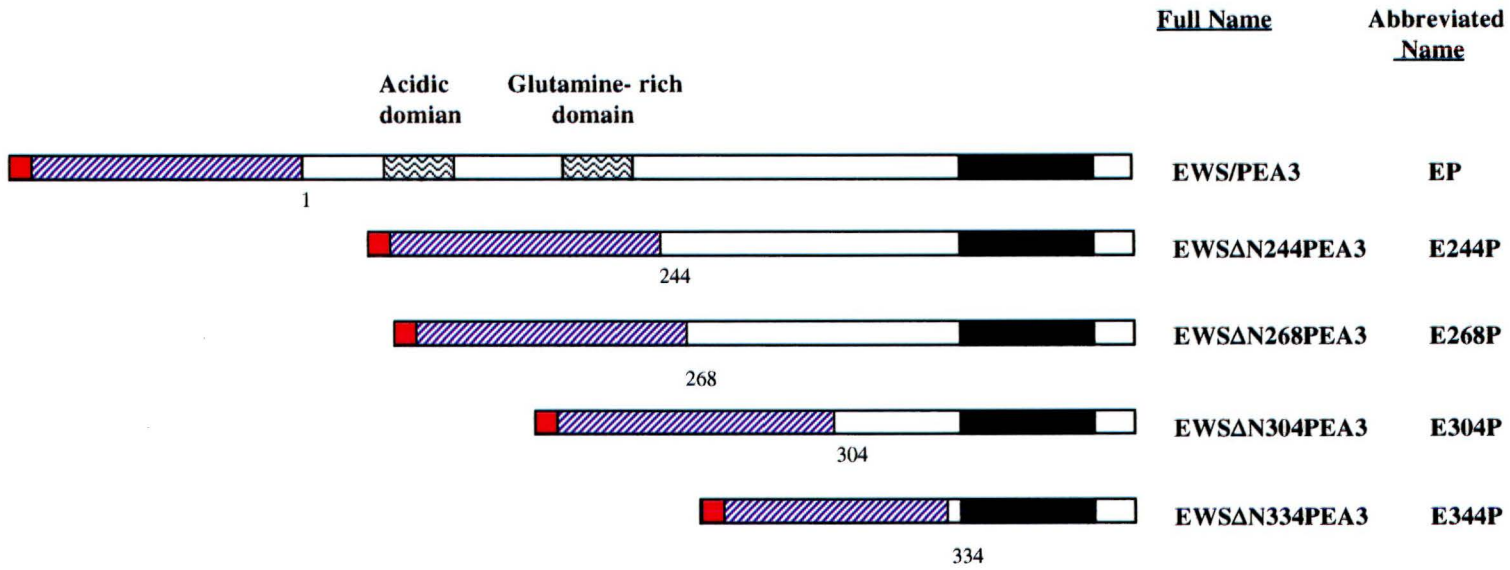
3.0 Introduction

To create a constitutively activated PEA3 protein with increased transcriptional activity and lacking responsiveness to normal regulatory signals, the potent transactivation domain of the EWS protein was artificially fused to the amino terminal domain of PEA3. The motivation for this approach was the isolation of EWS-Ets fusion proteins in the human cancer syndrome, Ewing's sarcoma. These chimeric proteins, composed of the EWS transcriptional activation domain juxtaposed to the DNA binding domains of the involved Ets proteins, appear to function *in vivo* as activated and/or deregulated Ets transcription factors. It was conjectured that the construction of analogous fusions between EWS and murine PEA3 would result in a protein with similar functional attributes.

In all, five EWS-PEA3 chimeric genes were created as previously described (Section 2.0) and are schematically represented in Figure 3.1. The PEA3 gene and four PEA3 gene truncations were fused in frame to the minimum segment of the EWS gene common to all the Ewing's sarcoma translocations. Initially only four EWS-PEA3 chimeras were created, three encoding amino terminal deletions of PEA3 (EWSΔN244PEA3, EWSΔN304PEA3 and EWSΔN334PEA3) and one fusion to the full length PEA3 cDNA (EWS/PEA3). These original PEA3 truncations were designed to

Figure 3.1. Schematic representation of the EWS-PEA3 chimeras.

The five EWS-PEA3 chimeric genes were modelled after the fusion genes resultant from the chromosomal translocations causative of Ewing's sarcoma. In particular, EWSΔN268PEA3 (E268P) is the murine analog of the EWS-hPEA3 fusion reported in Urano *et al.*, 1996. EWS sequences amplified from an EWS-Fli-1 cDNA were fused directly upstream and in-frame with PEA3 and four PEA3 5' deletion mutants. The ensuing chimeric EWS-PEA3 genes encode proteins containing the first 264 amino acids of EWS juxtaposed to complete or partial segments of PEA3. The name of each construct signifies the first PEA3 amino acid present in the construct. All the EWS-PEA3 chimeras were subcloned into three eukaryotic expression vectors: pCanMyc1, pRc-RSV and MMTV (Figure 2.1-A,B,C). All are permanently tagged at their amino terminus with the myc epitope sequence to enable subsequent protein detection.


Full Name
Abbreviated Name
Acidic domian
Glutamine- rich domain
1
244
268
304
334
EWS/PEA3
EP
EWSΔN244PEA3
E244P
EWSΔN268PEA3
E268P
EWSΔN304PEA3
E304P
EWSΔN334PEA3
E344P
Myc tag (MEQKLISEEDG)
EWS (amino acids 1-264)
PEA3 (variable)
PEA3 ETS domain (amino acids 335-416)

remove the acidic and glutamine rich domains, which previous studies had broadly identified as being responsible for the transcriptional activation properties of PEA3. Furthermore, it had been shown previously that amino terminal deletions encompassing both domains resulted in a 30% reduction in PEA3-dependent reporter gene transcription in comparison to full length PEA3 (Cowie and Hassell, unpublished). Thus, other than the removal of both the acidic and glutamine-rich domains, the original three truncations were determined at random.

The fifth chimeric gene was engineered following the report of a fusion between the human PEA3 (hE1A-F) gene and EWS in a rare t(17;22) Ewing's sarcoma translocation (Urano *et al.*, 1996). The characterized breakpoint within the novel EWS-hPEA3 gene (Figure 3.2-A) was duplicated using an overlap extension PCR protocol to delete the sequences encoding PEA3 amino acids 244 and 267 from the EWSΔN244PEA3 construct. The resulting chimera, EWSΔN268PEA3 (E268P), also encodes the novel asparagine residue found at the junction of the EWS and PEA3 in the human translocation chimeric gene (Figure 3.2-B). Note that all of the other EWS-PEA3 fusions are separated by a 6 base pair sequence that was created during their joining by BamHI and BglII restriction endonuclease sites. The intermediate sequences encode two novel glycine and serine residues between EWS and PEA3.

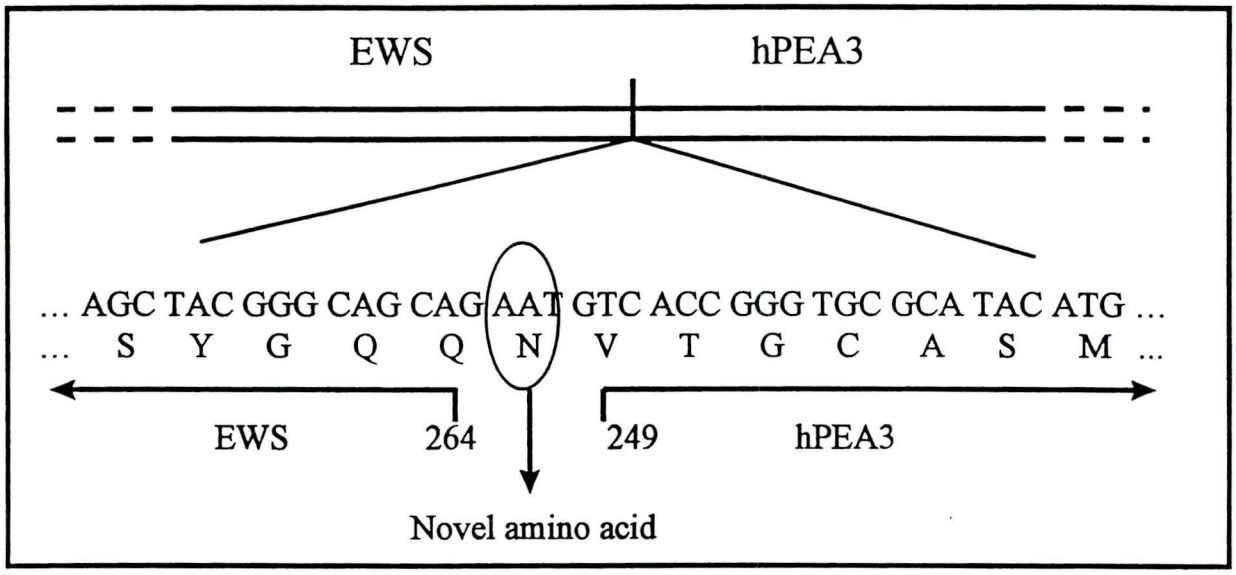
All the EWS-PEA3 chimeric genes were originally constructed and cloned into the pCanMyc1 eukaryotic expression vector which possesses an in-frame myc sequence tag upstream of the cloning site (Figure 2.1-A). The presence of a myc epitope (MEQKLISEEDLG) at the amino terminus of all the fusion proteins allows for their

Figure 3.2 Nucleotide and Predicted Protein Sequence of the Fusions between EWS and PEA3

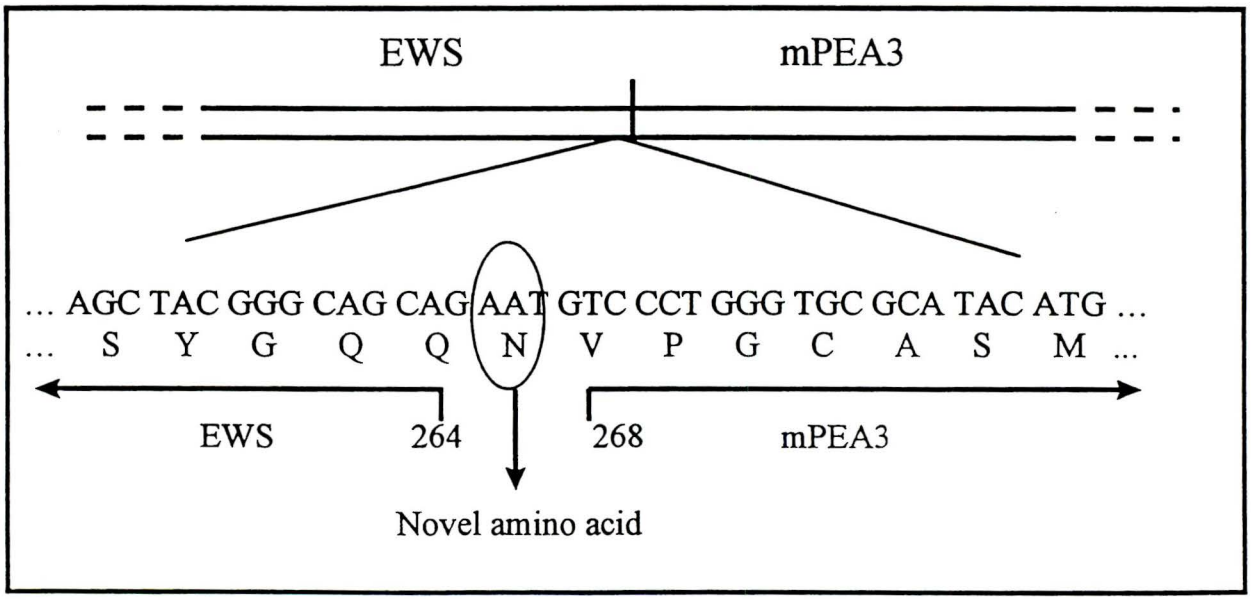
A. Schematic representation of the breakpoint region fusing the EWS and hE1A-F (hPEA3) genes isolated from a Ewing's sarcoma tumor sample (reported in Urano *et al.*, 1996). The isolated fusion gene encodes for a chimeric protein which possesses the first 264 amino acids of EWS fused in frame to an amino terminal truncation of the hPEA3 protein beginning at amino acid 249. The circled region denotes the exact location of the junction between the two genes and represents the insertion of a novel asparagine residue between the two coding regions.

B. Schematic representation of the EWSΔN268PEA3 (also E268P, in short) junction region. Modeled after the human EWS/PEA3 fusion gene depicted in (A), E268P contains the analogous murine PEA3 sequences juxtaposed to the partial human EWS gene. The chimera was constructed by the removal of sequences from the EWSΔN244PEA3 template and the addition of an asparagine codon between the two genes. The '268' reference within the name of the construct denotes the first intact PEA3 residue encoded.

A


EWS
hPEA3
... AGC TAC GGG CAG CAG AAT GTC ACC GGG TGC GCA TAC ATG ...
... S Y G Q Q N V T G C A S M ...
EWS
264
249
hPEA3
Novel amino acid

B


EWS
mPEA3
... AGC TAC GGG CAG CAG AAT GTC CCT GGG TGC GCA TAC ATG ...
... S Y G Q Q N V P G C A S M ...
EWS
264
268
mPEA3
Novel amino acid

detection using the monoclonal antibody, 9E10, which specifically recognizes this epitope (Evans *et al.*, 1985). In addition, all the myc-tagged EWS-PEA3 chimeras were subcloned from the pCanMyc1 vector into the pRc/RSV eukaryotic expression vector. This was done to enable expression of the chimeras from two different eukaryotic promoters: the human cytomegalovirus promoter (pCanMyc1 vector) and the Rous sarcoma virus long terminal repeat (RSV LTR).

3.1 Protein Expression of the Chimeras

To learn whether the EWS-PEA3 chimeric genes were expressed in eukaryotic cells, COS cells were transiently transfected with 1 μg of each DNA encoded within either the pCanMyc1 or pRc/RSV expression vectors. Protein lysates were separated by polyacrylamide gel electrophoresis and transferred to a PDVF membrane for Western analysis. A representative Western blot illustrating the expression of the constructs in COS cells is depicted in Figure 3.3. As is evident in (A) and (B), the recombinant proteins are expressed in COS cells and are readily detected using the myc 9E10 monoclonal antibody. The detection of PEA3 expression directed from the pRc/RSV plasmid (C) was achieved with the PEA3-specific monoclonal antibody, MP16, which recognizes epitope sequences between amino acids 256 and 268 of murine PEA3. This was necessitated by the fact that PEA3 encoded within the pRc/RSV plasmid does not possess a myc epitope tag since it was constructed by U. Ashraf prior to the start of this project. It should be noted that the MP16 monoclonal antibody failed to detect expression of the E268P, E304P, and E334P chimeras since the epitope region of PEA3 recognized by this antibody

Figure 3.3 Detection of Recombinant Protein Expression by Western analysis.

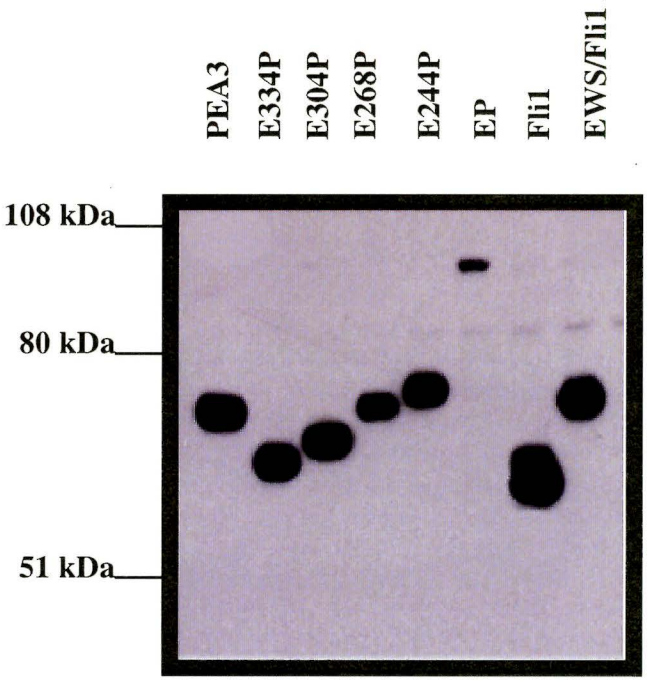
A. Autoradiogram of an immunoblot showing the abundance of the myc-tagged PEA3, EWS-PEA3, Fli-1 and EWS/Fli-1 proteins expressed from the pCanMyc1 vector containing the human cytomegalovirus promoter. Prepared COS cell lysates, following transiently transfection with 1 μg of each plasmid DNA, were electrophoresed in an SDS-polyacrylamide gel. Proteins were transferred to a PDVF membrane and probed with the myc-specific monoclonal antibody, 9E10.

B. Autoradiogram of an immunoblot showing expression of E334P, E304P, E268P, E244P, EP, Fli-1 and EWS/Fli-1 from the pRc/RSV eukaryotic expression vector which directs gene transcription from the Rous sarcoma virus long terminal repeat (RSV LTR). The myc tag present at the amino-terminus of all the proteins enabled their detection in COS cell lysates using the myc 9E10 monoclonal antibody.

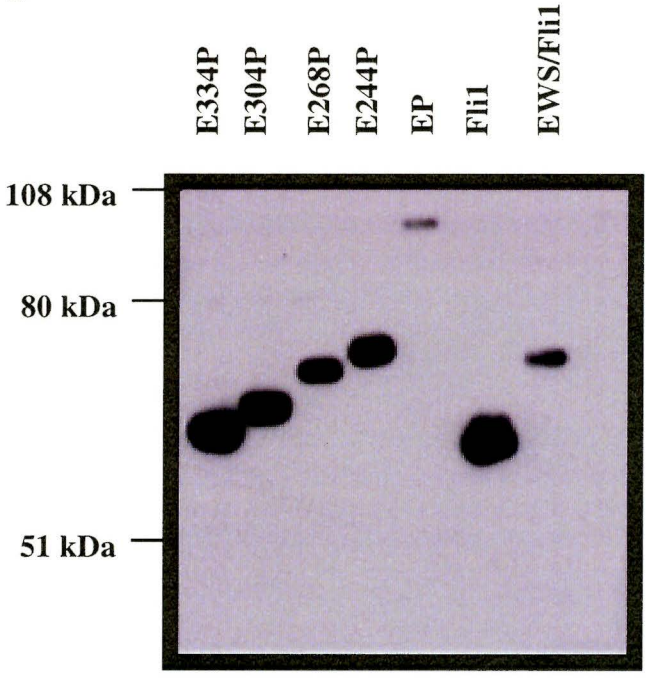
C. Autoradiogram illustrating the expression of PEA3 encoded within the pRc/RSV vector in COS cell lysate. The pRc/RSV-PEA3 plasmid does not contain a myc epitope tag and thus the resultant PEA3 protein can not be detected using the 9E10 antibody. To illustrate PEA3 expression, the immunoblot depicted in (B) was stripped and re-probed with the PEA3-specific monoclonal antibody, MP16. Single lane shown.

The relative molecular weight of each protein can be assessed in reference to pre-stained molecular weight markers whose migration in SDS-PAGE is indicated on the left.

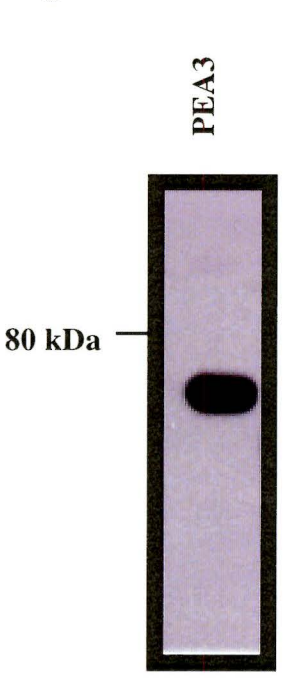
A


PEA3
E334P
E304P
E268P
E244P
EP
Fli1
EWS/Fli1
108 kDa
80 kDa
51 kDa

B


E334P
E304P
E268P
E244P
EP
Fli1
EWS/Fli1
108 kDa
80 kDa
51 kDa

C


PEA3
80 kDa

is not contained in any of these proteins (data not shown). Furthermore, an antibody raised to the ETS domain of PEA3 (MP113), a region common to all the chimeric proteins, also failed to detect their protein expression by Western analysis (data not shown), likely owing to poor antibody affinity. Thus, a PEA3-specific antibody which could detect the expression of all the chimeras simultaneously was not available.

With the exception of EWS fused to full length PEA3, EWS/PEA3, protein abundance of all the other chimeras are detected at equivalent levels to wild type PEA3 in COS cells. The detection of significantly lesser quantities of EWS/PEA3 protein in transfected COS cell lysates is consistent between experiments. It is not known whether this observation is due to an actual decrease in EWS/PEA3 expression either at the transcription or translation level, decreased protein stability, decreased sensitivity in detection caused by protein conformation or inefficient transfer to PDVF membrane.

Comparison of detected protein levels between COS cell lysates transfected with DNA encoded within either the pCanMyc1 or pRc-RSV expression vectors reveals no significant difference in expression. This suggests that in COS cells, transcription directed from the human cytomegalovirus promoter (hCMV) or Rous sarcoma virus long terminal repeat (RSV LTR) occurs at similar rates. The presence of an SV40 origin of replication element within both the pCanMyc1 and RSV vectors enables plasmid replication in COS cells, a kidney fibroblast-like cell line which expresses the SV40 tumor (T) antigens required for SV40 DNA replication (Cell 23: 1981). Thus, expression levels of recombinant proteins encoded within these plasmids are significantly higher in COS cells than would be observed in cell lines that do not support plasmid replication.

Finally, all the EWS-PEA3 chimeras migrate at higher molecular weights in SDS-PAGE than would be predicted based on sequence alone. This anomalous migration in SDS-PAGE has also been observed with PEA3 (Xin *et al.*, 1992). It is possible that the differences in predicted and observed molecular mass may be attributed to post-translational modifications of the proteins *in vivo* or the proteins may assume secondary structures which affect their migration in SDS-PAGE.

3.2 Transcriptional Activation Studies

To determine whether the EWS-PEA3 chimeras possessed altered transcriptional activation properties in comparison to wild type PEA3, the recombinant proteins were assayed for their ability to direct PEA3-dependent gene transcription. Several different reporter constructs containing both artificial and natural Ets-responsive promoter elements were assayed for their ability to be transactivated by PEA3 and the E244P protein. All the reporters tested exhibited increased reporter gene transcription in the presence of E244P to varying extents (data not shown). The E.18-luciferase reporter was chosen for subsequent analysis because it was transactivated to a suitable extent by both proteins in COS cells and the difference in transcriptional activation of the luciferase gene specifically by the activity of PEA3 or E244P was the greatest among all the reporters surveyed. The E.18-luciferase reporter possesses an artificial element analogous to the ETS and Ras responsive region of the stromelysin promoter (Figure 3.4-A). The two inverted and overlapping ETS-binding elements (GACC**GGAACTACTTCC**GGTC) are positioned

directly upstream of the minimal *c-fos* promoter (-56 to +109) to direct ETS-responsive transcription of the luciferase gene (Yang *et al.*, 1996; Galang *et al.*, 1994).

3.2.1 Reporter and Effector Titrations for Transactivation Studies

To assess the relative activity of the E.18-Luciferase reporter and determine the optimal quantity for use in transactivation assays, COS cells were transiently transfected with increasing quantities of the reporter DNA. The relative activity of the reporter in the absence of exogenous effector DNA was determined by measurement of luciferase activity followed by normalization to protein concentration. The resultant titration curve illustrates a linear increase in luciferase activity with increasing quantity of the E.18-luciferase reporter from 0.04 μg to 0.16 μg (Figure 3.4-B). These results suggest that within the tested parameters, the E.18-Luciferase reporter elements do not saturate and could theoretically sustain increased transactivation. 0.10 μg of the E.18-luciferase reporter was used for subsequent transient transactivation studies in COS cells.

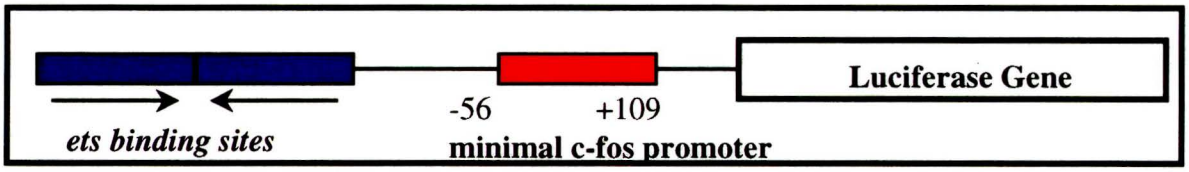
To determine the optimal quantity of effector DNA to use in these experiments, COS cells were co-transfected with 0.1 μg of E.18-luciferase reporter and increasing quantities of the effectors, pCanMyc1-PEA3 and pCanMyc1-E244P. This revealed that both the PEA3 and E244P proteins transactivated the E.18-Luciferase reporter in a dose-dependent manner over the range 0.0 μg to 1.50 μg of effector DNA (Figure 3.5). Furthermore, the E244P protein consistently exhibited at least a 4 fold greater ability to transactivate the E.18-Luciferase reporter than wild type PEA3 at each DNA

Figure 3.4 Titration of the E.18-luciferase Reporter Activity in COS cells

A. Structure of the E.18-luciferase reporter as described in Yang *et al.*, 1996. The two inverted *ets* binding sites inserted upstream of a minimal c-fos promoter is an artificial reconstruction of the *ets*-responsive region contained within the human stromelysin gene promoter. The entire reporter element is cloned upstream of the luciferase gene within the pGL2-basic expression vector (Promega).

B. The endogenous activity of the E.18-luciferase reporter observed in COS cells. Increasing quantities of the E.18 reporter plasmid from 0.0 μg to 0.20 μg (0.02 μg increments) was transfected in COS cells and the luciferase activity measured. The data shown represents the average results obtained from a single experiment performed in duplicate. Error bars represent the standard error associated with the mean.

A


Luciferase Gene
-56
+109
ets binding sites
minimal c-fos promoter

B

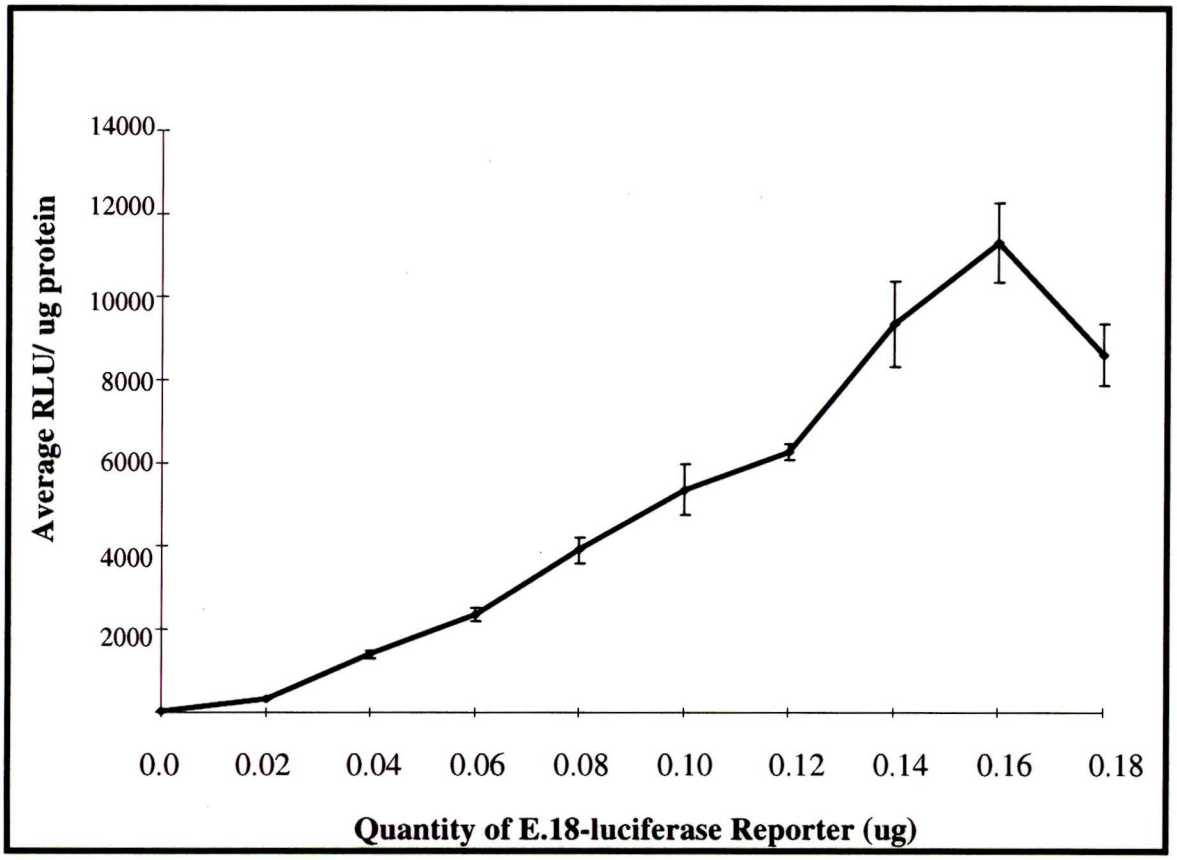
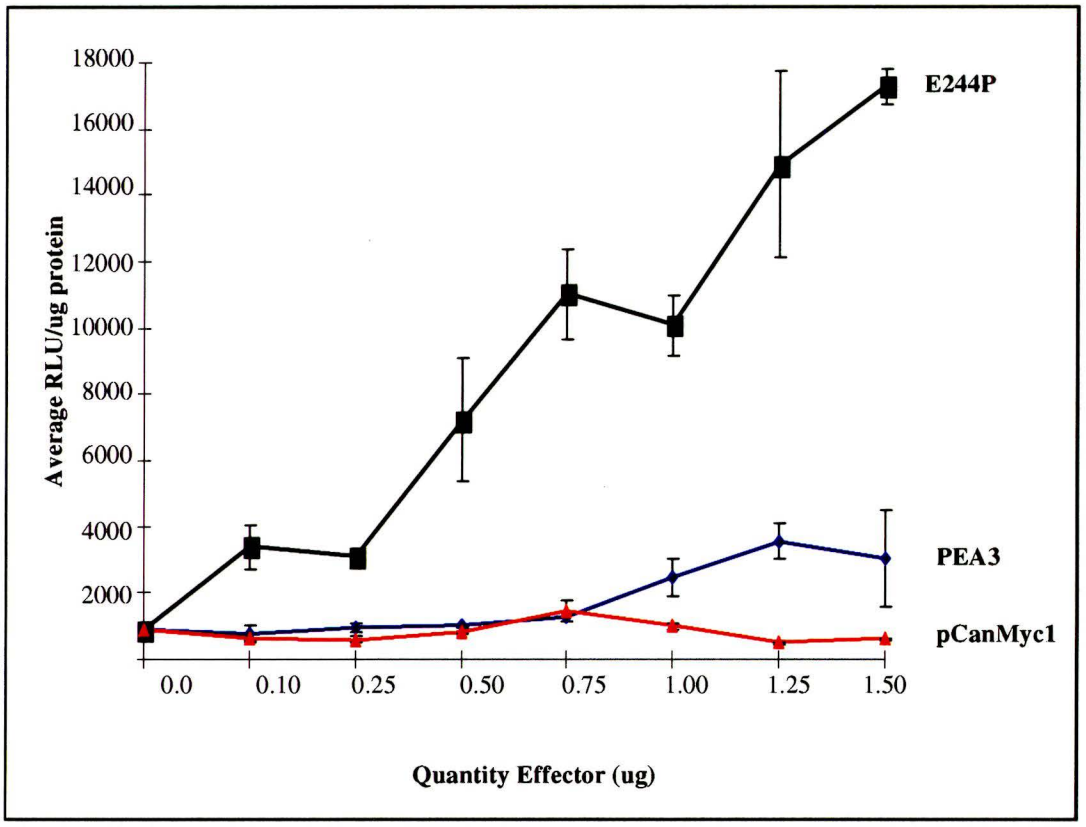

Average RLU/ ug protein
14000
12000
10000
8000
6000
4000
2000
0.0
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
0.18
Quantity of E.18-luciferase Reporter (ug)

Figure 3.5 Transactivation of the E.18-Luciferase reporter with increasing quantities of PEA3, E244P and pCanMyc1 empty vector.

COS cells were transiently transfected with 0.1 μg of the E.18-Luciferase reporter and increasing quantities (0.0 μg to 1.5 μg) of each effector (E244P, black; PEA3, blue; and pCanMyc1 empty vector, red) encoded within the pCanMyc1 expression plasmid. Lysates were collected 24 hours post transfection, assayed for luciferase activity and normalized to protein levels. The data shown is compiled from two independent transfections performed and assayed in duplicate; and represents the average luciferase activity determined for each effector at each DNA concentration. Standard error is depicted by error bars.


18000
16000
14000
12000
10000
8000
6000
4000
2000
Average RLU/ug protein
0.0
0.10
0.25
0.50
0.75
1.00
1.25
1.50
Quantity Effector (ug)
E244P
PEA3
pCanMyc1

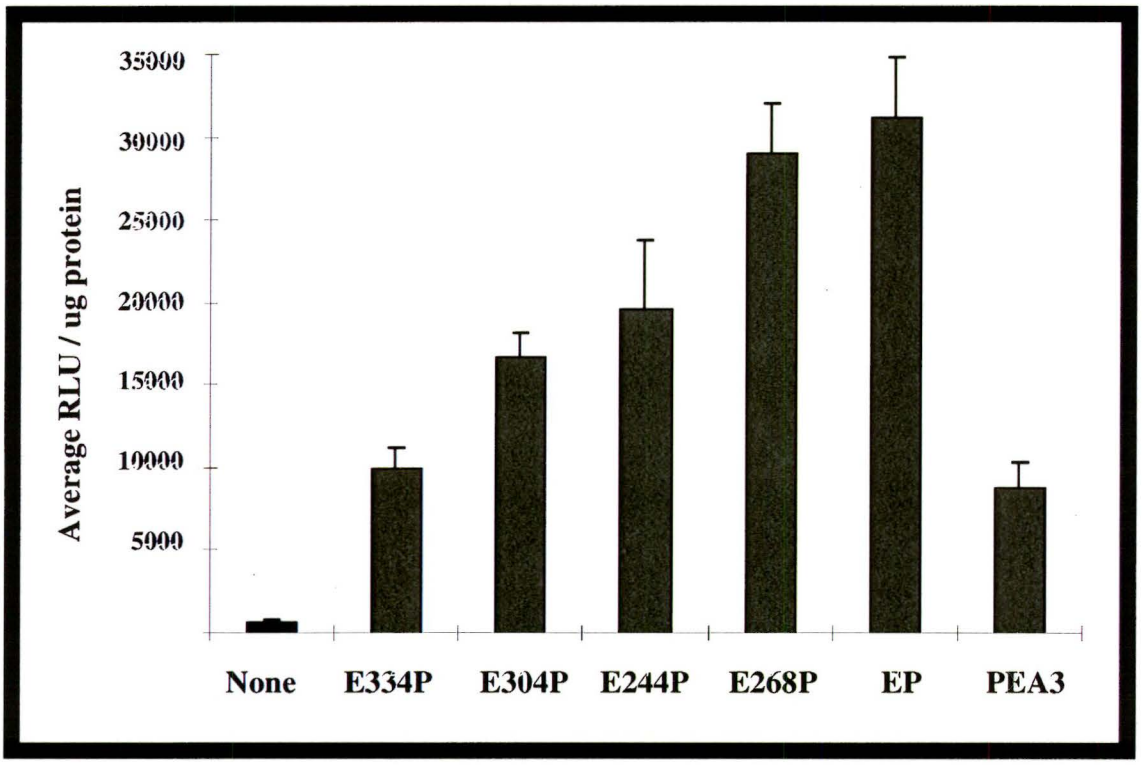
concentration tested. Saturation of the reporter, which would have appeared as a plateau of average relative luciferase activity, did not occur at the highest effector DNA concentrations used in these experiments. 1.5 μg of effector DNA was used in all subsequent transcriptional activation experiments.

3.2.2 Assessment of the Transactivation Properties of the EWS-PEA3 chimeras

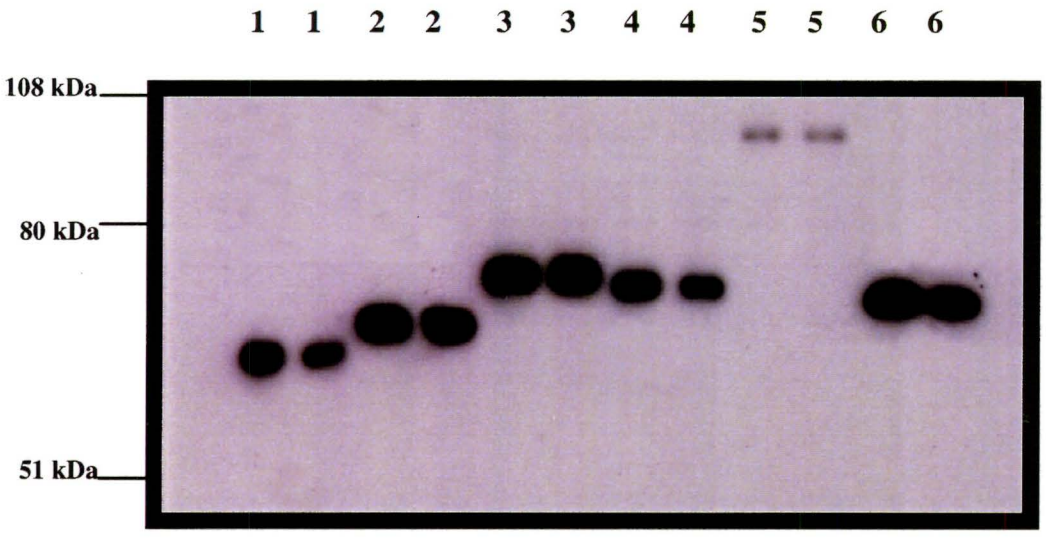
To determine the specific transcriptional activity of all the EWS-PEA3 recombinant proteins in comparison to that of full length PEA3, COS cells were co-transfected with 1.5 μg of each effector DNA and 0.1 μg of the E.18-luciferase reporter. The lysates were assayed for luciferase activity and normalized to corresponding total protein levels in the same experiment to determine the specific transcriptional activity of each protein. Figure 3.6-A depicts the average of the results obtained from three independent experiments. With the exception of E334P, all the EWS-PEA3 chimeras possessed increased ability to transactivate the E.18-luciferase reporter in comparison to wild type PEA3. The increased activity varied between 2 to 4 fold, with the greatest transcriptional activity exhibited by the fusion of EWS to full length PEA3 (EP).

To ensure that the differences in transactivation properties of the EWS-PEA3 chimeras was not due to variation in protein expression levels, Western analysis was performed. The protein lysates were prepared using a fraction of the transiently transfected COS cells assayed for luciferase activity and thus are directly correlative with the transactivation results. A representative Western blot from one experiment is presented in Figure 3.6-B. As is evident, the E304P, E268P and E244P chimeras are

A


35000
30000
25000
20000
15000
10000
5000
Average RLU / ug protein
None
E334P
E304P
E244P
E268P
EP
PEA3

B

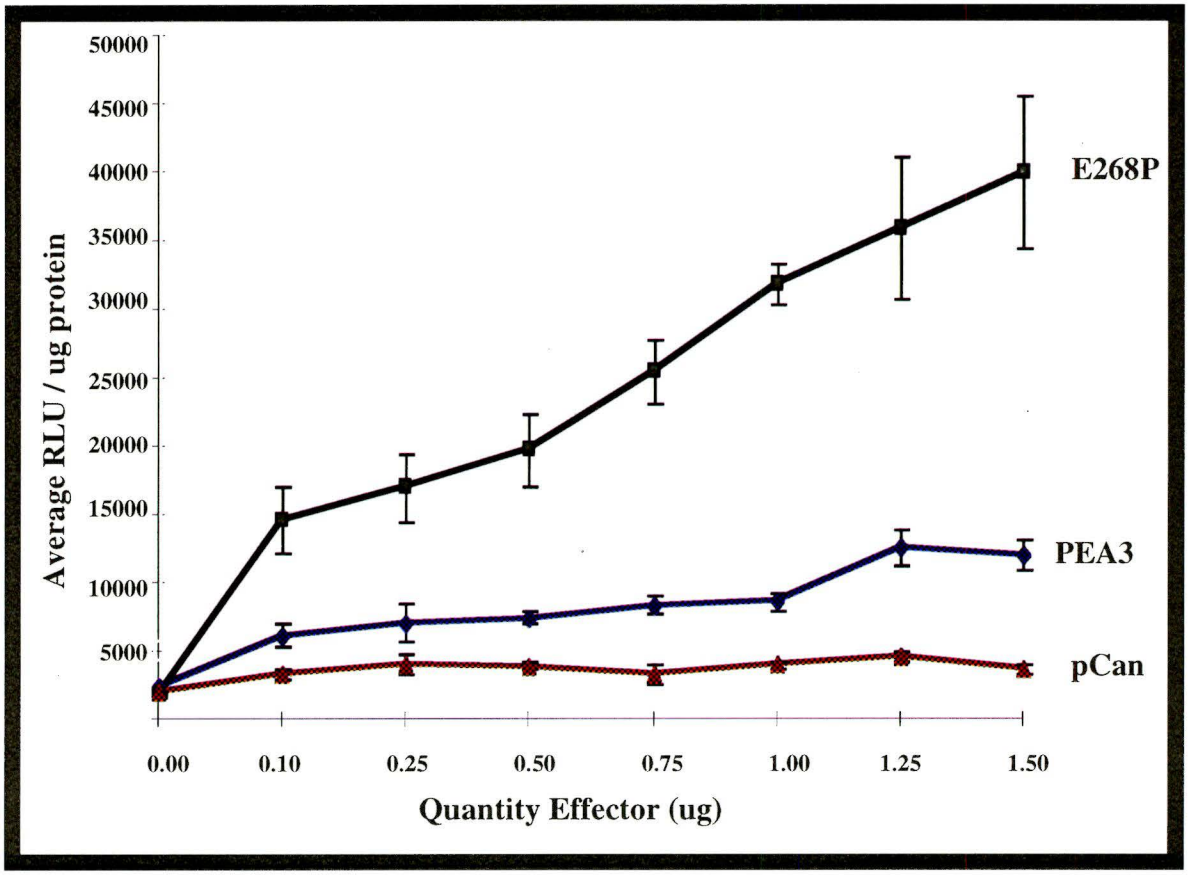

1 1 2 2 3 3 4 4 5 5 6 6
108 kDa
80 kDa
51 kDa

detected at protein expression levels similar to PEA3. In contrast, the E334P and EP constructs show decreased protein expression in COS cells within this experiment. Quantitation of protein expression levels obtained in other experiments by Western blot using a radiolabelled secondary antibody (^{125}I- goat α- mouse) indicate that E334P and EP are detected at levels 2 and 10 fold less than PEA3, respectively (data not shown). Consequently, it may be suggested that the actual specific activity of these two proteins may in fact be higher than indicated by reporter gene assay alone. In other words, if both E334P and EP proteins were endogenously expressed at levels equivalent to those observed for PEA3, then their respective transcriptional activities may be higher than currently observed.

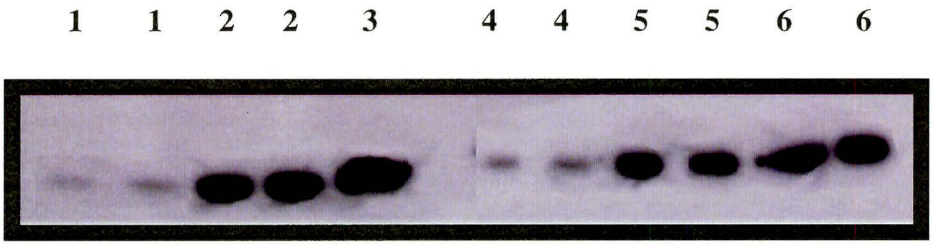
3.2.3 Assessment of E268P activity

The E268P chimera was constructed to represent a murine analog of the human EWS-hPEA3 transcription factor isolated from a Ewing's sarcoma. E268P has a 2 to 3 fold increased capacity to activate luciferase expression from the E.18 reporter in comparison to PEA3 (Figure 3.6-A). To carefully assess the relative transcriptional activities of E268P and PEA3, COS cells were transiently transfected with increasing quantities of either the E268P or the wild type PEA3 effector plasmids. The average results from 4 independent measurements (Figure 3.7-A) illustrate a consistent increase in the ability of E268P to transactive the E.18-Luciferase reporter in comparison to PEA3 at all DNA concentrations. Furthermore, the differential between the two proteins is maintained at a magnitude of 3 to 4 fold, consistent with previous results. Western

A


50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
Average RLU / ug protein
E268P
PEA3
pCan
0.00
0.10
0.25
0.50
0.75
1.00
1.25
1.50
Quantity Effector (ug)

B


1 1 2 2 3 4 4 5 5 6 6

analysis on lysates collected from COS cells transiently transfected with equivalent amounts of DNA revealed no significant variation in protein expression levels between PEA3 and E268P (Figure 3.7-B), indicating that differences in transactivation ability are attributable to the specific activity of the two proteins.

3.2.4 Effect of Ras Stimulation on E268P Transactivation Ability

Recently, the transcriptional activity of PEA3 has been shown to be regulated through the activity of Ras and proteins downstream of Ras within both the MAPK/ERK and SAPK/JNK signalling cascades (O'Hagan *et al.*, 1996). In transient assays, the ability of PEA3 to direct PEA3-dependent transcription has been shown to be increased about 10 fold in the presence of RasV12, a constitutively-activated Ras protein. The region of PEA3 which is responsive to Ras stimulation (amino acids 85-129) has been identified in deletion studies (O'Hagan and Hassell, unpublished) and found to contain three putative MAPK phosphorylation sites. PEA3 possesses eight consensus MAPK kinase sites (proline-directed serines) and is phosphorylated *in vivo* exclusively on serine residues in response to Ras stimulation; predominantly on serine 101 and to a lesser extent, on serines 90 and 134 (Tozer and Hassell, unpublished). Based on these results, it has been postulated that the transcriptional activity of PEA3 may be regulated by direct phosphorylation induced in response to specific extracellular signals communicated through Ras.

With the exception of EP, the EWS-PEA3 fusions contain amino-terminal deletions of PEA3, which remove the Ras-responsive region and six of the eight putative

MAPK phosphorylation sites. In addition, the chimeric EWS-PEA3 proteins also lack the three serine residues (101, 90 and 134) which account for the majority of phosphorylation observed *in vivo*. To learn whether the EWS-PEA3 chimeras are Ras-responsive, the ability of E268P to transactivate a PEA3-dependent reporter in the presence and absence of activated Ras was measured. The E268P chimera was chosen for analysis because of the involvement of its human homolog in Ewing's sarcoma.

The results obtained from experiments performed as outlined in O'Hagan *et al.*, 1996, failed to show an increase in the transcriptional activity of either E268P or PEA3 in the presence of activated Ras. At best, the ability of PEA3 and E268P to transactivate the reporter, pPyOriCAT(PEA3)4, increased only two to three fold in the presence of activated Ras (data not shown). This result was contradictory to previously reported results which demonstrated that the ability of PEA3 to transactivate this reporter in COS cells was stimulated approximately 10 fold in the presence of activated Ras under identical experimental conditions. Furthermore, quantitative Western analysis revealed increased amounts of both proteins in COS cell lysates concurrently transfected with the plasmid encoding activated Ras (data not shown). Together, these results suggested that increases in protein abundance were likely responsible for the observed stimulation of PEA3 activity in this assay system.

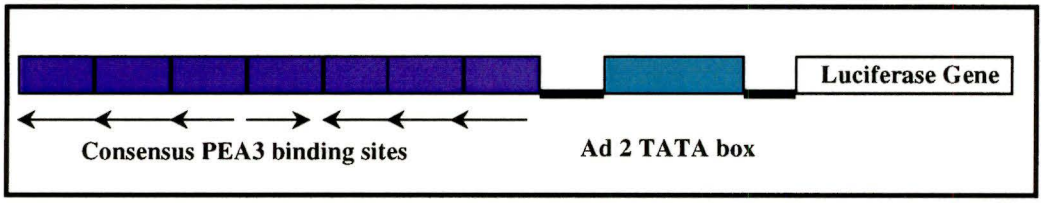
To circumvent any potential complications associated with replication of the effector plasmids in COS cells, subsequent Ras experiments were performed in HepG2 cells (human hepatoma cell line) which express negligible amounts of endogenous PEA3 protein. In addition, a reporter bearing seven PEA3 consensus binding sites, 7x-PEA3-

luciferase, was used to specifically measure the transcriptional activity attributable to the PEA3 and E268P proteins (Figure 3.8-A); hence, to minimize the transactivation of the reporter gene by any endogenous HepG2 factors.

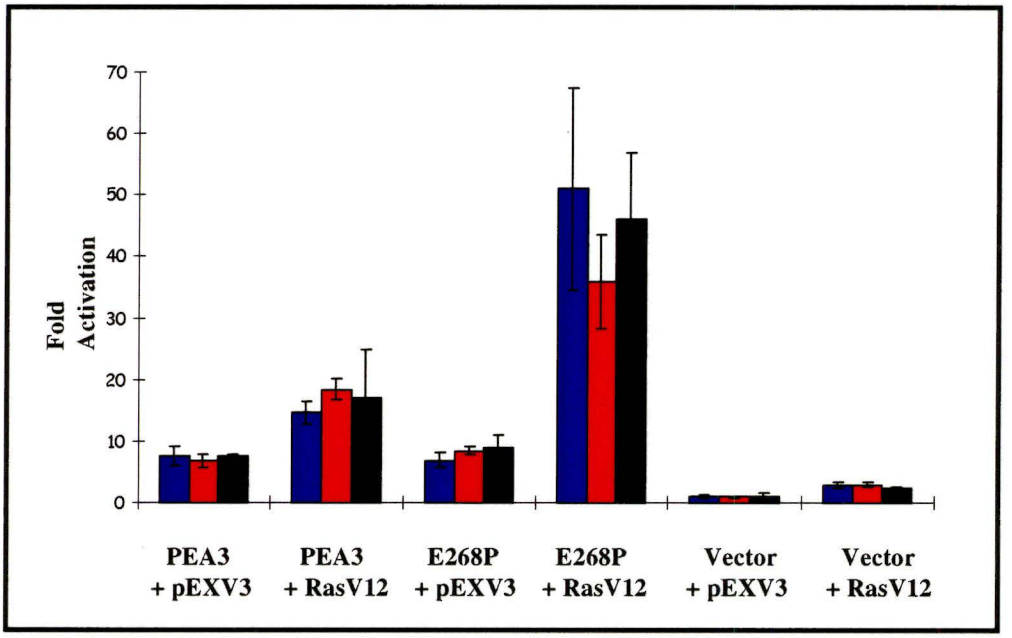
HepG2 cells were transiently transfected with 0.1 μg of the 7x-PEA3-Luciferase reporter, 0.05 μg or 0.10 μg of the effectors RSV-PEA3, RSV-Myc1-E268P or RSV vector (empty), and either 0.1 μg or 0.3 μg of activated Ras (RasV12) or an empty vector control, pcEXV3. The results depicted in Figure 3.8-B are compiled from one experiment performed using three different ratios of effector (PEA3 or E268P) to activator (RasV12) plasmid DNA. As is evident, both PEA3 and E268P transactivated the PEA3-dependent reporter 7 to 8 fold over the RSV empty vector in the absence of an activating signal. In the presence of RasV12, the transcriptional activity of PEA3 increased two fold, while the ability of E268P to transactivate the 7xPEA3-luciferase reporter was increased by a magnitude of 5 to 6 fold. These results were consistent between experiments in which the effector to activator ratio was altered (50 ng effector + 300 ng activator; 100 ng effector + 100 ng activator; 100 ng effector + 300 ng activator) and implied that RasV12 activated the E268P protein to a greater extent than it did PEA3.

Since PEA3 expressed from the pRc-RSV plasmid does not possess a myc epitope at its amino-terminus and a PEA3-specific antibody capable of detecting both proteins simultaneously was not available, then the relative abundance levels of the proteins in the presence and absence of Ras could not be assessed. To ensure that the increase in the specific activity of the PEA3 and E268P proteins observed in the presence of Ras was not

A


Luciferase Gene
Consensus PEA3 binding sites
Ad 2 TATA box

B


Fold Activation
70
60
50
40
30
20
10
0
PEA3 + pEXV3
PEA3 + RasV12
E268P + pEXV3
E268P + RasV12
Vector + pEXV3
Vector + RasV12

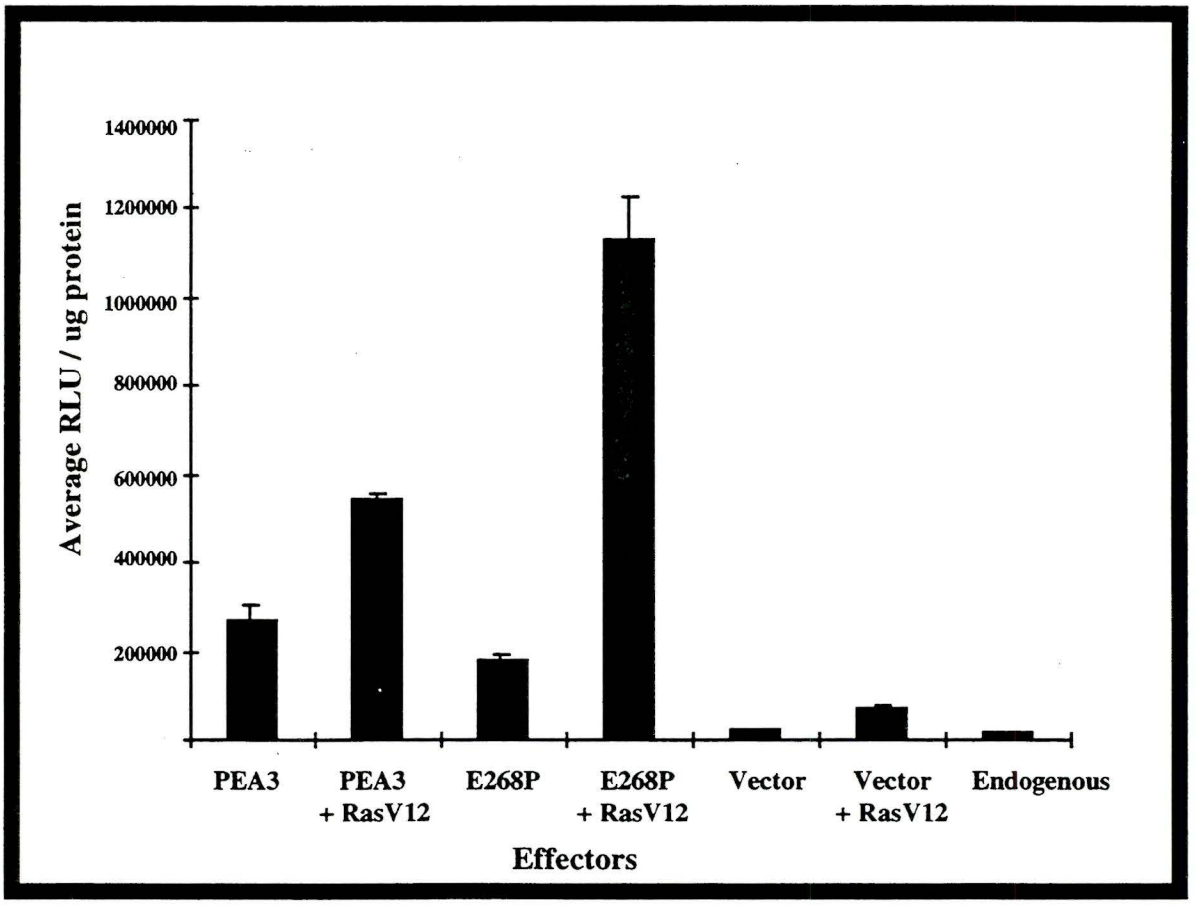
due to variation in protein expression levels, the experiment was repeated using effectors encoded within the pCanMyc1 plasmids. Consequently, this allowed for the simultaneous detection of PEA3 and E268P with the myc monoclonal antibody. HepG2 cells were transiently co-transfected with 0.1 μg of the 7x-PEA3-Luciferase reporter, 0.1 μg of each effector DNA (pCanMyc1) and 0.3 μg of either the empty pcEXV3 plasmid or pcEXV3 encoding RasV12. The results obtained from one experiment performed in triplicate is depicted in Figure 3.9. As observed previously, PEA3 was demonstrated to have a 2 fold increased capacity to transactivate the 7x-PEA3-luciferase reporter in the presence of RasV12. Similarily, the activity of E268P increased 6 fold when co-transfected with a plasmid encoding activated Ras. To ensure that the increased transcriptional activity of the two proteins observed in the presence of activated Ras was not due to increased protein levels, Western analysis on the lysates was performed. Unfortunately, the expression of PEA3 and E268P could not be detected in HepG2 cells under the given transfection conditions, likely due to low expression levels.

3.3 Transformation Properties of Chimeras

The reciprocal chromosomal translocations identified in Ewing's sarcoma tumors results in the expression of chimeric genes whose commonality is the juxtaposition of a truncated EWS gene to the ets genes Fli-1, Erg, ETV-1 and hE1A-F (hPEA3). Evidence obtained from the analysis of several EWS-Fli-1 isoforms indicated that their expression is sufficient to cause cellular transformation in culture. This suggests that EWS-Fli-1, and by

Figure 3.9 Transactivation of the 7x-PEA3-Luciferase Reporter in HepG2 cells by PEA3 and E268P in the Presence and Absence of Activated Ras.

Transactivation of the 7x-PEA3-Luciferase reporter in HepG2 cells. HepG2 cells were transiently co-transfected with 0.1 μg of the reporter, 0.1 μg of effector DNA (pCanMyc1-PEA3, pCanMyc1-E268P or pCanMyc1 empty vector) and either 0.3 μg of activated RasV12 (pEXV3-RasVal12) or pEXV3 empty vector. Lysates were harvested 24 hours post transfection and assayed for luciferase activity and protein levels. The results are expressed as the average luciferase activity obtained from four independent HepG2 cell transfections. Standard errors are as indicated by error bars.


Average RLU / ug protein
1400000
1200000
1000000
800000
600000
400000
200000
PEA3
PEA3 + RasV12
E268P
E268P + RasV12
Vector
Vector + RasV12
Endogenous
Effectors

extention, all other EWS-ETS chimeras, are oncogenic and possibly directly contribute to the transformation of cells possessing the chromosomal translocation.

3.3.1 Focus Assays

To determine whether the chimeras were able to transform, NIH 3T3 and Rat-1 cells were transfected with plasmids encoding the EWS-PEA3 recombinant genes, as well as, the murine PEA3, EWS-Fli-1, and Fli-1 cDNAs. A plasmid encoding the polyomavirus (Py) middle T antigen, pPyMT-1, was utilized as a positive control; Py middle T antigen transforms fibroblast cells with high efficiency (Mess *et al.*, 1982). NIH 3T3 or Rat-1 cells were transfected by the calcium phosphate method (Graham *et al.*, 1973) with 0.1 μg, 1.0 μg or 5.0 μg of the effector plasmids pCanMyc1-E268P, pCanMyc1-PEA3, or pCanMyc1 vector. In addition, both cell lines were transfected with either 5.0 μg of pCanMyc1-EWS-Fli-1or 5.0 μg of pCanMyc1-Fli-1 to reproduce the published results (May *et al.*, 1993). To measure transfection efficiency both within and between experiments, 0.1 μg of pPyMT-1 plasmid DNA was used. The results of two independent experiments per cell line (10-15 transfected plates) produced no evidence of focus formation for any effector DNA at any concentration excluding pPyMT-1. Expression of Py middle T antigen in NIH 3T3 cells resulted in an average 69.3 ± 6.7 foci/100mm plate and 91.7 ± 9.2 foci/100mm plate in Rat-1 cells. In addition, focus assays performed with NIH 3T3 and Rat-1 cells transfected with pCanMyc1-E334P,

pCanMyc1-E304P, pCanMyc1-E244P or pCanMyc1-EP also did not result in cellular transformation.

Expression from the pCanMyc1 vector plasmids is directed from the human cytomegalovirus (hCMV) promoter. To learn whether the lack of transformation by the effectors could be attributed to relative promoter strength and/or activity in the cell lines, effectors subcloned in the pRc-RSV (Invitrogen) expression plasmid were utilized in focus assays. Transcription from the pRc-RSV expression plasmid is directed from the Rous sarcoma virus long terminal repeat (RSV LTR). Identical conditions and quantities of each of the effectors RSV-Myc1-E268P, RSV-PEA3, RSV-Myc1-EWS/Fli-1, RSV-Myc1-Fli-1, and pPyMT-1 were transfected by calcium phosphate into NIH 3T3 and Rat-1 cells. The results of two experiments performed in each cell line did not demonstrate that the expression of any effector from the RSV LTR induced focus formation in either NIH 3T3 or Rat-1 cells. As expected, Py middle T antigen expression in NIH 3T3 and Rat-1 cells resulted in similar transfection efficiencies as observed in previous experiments.

Infection of NIH 3T3 cells with retroviral stocks expressing EWS/Fli-1 protein has been reported to transform NIH 3T3 cells (May *et al.*, 1993). Since this study utilized an alternate method to introduce the effector DNA into the cells it was possible that the inability of EWS/Fli-1 to transform cells was due to low transfection efficiencies. To test this hypothesis, a commercial liposome formulation, LipofectAMINE™ (Gibco BRL), which transfects NIH 3T3 and Rat-1 cells with a 20-30% efficiency rate (Crnac and Hastings, unpublished) was used. NIH 3T3 and Rat-1 cells were transfected with 2 μg of either pCanMyc1-E268P, pCanMyc1-PEA3, pCanMyc1-EWS/Fli-1, pCanMyc1-Fli-1 or

pCanMyc1 plasmid, as well as, 40 ng of pPyMT-1 plasmid. No foci appeared on the NIH 3T3 or Rat-1 monolayers which had been transfected with either E268P, PEA3, EWS/Fli-1 or Fli-1. In contrast, liposome transfection with 40 ng of the pPyMT-1 plasmid produced large numbers of foci (greater than 300 colonies per 35 mm plate) on NIH 3T3 and Rat-1 monolayers.

Thus, the focus assay results did not provide evidence to substantiate a transforming activity for E268P, or any of the other EWS-PEA3 chimeras in either NIH 3T3 or Rat-1 cells. It should also be noted that these experiments were performed using numerous sources of NIH 3T3 and Rat-1 cells to ensure that results were not reflective of inherent alterations in clonal profile.

3.3.2 Soft Agar Assays

To assess whether expression of either the EWS-PEA3 or EWS/Fli-1 chimeras in NIH 3T3 cells was sufficient to induce anchorage independent growth (a marker of transformation), cell lines potentially expressing the chimeras were seeded in soft agar assays. To create stable cell lines, 1 μg of either RSV-Myc1-E268P, RSV-PEA3, RSV-Myc1-EWS/Fli-1, RSV-Myc1-Fli-1 or RSV plasmid vector was introduced into NIH 3T3 cells by liposome-mediated transfection. Following G418 selection, individual colonies from each transfection were isolated and propagated. In total, eight E268P-derived cell lines, seven PEA3-derived cell lines, three EWS/Fli-1-derived cell lines, and three Fli-1-derived cell lines were seeded in soft agar at a concentration of $1x10^5$ cells per plate. None of the cell lines tested formed colonies in soft agar within a 21 day period. In

contrast, a neu-transformed Rat-1 cell line readily produced macroscopic colonies in soft agar that were evident within 10 days. These results suggested that the inability of the derived cell lines to support growth in soft agar was not due to experimental difficulty, and futhermore, that none of the cell lines possessed a transformed phenotype. To determine that the cell lines stably expressed the chimeric proteins of interest, lysates from each cell line were immunoprecipitated with the myc 9E19 monoclonal antibody conjugated directly to Sepharose ®4B beads. Several attempts using various modifications of the immunoprecipitation protocol failed to show the stable expression of the chimeric proteins, and in particular, E268P protein. These results suggested that the cell lines either expressed the chimeric proteins at extremely low levels or not at all. Time constraints prohibited further investigation into this avenue.

DISCUSSION

The construction of an activated allele of the PEA3 transcription factor was motivated by the potential contribution its use would make to the study and elucidation of PEA3 function. In particular, aberrant PEA3 activity has been associated with the development, progression and metastasis of various types of cancer. Since the activity of PEA3 is normally highly regulated, the use of an activated and/or deregulated PEA3 protein could potentially assist in the investigation of the specific role PEA3 plays in these processes.

To achieve this end, several EWS-PEA3 fusions were constructed to mimic the chimeric genes isolated in the human cancer syndrome, Ewing's sarcoma. These tumors harbor various chromosomal translocations which result in the positioning of several different Ets genes downstream and in-frame of a truncated EWS gene. Expression of these mutant genes consequently leads to the generation of novel EWS-Ets fusion proteins. The retention of the Ets DNA binding domain in all the EWS-Ets proteins suggest that these chimeras function as transcription factors whose target specificity is conferred by the Ets protein. The EWS segment of these proteins contributes a strong transcriptional activation domain which effectively increases the activity of the EWS-Ets factors.

The PEA3 gene and four PEA3 gene truncations were fused in frame to the minimum segment of the EWS gene common to all the Ewing's sarcoma translocations. The truncations were designed to remove the acidic and glutamine rich domains which previous studies had broadly identified as being responsible for the transcriptional activation properties of PEA3. An additional criteria in border selection was the Fli-1 and ERG truncations isolated in the characterized EWS-Fli-1 (Delattre *et al.*, 1992) and EWS-ERG (Sorensen *et al.*, 1994) translocation products. However, since PEA3 bears significant sequence and amino acid divergence from both Fli-1 and ERG outside of the ETS domain, determination of a PEA3 truncation analogous to the previously isolated ETS truncations was difficult. The decision to create the PEA3 mutant which resulted in the deletion of the first 267 amino acids of the protein (ΔN268PEA3) was based on the report of a Ewing's sarcoma tumor possessing a fusion between EWS and human PEA3 (Urano *et al.*, 1996). The EWSΔN268PEA3 (E268P) chimera, thus represents a murine mimic of the human EWS-PEA3 translocation product.

Transcriptional Activation Properties of the EWS-PEA3 Chimeras.

The five EWS-PEA3 chimeric proteins and wild type PEA3 were assayed for the ability to direct PEA3-dependent reporter gene transcription. Transient assays showed that the E304P, E268P, E244P and EP proteins all stimulated luciferase expression from the the E.18 reporter at levels two to four fold higher than that observed with full length PEA3. To ascertain that the apparent increases in transactivation ability of the chimeras could be ascribed to increases in specific activity rather than variation in protein

expression, their relative abundance levels were measured. The E304P, E268P and E244P proteins were all found to be expressed at equivalent levels to PEA3. However, the chimeras, E334P and EWS/PEA3, were detected at protein levels two and ten fold less than full length PEA3, respectively; suggesting that the specific activity of these EWS-containing fusions may be higher than attributed by transcriptional activation assays alone. In this respect, normalization of the transcriptional activity of EWS/PEA3 to quantitated expression levels (as observed in Western analysis) suggests that the fusion of EWS to full length PEA3 may confer an estimated 40 fold increase in the ability of PEA3 to direct PEA3-dependent reporter gene transcription. However, owing to the fact that the low expression levels of EWS/PEA3 detected in Western analysis have not been shown to be representative of an actual decrease in protein expression *in vivo*, conclusive statements on the validity of this inference can not be made at this time.

Additional characterization of two EWS-PEA3 constructs showed a constant difference in transcriptional activity between the chimeric and PEA3 proteins. Both E268P and E244P consistently transactivated the E.18-luciferase reporter at levels three to four times greater than PEA3 over a range of DNA concentrations. Furthermore, the observed increase in reporter transactivation by E268P and E244P was not due to increased protein abundance since Western analysis revealed equivalent amounts of PEA3 and chimeric protein in COS cell lysates transiently transfected with equal amounts of DNA. Together these results suggest that the E268P and E244P possess an inherently higher transcriptional activity than PEA3.

The EWS-PEA3 chimeras were demonstrated to be more potent transcriptional activators than PEA3 in the assay system studied. The observed differences in activity between PEA3 and the EWS-PEA3 chimeras were as expected based on published comparative analysis of the transactivation abilities of the EWS/Fli-1 and Fli-1 proteins. Previous studies report a five to ten fold increase in the transactivation of reporter constructs bearing the ets-responsive element of the human T-cell leukemia virus long terminal repeat (HTLV LTR) by EWS/Fli-1 in comparison to normal Fli-1 protein (Bailley *et al.*, 1994).

Whether the measured transcriptional activation properties of the EWS-PEA3 chimeras, particularily, E268P, in this transient system similarily represent the actual increase in protein activity caused by the replacement of the PEA3 activation domain with the EWS activation domain is unknown. Although previous analysis has shown the EWS transactivation domain to be 30 times more potent than the activation domain of Fli-1 in transient assays (May *et al.*, 1993), it is unlikely that the fusion of the EWS amino terminal domain to ETS proteins results in an equivalent increase in transcriptional activation activity *in vivo*.

Effect of Ras Stimulation on E268P transcriptional activity.

Recent evidence suggests that the transcriptional activity of PEA3 is regulated by Ras and proteins downstream of Ras within both the MAPK/ERK and SAPK/JNK signalling cascades (O'Hagan *et al.*, 1996). Although the precise mechanism responsible for Ras-induced activation of PEA3 has not been fully determined, protein

phosphorylation has been implicated. PEA3 contains eight potential phosphorylation sites, which correspond to the consensus protein sequence required for phosphorylation by MAP kinases. The E268P chimera lacks six of the eight candidate MAPK phosphorylation sites found within PEA3; including the three serines residues (101, 90 and 134) which account for the majority of phosphorylation observed *in vivo* (Tozer and Hassell, unpublished). Based on this evidence, it was postulated that if the phosphorylation of these serine residues were required for Ras-activation of PEA3, then the activity of the E268P chimera could not be similarily induced by Ras.

To determine whether the E268P chimeric protein is regulated by Ras, plasmids encoding both E268P and PEA3 were co-transfected with constitutively-activated Ras (RasV12). Interestingly, experiments performed in HepG2 cells demonstrated that the transcriptional activity of the E268P chimeric protein was increased to a greater extent than PEA3 in response to Ras stimulation. Although preliminary, these results nonetheless suggest that the activity of E268P might also be regulated by Ras. There are several hypotheses which could account for this finding. The first is that Ras-stimulation of PEA3 transcriptional activity may not be caused by direct phosphorylation of PEA3; particularily on the first six MAPK consensus serine residues. The fact that these sites are absent from the E268P chimera and its transcriptional activity is still increased in the presence of Ras suggests that the activation of PEA3 activity must occur either through the phosphorylation of the remaining sites or by a different mechanism. With respect to the former, one of the remaining candidate MAPK phosphorylation sites in PEA3 (serine 458) is not phosphorylated *in vivo* in response to Ras (Tozer and Hassell, unpublished). This

suggests that if direct phosphorylation of PEA3 is required for Ras-stimulation of its activity, then the serine at position 285 may be the most likely candidate.

However, even if PEA3 is phosphorylated on serine 285, it does little to explain why the activity of E268P increased five to six fold in the presence of Ras while under similar conditions, PEA3 transcriptional activity increased only two fold. If phosphorylation of a specific residue was the sole factor governing responsiveness to Ras, then this would suggest that the presence of that residue within both PEA3 and E268P would allow for equivalent inductions of their respective activities following stimulation. However, the activity of E268P was stimulated to an extent two to three fold higher than observed for PEA3. This result implies that other factors or mechanisms may be contributing to the Ras-induced stimulation of E268P activity. One possibility is that additional responsiveness to Ras may be conferred from the segment of EWS present in E268P. Analysis of the first 264 amino acids of EWS for potential regulatory motifs did not reveal the presence of any candidate ERK or SAPK phosphorylation sites; suggesting that EWS is not likely phosphorylated by kinases whose activities are Ras-regulated. However, this finding does not exclude the possibility that Ras or other signal modulators may somehow effect the transactivation properties of EWS, and by extension, the EWS-PEA3 chimeras. To date, little is known regarding the function of the EWS protein and particularily, the mechanisms (if any) through which its activity may be regulated. Furthermore, even less is known about the actual regulation of the EWS-Ets chimeras found in Ewing's sarcoma. It has been postulated that the increased transcriptional activity of the EWS-Ets chimeras could be attributed to two phenomenon: their severance

from the regulatory mechanisms controlling normal Ets activity (by the loss of Ets sequences) and the addition of a stronger transcription activation domain from the fusion to EWS. No other function has been attributed to the segment of EWS found in the chimeric factors. In light of the finding that Ras increased E268P activity to a greater extent than it did PEA3 activity, it is interesting to speculate that EWS may also contribute additional regulatory control to the EWS-Ets chimeras, which consequently would affect their function.

Transformation Properties of the EWS-PEA3 Chimeras.

Results from focus assays and experiments performed in soft agar did not identify any transforming activity attributable directly to expression of the EWS-PEA3 chimeras, including E268P. Perhaps more significant, however, was the finding that under identical assay conditions, EWS/Fli-1 was also found to have no detectable transforming activity. This result is in direct contrast to the data published in May *et al.*, 1993, which clearly illustrates the transformation of NIH 3T3 cells by expression of EWS/Fli-1. Several reasons can be postulated to account for these differences. The first issue regards the effect of promoter strength and protein expression levels on the induction of transforming ability. In the original report, retroviral-driven expression of EWS/Fli-1 was found to potently transform NIH 3T3 cells (May *et al.*, 1993). However, within the context of these experiments, gene expression was directed either from the human cytomegalovirus promoter (hCMV) or the Rous sarcoma virus (RSV) LTR. It is possible that insufficient proteins levels were produced under the transcriptional control of either the hCMV

promoter or RSV LTR to induce transformation of NIH 3T3 cells in these experiments. The correlation of transformation and protein expression levels has also been observed in studies performed to assess the effect of c-erbB-2/neu protein overexpression in NIH 3T3 cells. Expression of c-erbB-2/neu genes under the control of the Moloney murine leukemia virus long terminal repeat (MoMLV LTR) results in the induction of protein levels sufficient to transform NIH 3T3 cells, while SV40-driven gene expression does not (Di Fiore *et al.*,; DiMarco *et al.*, 1990; Hudziak *et al.*, 1987). Thus, it is likely that a similar mechanism is responsible for the inability of EWS/Fli-1 to transform NIH 3T3 cells when its gene expression in under the transcriptional control of the hCMV promoter or RSV LTR. Unfortunately, time constraints prohibited the investigation of this avenue.

In addition, it should be noted that difficulties in reproducing the results reported in May *et al.*, 1993 have also been reported by other researchers (communication from M. Roussel to J. A. Hassell). Given this information, it is possible that variations between cell lines may have contributed to the observed lack of transforming activity attributable to EWS/Fli-1. Anecdotally, differences in the properties of cell lines, particularly NIH 3T3 cells, have been observed for lines derived from different sources; suggesting that culture conditions may influence phenotypic properties. Thus, it is likely that the NIH 3T3 cell line used in the original study of EWS/Fli-1 transforming ability may have possessed distinct properties which supported its transformation following transfection with EWS/Fli-1. Since the original NIH 3T3 cell line in which the original focus assays were performed (May *et al.*, 1993) could not be obtained, this hypothesis could not be tested.

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