SPECTRUM OF *ETS* GENES EXPRESSED IN HER2/NEU-INDUCED TUMORS

DETERMINATION OF THE SPECTRUM OF *ETS* GENES EXPRESSED IN HER2/NEU-INDUCED MOUSE MAMMARY TUMORS

By

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A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

for the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (1999)
(Biology)

McMaster University
Hamilton, Ontario

TITLE: Determination of the Spectrum of *ets* Genes Expressed in HER2/Neu-Induced Mouse Mammary Tumors

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NUMBER OF PAGES: x, 119

ABSTRACT

The *ets* gene family of transcription factors has been widely implicated in a variety of human tumors. PEA3, the founding member of the PEA3 subfamily of *ets* genes is overexpressed in a mouse model of mammary tumorigenesis as well as in primary human breast tumors. PEA3 deregulation in these tumors is thought to increase the metastatic potential of mammary tumors by increasing the expression of various matrix metalloproteinases. The identification of other *ets* gene's expression using a mouse model of HER2/Neu-induced mammary tumorigenesis would provide insight into the mechanisms behind these mammary tumors. Degenerate RT-PCR analysis was used to screen for expression of all known *ets* genes in these tumors. A large spectrum of *ets* genes was identified as being expressed in these tumors. Quantitative analyses including semi-quantitative RT-PCR and ribonuclease protection assays, indicate that the PEA3 subfamily of *ets* genes, including PEA3, ERM and ER81, as being overexpressed in these tumors, while other *ets* genes, Ets-1, Ets-2 and GABPα were not. These results imply a specific role for the PEA3 subfamily in this model of mammary tumorigenesis and isolate this subfamily of *ets* genes as a possible therapeutic target.

ACKNOWLEDGEMENTS

The author would like to thank John Hassell for his continuous support, ideas and encouragement as well as Michael Rudnicki and Ana Campos for sitting on her committee. Both past and current members of the Hassell lab have been extremely helpful and are to be thanked. In particular, my thanks go to Lesley MacNeil and Trevor Shepherd for their unrelenting support, help and ideas. Brian Allore and Dinsdale Gooden must also be thanked for their efforts in the Central Facility.

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Introduction

The use of transgenic animals in cancer research provides a means to investigate and mimic human conditions. These animals are used in therapy trials as well as for molecular analysis of the cellular mechanisms by which tumor formation occurs (reviewed in Muller WJ, 1991). Primary human tumors are often difficult to analyze because of limitations in acquisition and sample quantity and quality.

The Mouse Mammary Tumor Virus (MMTV) Long Terminal Repeat (LTR) is used in transgenic mouse research to investigate the role of genes in mouse mammary gland tumorigenesis. The MMTV LTR directs high levels of transgene expression to the luminal epithelium of the mouse mammary (Choi *et al.,* 1987). Using this promoter element to direct high levels of HER2/Neu to the mouse mammary gland, a line of transgenic mice were created that develop focal mammary tumors after a long latency period (Guy *et al.,* 1992). These mice will also develop lung metastases usually after having borne the primary tumor for at least two months. Due to the long latency period prior to tumor development, it is thought that these tumors require an activation of HER2/Neu for tumor onset. The tumors that develop in these mice histologically resemble those of human

breast carcinomas. This line of mice is therefore a good model for human breast cancer progression.

Tumors that develop in the MMTV LTR-HER2/Neu line of transgenic mice have elevated levels of PEA3 expression (Trimble *et al.,* 1993). PEA3 is the founding member of a subfamily of a large family of transcription factors known as Ets proteins. PEA3 is also elevated in primary human breast tumors (Benz *et al.,* 1997). Putative PEA3 target genes include matrix metalloproteinases, whose deregulated expression is considered to be important in increasing the metastatic potential of cancers (reviewed in Westermarck and Kahari, 1999). There is also evidence to suggest that matrix metalloproteinases are involved in the early stages of cancer development as well (Basset *et al.*, 1990; Witty *et al.*, 1994; d'Armiento *et al.*, 1995; Wilson *et al.*, 1997). Identifying transcription factors that are involved in the initiation, progression or metastasis of cancer and their target genes is an invaluable tool in developing therapeutic strategies.

1.0 The Ets Family of Transcription Factors

Membership to this family of transcription factors is based on sequence similarity within the ETS domain, a ~85 amino acid DNA binding domain. Placement into one of several *ets* subfamilies is based on high sequence similarity within the ETS domain as well as the presence of other domains. The *ets* family consists of over 30 members, and includes homologues from a variety

of species, ranging from *Drosophila* to human. The founding member of this family, v-ets, was originally discovered as a sequence within a retrovirus, E Twenty-Six, capable of inducing myeloblastosis and erythroblastosis in chickens (Leprince *et al.*, 1983).

The ETS domain recognizes and binds to the EBS, or Ets Binding Site, a ~10 base pair sequence found in gene promoters. The EBS consists of a core C/A GGA A/T motif and flanking sequences help determine the specificity with which ETS proteins bind (reviewed in Graves and Petersen, 1998; Wang *et al.*, 1992). Although each ETS protein preferentially binds to a specific sequence, more than one protein is capable of binding to the same sequence. For example, the sequence Ets-1 binds with greatest affinity, 5'-ACCGGAACG-3', (Nye *et al.*, 1992) is also bound by a variety of other *ets* genes (Gunther and Graves, 1994; Graves *et al.*, 1996). The ETS domain is sufficient for DNA binding [Elk-1 (Janknecht and Nordheim, 1992), Ets-1 (Lim *et al.*, 1992), Ets-2 (Wasylyk *et al.*, 1992), GABPα (Thompson *et al.*, 1991); PEA3 (Xin *et al.*, 1992); PU.1 (Klemsz *et al.*, 1990)].

Ets proteins are localized to the nucleus and regulate transcription of their respected target genes. The vast majority of ets transcription factors activate transcription, however, there are a few examples of ets proteins acting as repressors. Fli-1 can bind to an EBS in the promoter of the *Rb* gene and repress its transcription (Tamir *et al.*, 1999). Tel represses MCSFR promoter activation

by CBFA2B and C/EBPa (Fears *et al.*, 1997). ERF can repress transcription from a variety of promoters, including the Ets-2 and GATA1 promoters in transient expression experiments (Sgouras *et al.*, 1995). ERF can also block NIH 3T3 cell transformation by a ME26 virus that expresses the p135 $^{gag\text{-}myb\text{-}ets}$ fusion protein (Sgouras *et al.*, 1995). The Net ets protein is a transcriptional repressor unless activated, probably post-translationally, by Ras (Giovane *et al.*, 1994). Yan, a *Drosophila ets* gene, negatively regulates photoreceptor cell differentiation by competing with an activator, Pnt P2 (Lai and Rubin, 1992; O'Neill *et al.*, 1994; Rebay and Rubin, 1995).

NMR analyses of Ets-1 and Fli-1 illustrate that the ETS family belongs to the winged helix-turn-helix class of proteins. The ETS domain consists of three alpha helices (α) and four beta strands (β) that are arranged α1-β1-β2-α2-α3-β3-β4 (Donaldson *et al.*, 1994; Liang *et al.*, 1994a). The alpha helices fold into a helix-turn-helix structure which is set next to a four-stranded antiparallel beta-sheet (Liang *et al.*, 1994b; Werner *et al.*, 1995; Donaldson *et al.*, 1996; Kodandapani *et al.*, 1996). Conserved residues within the ETS domain of most ets proteins reside in the helices and strands, while non-conserved amino acids lie in the loops and turns. The conserved amino acids therefore, probably have important roles in the proper folding of the ETS domain (Donaldson *et al.*, 1996).

1.0.1 *Ets* genes are involved in cellular proliferation

Transcription factors are important in the control of cellular division and proliferation. A change in normal function of a transcription factor would have serious consequences in the loss of cellular control. Several ets proteins have been implicated in the regulation of cellular proliferation (reviewed in Hromas and Klemsz, 1994). Ets proteins would therefore be important in whether a cell becomes tumorigenic or not. Some *ets* genes have been shown to be involved in the early signals for cell growth and division. Ets-1 and Ets-2 both activate transcription by cooperating with the AP-1 transcription factor after stimulation by phorbol esters or serum growth factors (Wasylyk *et al.*, 1990). The Serum Response Factor (SRF) recruits the *ets* gene SAP-1 to activate the serum response element (SRE) in the c-fos promoter (Dalton and Treisman, 1992). This SRE can also be bound by the ets protein Elk-1 (Hipskind *et al.*, 1991). Environmental stimuli can also signal through ets proteins. For example, dominant-negative Ets-2 can block transformation of NIH 3T3 cells by the CSF-1 receptor (Langer *et al.*, 1992).

1.0.2 Translocations Involving *ets* Genes Result in Human Cancer.

Several *ets* genes are involved in the pathogenesis of human cancers. Over 90% of the Ewing family of tumors are due to a translocation between EWS and the Fli-1 *ets* gene (Turc-Carel *et al.*, 1988). This family of tumors includes

Ewing's sarcoma and neuroepitheliomas. The EWS-Fli-1 fusion protein can transform NIH3T3 cells and this ability is lost following the deletion of either the EWS or Fli-1 segments (May *et al.*, 1993). The remaining Ewing related tumors are due to translocations between EWS and Erg, PEA3 and ER81 (Sorensen *et al.*, 1994; Kaneko *et al.*, 1996 and Jeon *et al.*, 1995 respectively). The *ets* gene *Tel* was first identified as part of a translocation breakpoint in a subset of chronic myelomonocytic leukemia patients. This translocation involved the fusion of the helix-loop-helix domain of TEL with the transmembrane and tyrosine kinase domains of platelet-derived growth factor receptor β (Golub *et al.*, 1994). Tel was later identified as part of a fusion protein including the DNA binding and transactivation domains of the transcription factor AML1 in acute lymphoblastic leukemia patients (Golub *et al.*, 1995).

1.0.3 Deregulation of *ets* Gene Expression in Cancer.

Deregulation of *ets* gene expression has also been implicated in human cancers. *Ets-2* and *Erg* mRNA are overexpressed in a subset of HPV-immortalized and human cervical carcinoma cell lines (Simpson *et al.*, 1997). Ets-2 overexpression has been detected in primary human prostate tumors (Liu *et al.*, 1997) and in well-differentiated prostate adenocarcinomas cell lines (Sementchenko *et al.*, 1998). Ets-1 protein has been detected at high levels in

well-differentiated to moderately differentiated pancreatic adenocarcinomas (Ito *et al.*, 1998) and in invasive gastric adenocarcinomas (Nakayama *et al.*, 1996).

Ets deregulation caused by Friend retroviral insertion causes leukemia in mice. Constitutive expression of PU.1 and Fli-1 as a result of SFFV and F-MuLV insertion respectively results in inappropriate signaling during hematopoietic development resulting in erythroid tumors and erythroleukemias (Moreau-Gachelin *et al.*, 1989; Ben-David *et al.*, 1991). Transgenic mice with PU.1 directed by the SFFV LTR also develop erythroleukemia (Moreau-Gachelin *et al.*, 1996).

1.0.4 The PEA3 subfamily of *ets* genes

The PEA3 subfamily of *ets* genes includes PEA3, ERM and ER81. These genes share 95% sequence identity within their ETS domains and share over 50% sequence similarity overall. PEA3 was originally identified as an element capable of binding to a motif in the polyomavirus enhancer in mouse 3T6 cell nuclear extracts (Martin *et al.*, 1988) and was cloned from a mouse FM3A cell cDNA library (Xin *et al.*, 1992). ERM was isolated from a human testis cDNA library using the PEA3 ETS domain as a probe (Monté *et al.*, 1994). ER81 was cloned after screening an 8.5 day mouse embryo library with degenerate oligonucleotides homologous to conserved regions within the ETS domain (Brown and McKnight, 1992). This subfamily has been implicated in several

early embryonic developmental processes. *In situ* hybridization during embryonic mouse development revealed that the expression patterns of *PEA3* and *ERM* are very similar, whereas that of *ER81* is distinct (Chotteau-Lelievre *et al.*, 1997; Laing and Hassell, in preparation). Chotteau-Lelievre *et al.* showed that while ERM and PEA3 are preferentially expressed in epithelial cells, ER81 expression is often limited to the cells of mesenchymal origin. The expression of all three genes were commonly expressed in organs where epithelial-mesenchymal interactions occur, at sites of cell migration and proliferation. The expression profiles of *ERM* and *ER81* in adult mouse organs is broad, whereas that of *PEA3* is much more restricted, exhibiting the highest levels in the brain and epididymis (Xin *et al.*, 1992). Expression of ERM mRNA can be detected in virtually all human tissues except liver and kidney and is highly expressed in the brain and placenta (Monté *et al.*, 1994). ER81 expression has been detected in the human brain, heart, lung, testis, colon, pancreas, small intestine, spleen, kidney, liver, ovary, prostate, skeletal muscle and thymus (Brown and McKnight, 1992; Jeon *et al.*, 1995; Monté *et al,*. 1995).

The overexpression of *PEA3*, *ERM* and *ER81* has been detected in human breast tumor cell lines (Baert *et al.*, 1997). Earlier work in our lab has demonstrated the overexpression of *PEA3* mRNA in HER2/Neu-induced mouse mammary tumors (Trimble *et al.*, 1993). These MMTV LTR-HER2/Neu transgenic mice develop focal mammary tumors after a long latency period and

develop lung metastases which also overexpress *PEA3* mRNA. In addition, *PEA3* mRNA overexpression has been detected in 93% of HER2/Neu positive human breast cancers and 76% of all human breast cancers (Benz *et al.*, 1997).

1.0.5 *Ets* Genes are Downstream of Various Signaling Cascades

Ets proteins have been described as signaling downstream of various signaling pathways. ERM-mediated transactivation through both the Ras/Raf-1/MAPK and PKA pathways has been illustrated, as well as its phosphorylation by activated ERK2 and activated PKA (Janknecht *et al.*, 1996). ER81 can be phosphorylated by ERK1 and ER81 transcriptional activity is also regulated by the Ras/Raf-1/MAPK pathway (Janknecht, 1996). PEA3 can bind to and regulate the HER2/Neu promoter (Benz *et al.*, 1997). As both PEA3 and HER2/Neu are overexpressed in breast carcinomas, an autostimulatory feedback loop involving PEA3 and HER2/Neu would deregulate the expression of HER2/Neu downstream target genes contributing to breast tumorigenesis.

1.0.6 Ets Proteins Transactivate Genes Implicated in Cancer

There are few *bone fide* PEA3 subfamily target genes known. However, there are several putative target genes based on sequence analysis of their promoters revealing consensus ets binding sites. Many of these include matrix metalloproteinases, thought to be involved in cancer by increasing metastatic

potential. PEA3 has also been shown to transactivate three matrix metalloproteinases: stromelysin, type I collagenase and type IV collagenase (Higashino *et al.*, 1995). PEA3 can activate collagenase-1, stromelysin-1 and gelatinase B promoters, inducing an invasive phenotype in MCF-7 cells (Kaya *et al.*, 1996). There are PEA3 EBSs in the promoters of collagenase-1 and –3, stromelysin-1, –2 and -3, matrilysin, metalloelastase and gelatinase B (reviewed in Westermarck and Kahari, 1999). Although matrix metalloproteinases are thought to increase the ability of a cancer cell to metastasize by breaking down components of the basement membrane there is evidence to suggest that matrix metalloproteinase activity is important in the early stages of cancer development. For example, the expression of collagenase in the suprabasal layer of skin in a line of transgenic mice results in epidermal hyperplasia and leaves the skin susceptible to chemical carcinogenesis (D'Armiento *et al.*, 1995).

Stromelysin-3 is a matrix metalloproteinase that is expressed in mouse mammary glands during the process of involution but not during other stages of mammary gland development (Lefebvre *et al.*, 1992). Expression was detected in the fibroblasts that surround the degenerating ducts implicating stromelysin-3 expression is important in basement membrane remodeling. Since it is also found in fibroblasts that surround invasive neoplastic cells of human breast carcinomas stromelysin-3 activity is most likely important in breast cancer progression as well (Basset *et al.*, 1990).

Usually, matrix metalloproteinases are expressed in the stromal compartment of cancerous lesions (reviewed in Powell and Matrisian, 1996), matrilysin however has been detected in tumor cells of epithelial origin (reviewed in Wilson and Matrisian, 1996). Matrilysin is a putative PEA3 target gene as there is a PEA3 EBS in the promoter. Matrilysin is expressed in primary early-stage human colorectal tumors and in human breast cancers. As well, ectopic expression of matrilysin in a colorectal carcinomas cell line increased it's tumorigenicity in nude mice but did not increase the metastatic potential of these cells (Witty *et al.*, 1994). Mice carrying a germline mutation in the *Apc* gene (*Min* mice) normally develop many spontaneous intestinal tumors, however, when crossed to matrilysin-deficient mice they developed fewer and smaller tumors (Wilson *et al.*, 1997).

Vimentin is an intermediate filament present in the cytoskeleton of mesenchymal cells (Steinert and Liem, 1990; Fuchs and Weber, 1994). PEA3 can bind to and activate the vimentin promoter and PEA3 expression is correlated with vimentin upregulation in both human and mouse mammary tumor cells compared to normal mammary epithelium (Chen *et al.*, 1996). Vimentin is thought to play a role in cell motility and has been used as a prognostic factor for metastatic breast cancer (Raymond and Leong, 1989).

1.1 Neu is a Receptor Tyrosine Kinase Originally Characterized as a Transforming Activity

Neu was originally identified as p185 , a protein isolated from transformed NIH3T3 cells transfected with DNAs from nitrosoethylurea-induced rat neuroblastomas. When these transfectants were injected into mice, fibrosarcomas developed (Charan Padhy *et al.*, 1982). The neu protein was characterized as a 1260 aa transmembrane protein similar to the EGF receptor (Bargmann *et al.*, 1986). These two proteins share over 80% aa identity in the tyrosine kinase domain and 50% aa identity overall. The position of two cysteine-rich domains in the extracellular domain is also highly conserved. Neu is also serologically related to the EGF receptor as polyclonal antibodies against the EGF receptor are capable of binding neu (Schechter *et al.*, 1984). A partial cDNA sequence was isolated from a human mammary carcinoma using the v-erbB gene as a probe. This sequence was found to be 5-10 fold amplified in the carcinoma compared to human placental DNA (Richter King *et al.*, 1985). The full length cDNA was isolated from NIH3T3 cells that were transformed by DNA isolated from a B104 rat neuroblastoma cell line known to express high levels of p185 (Bargmann *et al.*, 1986).

1.1.1 HER2/Neu lies Upstream of Ras Signaling Cascades

HER2/Neu stimulates transcription of various target genes via the Ras pathway. For example, the GlcNAc transferase V promoter is activated by the HER2/Ras/Raf/Ets pathway (Chen *et al.*, 1998). This enzyme is responsible for the addition of β(1,6) branches on specific glycoproteins. The presence of these branches is often seen at increased levels in transformed cells and in primary human tumors. For example, cells transformed by the Rous sarcoma virus, polyoma virus, Ras or fps/yes oncogenes display an increase in N-linked oligosaccharides synthesized by GlcNAc-TV (Pierce and Arango, 1986; Dennis *et al.*, 1989). The overexpression of this enzyme in Mv1Lu mink lung cells alters cell migration, induces the loss of contact-inhibition of cell growth, relaxes growth controls and changes adhesion properties (Demetriou *et al.*, 1995). Increased levels of Glc-Nac T V enzymatic activity and the presence of high levels of β(1,6) branches has been correlated to higher metastatic potential (Dennis *et al.*, 1987). Using a plant lectin (L-PHA) that binds to β(1,6) linked lactosamine antenna with high affinity, these branches have been shown to be significantly elevated in human primary malignancies compared to normal human breast tissue and benign lesions (Dennis and Laferte, 1989). L-PHA staining is also correlated with disease progression (Fernandes *et al.*, 1991). Neu-transformed NIH 3T3 cells have a three-fold increase in GlcNAc T V enzyme activity and a corresponding increase in β(1,6) branches (Chen *et al.*, 1998). The region of the GlcNAc T V

promoter that responds to HER2 stimulation contains three PEA3 consensus sites that are bound by Ets2.

Overexpression of activated *neu* causes transcriptional activation of Ets, AP-1 and NK-κB-dependent reporter genes (Galang *et al.*, 1996). Wild type *neu* does not elicit these activations. Dominant-negative Ras and Raf both independently block neu-medicated transcriptional activation. Dominant-negative Ets-2 blocks *neu*-transformation in focus forming assays but does not inhibit the growth of normal cells.

1.1.2 Neu is Involved in Mammary Tumorigenesis

The percentage of human tumors bearing amplification of the *neu* gene and any correlation this has with prognostic factors is much debated. Neu amplification has been detected in 17% (Zhou *et al.*, 1987), 18% (Parkes *et al.*, 1990), 19% (Lacroix *et al.*, 1989), 25% (Berger *et al.*, 1988) and 28% (Slamon *et al.*, 1989) of human primary breast tumors. 8% of adenocarcinomas (Yokota *et al.*, 1986), 17% of invasive primary breast tumors (Borg *et al.*, 1990) and 19% of infiltrating ductal carcinomas (Varley *et al.*, 1987) also have *neu* gene amplification.

These analyses eventually extended to examining mRNA and protein levels. Generally, there is a strong correlation between the extent of gene amplification and mRNA and protein levels. In many cases however, mRNA and

protein levels are elevated without gene amplification, indicating alternate methods of gene activation other than gene amplification. *Neu* mRNA levels have been found to be elevated in 30% and 33% of human breast tumors (Parkes *et al.*,1990; Richter King *et al.*, 1989 respectively). Using either Western blot analyses or immunohistochemistry, elevated neu protein levels have been detected in 26% (Lacroix *et al.*, 1989) and as high as 49% (Berger *et al.*, 1988) of primary human breast tumors. 14% of stage II breast cancer patients (van de Vijver *et al.*, 1988) and 19% of more than 300 invasive primary breast tumors (Borg *et al.*, 1990) have elevated neu protein levels. The overexpression of neu appears to be specific to a subset of breast tumors. While 42% of ductal carcinomas *in situ* of the large-cell, comedo growth type stained positively for neu, no ductal carcinomas *in situ* of small-cell, papillary or cribriform growth type displayed neu protein overexpression (van de Vijver *et al.*, 1988).

The overexpression of neu has been correlated with axillary lymph node involvement (Zhou *et al.*, 1987; Borg *et al.*, 1990; Tandon *et al.*, 1989), poor tumor grade (Parkes *et al.*, 1990; Berger *et al.*, 1988), advanced staging (Zhou et al., 1987; Borg et al., 1990), the absence of steroid receptors (Borg *et al.*, 1990; Tandon *et al.*, 1989) and larger tumor size (van de Vijver *et al.*, 1988; Borg *et al.*, 1990). Regardless of lymph node involvement in disease, *neu* amplification has been shown to be significantly correlated with decreased overall survival time (Parkes *et al.*, 1990). *Neu* amplification, mRNA or protein overexpression has

been shown not to be correlated with disease outcome for patients with negative axillary lymph nodes, but is a significant independent predictor of early relapse and death in node positive patients (Tandon *et al.*, 1989; Richter King *et al.*, 1989; Borg et al., 1990; Slamon *et al.*, 1989). While some studies have found no correlation with *neu* overexpression and early disease recurrence but perhaps a trend towards poorer prognosis (Parkes *et al.*, 1990; Varley *et al.*, 1987). There is also evidence that neu amplification is not correlated with steroid receptor status (Parkes *et al.*, 1990).

1.1.3 Neu is Activated by Mutations in the Transmembrane Domain

Since amplification of wild type *neu* in NIH3T3 cells was non-transforming (Hung et al, 1986) and no gross gene rearrangements were detected in primary human breast tumors that displayed gene amplification (Yokota et al, 1986), a minor change in the DNA sequence was thought to be responsible for *neu* activation and transformating ability (Hung *et al.*, 1986). A mutation within the transmembrane domain was found in four independent cell lines derived from nervous system tumors developed in BDIX rats after treatment with ethylnitrosourea. This mutation, at positon 644, replaced valine with glutamic acid (Bargmann *et al.*, 1986). This mutation is specific in it's ability to activate *neu*, as other amino acids substituted at position 644 are non-transforming and

mutant proteins with glutamic acid introduced at either 643 or 645 are also non-transforming (Bargmann and Weinberg, 1988).

The oncogenic form of neu aggregates at the plasma membrane (Weiner *et al.*, 1989). The increased aggregation is thought to be due to the formation of two hydrogen bonds between alanine at position 661 and the glutamic acid introduced at position 644. This would stabilize what would otherwise be held together by van der Waals forces only (Sternberg and Gullick, 1989).

1.2 Project Objective

PEA3 is a transcription factor that is upregulated in HER2/Neu-induced mouse mammary tumors (Trimble *et al.,* 1993). PEA3 is thought to increase the metastatic potential of these tumors by subsequent deregulation of matrix metalloproteinase expression. The objective of this project was to determine if other *ets* genes are involved in this model of human breast cancer as well. Since the family of *ets* genes is very large in order to identify potential *ets* genes of interest, a degenerate RT-PCR approach was utilized. Briefly, degenerate primers were designed to amplify from highly conserved regions with the ETS domain. All members of the *ets* family were grouped according to high sequence similarity and divided into five groups. Using five different pairs of degenerate primers, sequences were cloned from mouse mammary tumor RNA and sequenced. Several *ets* genes were identified for further characterization based

on differences in frequency of recovery from normal mouse mammary glands (performed by M. Szrajber). These genes were tested for quantitative differences in gene expression between tumor and normal mouse mammary gland samples by semi-quantitative RT-PCR analysis and RNase protection analysis.

Material and Methods

2.0 Genotype Analysis of Transgenic Mice

The presence of the MMTV-HER2/Neu transgene in the FVB strain of mice was detected by Southern hybridization (adapted from Laird *et al.*, 1991). DNA was obtained from tail clippings which were incubated in lysis buffer [.1M Tris-Cl, 0.2M NaCl, .2% SDS, 5mM EDTA (pH 8.0), 100ug Proteinase K/ml] at 55º overnight. After vigorous vortexing and centrifugation at high speed for 10 minutes the supernatant was mixed with 500ul of isopropanol followed by another 10 minute centrifugation. The pellet was dried briefly, resuspended in 50ul of ddH_2O and incubated at 55°C for at least one hour, up to as long as overnight.

5 ul of each DNA sample was incubated at 37°C overnight in a 25ul reaction including 10X Buffer B and 1ul of high concentration BamHI (both from Boehringer Mannheim). The samples were run on a 1% agarose gel made using TPE buffer (1X: 0.09M Tris-phosphate, 0.002M EDTA) for approximately three hours at 70 volts. The DNA was then transferred overnight onto a nylon membrane (GeneScreen Plus, NEN™ Life Science Products). After UV crosslinking the membrane was incubated for at least 30 minutes at 65°C in an

appropriate amount of prehybridization solution (5X Denhardts' solution, 5X SSC, 1.5% SDS, 5mg sheared salmon sperm DNA). 50X Denhardts' solution contains 5g of Ficoll (Type 400, Pharmacia), 5g of polyvinylpyrrolidone, and 5g of bovine serum albumin (Fraction V; Sigma) in a final volume of 500ml.

The probe was made by incubating 5ul of the DNA template (SPA probe, from W. Muller) with 100U of random hexamers (Boehringer Mannheim), 10X React 2 (Gibco BRL) and 1mM of dATP, dTTP and dGTP each. This was boiled for 5 minutes and then 50uCi [α-^{32}P]dCTP (Amersham Pharmacia Biotech Inc.) and 1ul of Klenow (Large Fragment of DNA Polymerase I, Gibco BRL) was added and the reaction was incubated at 37°C for one to two hours. The probe was then purified by either a homemade Sephadex G-50 column or by using ProbeQuant™ G-50 Micro Columns from Amersham Pharmacia Biotech Inc.). After boiling for 5 minuts, 1X10^{6}cpm/ml of prehybridization solution was added to the membrane, which was then incubated at 65°C overnight. The membrane was exposed to film (Kodak Scientific Imaging Film, X-OMAT™ AR) for approximately one week before being developed.

2.1 RNA extraction

RNA was isolated from mouse mammary tumors by the guanidinium thiocyanate method (Ausubel *et al.,* 1995). Tissue was homogenized in 5 ml of tissue resuspension buffer (4M guanidinium isothiocyanate, 0.05M Tris-Cl pH 7.5,

0.01M Na_2EDTA pH 8.0, 0.72M β-mercaptoethanol) and layered over 7 ml of 5.7M cesium chloride solution and centrifuged at 32000 rpm at 22ºC overnight. The RNA pellet was resuspended in 500ul of resuspension buffer (.5% Sarkosyl, 5% β–mercaptoethanol, 5mM EDTA) and purified by phenol:chloroform and chloroform extractions. The RNA was precipitated by 100% ethanol followed by 75% ethanol 25% NaoAc solution and resuspended in an appropriate volume of DEPC-treated water.

2.2 Degenerate RT-PCR

2.2.1 Design of degenerate primers

The degenerate primers were designed by M. Loreto. The *ets* family was divided into five groups based on high sequence similarity within the ETS domain. Forward and reverse primers were designed to hybridize to regions of high sequence similarity within the ETS domain for each group. Forward and reverse primers for each group were:

group A: CA(G/A) CTI TGG CA(G/A) TT(T/C) (C/T)T(G/C/T) (G/C)T and C(G/T) (G/A)CT IAG (T/C)TT ITC (G/A)TA (G/A)TT C

group B: TA(T/C) (C/T)T(T/G) TGG GAG TT(T/C) (T/C)TI (C/T)T and CC CAT (G/T)GT (T/C)TC (A/G)TA (A/G)TT C

group C: ACI CTG TGG CAG TT(T/C) CT(G/C/T) (C/T)T and the same reverse primer used for group A

group D: CGC CTG TAC CAG TTG CTG (C/T)TG and G CGC CA(G/T) CTT CTG GTA GGT C

group E: C(T/A)(G/C) CT(T/G) TGG GA(T/G) T(A/T)C (G/A)TC and (G/C)(T/A) CA(T/G) (T/C)TT CTC (A/G)TA GGT C.

2.2.2 Reverse Transcription of Sample RNA

Reverse transcription of 1 ug of total RNA was performed using random hexamers and performed according to manufacturer's instructions (GibcoBRL SuperScript Preamplification System for First Strand cDNA Synthesis) with the following modifications. The reaction was incubated at room temperature for 15 minutes before addition of SuperScript II RT and cDNA synthesis occurred for 60 minutes at 42ºC. RNA was not removed by RNase H digestion. One µg of total RNA and 50 ng of random hexomers were used in each 60µl reverse transcription reaction.

2.2.3 PCR Amplification using Degenerate Primers

PCR was performed using these primers on cDNA reverse transcribed as detailed above. The PCR amplifications were performed in a 20 µl reaction containing 10X PCR reaction buffer (GibcoBRL), .25mM dNTPs (Pharmacia Biotech), 3 uM of each forward and reverse degenerate primers, 5 units of Taq polymerase and 1µl of cDNA. The reaction was performed over 5 MgCl titrations

(1.25mM to 4mM). The PCR conditions used for primer groups A, B, C and E was: 3 cycles of 95°C for 30 seconds, 42°C for 30 seconds and 72°C for 30 seconds, followed by 25 cycles of 95°C for 30 seconds, 48°C for 30 seconds and 72°C for 30 seconds. Conditions were identical for Group D primers except for annealing temperatures of 60ºC and 65ºC for 3 and 25 cycles respectively. The amplified products of expected size were extracted, pooled and gel purified using the Qiagen gel extraction kit.

2.2.4 Cloning and Isolation of the Amplified Products

Inserts were cloned using the Invitrogen TA cloning kit and the blue-white screening technique. White colonies were randomly picked and incubated in approximately 4ml of LB media overnight. Plasmid DNA was isolated as follows. Approximately half of the incubated mixture was centrifuged for 30 seconds to pellet the bacteria. After excess media was removed, 100ul of cold "Solution 1" [50mM glucose, 25mM Tris-Cl (pH 8.0) and 10mM EDTA (pH 8.0)] was added and the tube was vortexed to resuspend the bacterial pellet. 200ul of fresh "Solution 2" (1% SDS, .2N NaOH) was added and the tube was mixed by being rapidly inverted 5 times. 150ul of cold "Solution 3" (XM KOAc, 11.5% acetic acid) was added, vortexed (in an inverted position for approximately 10 seconds) and incubated on ice for 5 minutes. Following centrifugation at maximum speed for 10 minutes the supernatant was transferred to a new tube and an equal volume

of phenol:chloroform was added and vortexed. After a 5 minute centrifugation the supernatant was mixed with 0.1 volumes of 3M NaAcetate and 2 volumes of cold absolute ethanol. This was spun for 20 minutes at 4ºC. The supernatant was removed and the DNA pellet was rinsed with 500ul of 70% ethanol. After a 5 minute spin the pellet was resuspended in 30ul of resuspension buffer (1ml of TE buffer with 5ul of RNase H added).

The presence of inserts was confirmed by restriction endonuclease analysis. 10ul of the sample was incubated with 1ul of EcoR1, 10X buffer in a 20ul reaction at 37ºC for one hour. The samples were then run on a 1% agarose/TAE gel containing ethidium bromide. 1X TAE consists of .04 M Tris-acetate and .001 M EDTA. Inserts were visualized under ultraviolet light. Clones with inserts were sent to the MOBIX Central Facility for sequencing.

2.3 Semi-quantitative RT-PCR

2.3.1 Design of Gene Specific Primers

Primers specific to several *ets* genes (PEA3, ERM ER81, GABPα, Ets-1, and Ets-2) were designed to anneal to regions not in the ETS domain to avoid cross-amplification of different *ets* genes due to high sequence similarity in that region. The PEA3 primers were a gift from R. Tozer. The forward ERM primer was designed by L. MacNeil and the reverse primer was designed by L. Kockeritz. The ER81, rpL32 and cytokeratin 8 primers were designed by L. Kockeritz. The Ets-1, Ets-2 and GABPα primers were designed by M. Szrajber.

Primer sequences used in semi-quantitative RT-PCR analyses:

Gene	Forward Primer	Reverse Primer
PEA3	AGA CAA ATC GCC ATC AAG TCC	GAC TTC GCC TAC GAC TCA GAT
ERM	CAC TGT TTG AAC ATG GGG TCC	CAC TTT GAA GAC AAC CCT GCT
ER81	TCC AGA TCC CGA GGA AAT CTC	AGT GCC TGT ACA ATG TCA GTG
GABPα	GCT GAA TGT GTA AGC CAG GCC	GCT GCA CTG GAA GGC TAC AGA
Ets-1	CCG TCG ATC TCA AGC CGA CTC	CCA GAC AGA CAC CTT GCA GAC
Ets-2	ACA CTC AAG CGC CAG CCA GCC	CTG CAG CTC TGG GCT CTG AGC AAG
CK8	AGA AGC TGA AGC TGG AGG	CC ACC CTA GAG CTA GC
L32	AAA ACC AAG CAC ATG CTG CCC	CCA ATC CCA ACG CCA GGC TAC

2.3.2 Reverse Transcription of Sample RNA

Reverse transcription of sample RNA for semi-quantitative RT-PCR was performed as described earlier for degenerate RT-PCR.

2.3.3 PCR Amplfication Using Gene Specific Primers

The PCR reaction for each primer set was optimized for MgCl concentration and annealing temperature and performed at a final volume of 20ul using the same reagents as already described.

Gene	[MgCl] mM	Annealing Temp (°C)
PEA3	1.25	50
ERM	1.25	62
ER81	2.00	62
GABPα	1.25	62
Ets-1	1.25	65
Ets-2	1.25	65
CK8	1.25	55
rpL32	1.25	62

The range of exponential amplification was determined by performing PCR in duplicate every second or third cycle starting at 10 cycles up to 40. PCR was then performed in duplicate for at least three cycles within this range. Half of the PCR reaction was mixed with 1 ul of DNA loading buffer (0.25% xylene cyanol FF, 30% glycerol in water) and 10ul of this solution was run on 2% agarose gel. Transfers of DNA were performed as described previously. 100 bp DNA ladder was also run on the gel in DNA loading buffer that also contained 0.25% bromophenol blue. The bromophenol blue was disregarded from the samples because it migrated the same distance as the bands of expected size (~180 bp) and made visualization of these bands difficult at times.

The template for each probe was made by PCR amplification of plasmid cDNA using the gene specific primers detailed above. After Qiagen gel extraction 5ng of DNA was used as a template for probe synthesis which was performed as previously described. 7x10^6cpm of probe per 10ml of prehybridization solution was used in each Southern hybridization. The membrane were exposed on film (Kodak Scientific Imaging Film, X-OMAT™ AR) for an appropriate length of time and then quantified by PhosphorImager analysis.

PCR on the target genes (*ets* genes) and control genes (rpL32 and cytokeratin 8) was performed separately because it has been reported that in a similar experiment, simultaneous amplification using two sets of primers

generates competition within the reaction that results in lower levels of PCR products (Murphy *et al.,* 1990).

2.4 RNase Protection Analyses

2.4.1 Design of Riboprobe Templates

The PEA3 subfamily of riboprobes were designed by T. Shepherd. The Ets-1 and GABPα riboprobes were designed by M. Szrajber. The Ets-2 riboprobe was a gift from R. Oshima. The rpL32 and cytokeratin 8 riboprobes were from W. Muller.

2.4.2 RNase Protection

Riboprobes were made by incubating .5 ug of linearized template DNA in a 25 ul reaction containing 0.4 mM of each ATP, GTP and CTP (Pharmacia Biotech), .04mM UTP (Pharmacia Biotech), 1 ul of RNA guard (Pharmacia Biotech), 5 ul of the appropriate 5X buffer (GibcoBRL), 50 uCi α^{32}UTP (Amersham Pharmacia Biotech Inc.) and 2 ul of RNA polymerase (GibcoBRL) and incubated for 45 minutes at 37ºC (Ausubel *et al.,* 1995). 1 ul of 10mM UTP was added and incubated for an additional 10 minutes at 37°C. The mixture was DNase treated by adding: 20ul DEPC-treated water, 1ul .5mM $MgCl_2$ and 2ul of DNase/RNase free (Boehringer Mannheim) and incubated at 37°C for 15 minutes. The riboprobes were then purified by phenol:chloroform extractions and

precipitated using 2 volumes of 2.5M NH_4Oac and 7.5 volumes of absolute ethanol. The pellets were resuspended in 4ul of loading buffer (80ul formamide, 2ul 50mM EDTA, 10ul 1% xylene cyanol, 10ul 1% bromophenol blue), run on an acrylamide gel and gel extracted in elution buffer (0.625M NH_4OAc, .125% SDS, 1.25mM EDTA) and incubated at 37°C for at least one hour but up to overnight. The riboprobes were then precipitated using 2.5 volumes of absolute ethanol follwed by a final wash in 75% ethanol/NaOAc. The pellets were resuspended in 50ul of hybridization buffer (160ul 10X PIPES and 800ul formamide). 50 000 counts of each probe were hybridized with 10 ug of total sample RNA at 50ºC overnight. Acrylamide gel electrophoresis was performed following RNase digestion. The digestion buffer consisted of .3M NaCl, 10mM Tris and 5mM EDTA. The undigested yeast tRNA control was incubated in the above solution. All samples were incubated in a digestion buffer containing 5ul of 10mg/ml RnaseA and 1.5ul RNase T1 per ml of digestion buffer. This solution was incubated for 30 minutes at 37°C. 30ul of SDS/proteinase K (400ul of 10% SDS in 200ul of proteinase K) was added and incubated for 15 to 20 minutes at 37°C. The samples were purified by phenol:chloroform extraction and precipated using absolute ethanol and an incubation for one hour at -80°C. After a 30 minute centrifugation the pellet was dried and resuspended in loading buffer (previously described), denatured at 85°C for 5 minutes and run on an acrylamide gel. The gel was dried for at least 30 minutes after having run at 55W for approximately

three hours. The dried gel was exposed on film (Kodak Scientific Imaging Film, X-OMAT™ AR) for an appropriate length of time and then quantified by PhosphorImager analysis.

RESULTS

3. 0 Introduction

To identify *ets* genes that may play an important role in HER2/Neu-induced mouse mammary tumorigenesis it is important to isolate which *ets* genes are expressed in these tumors and if these genes are differentially expressed when compared to normal mouse mammary glands. To quickly identify the spectrum of *ets* gene expression in normal mouse mammary glands and in HER2/Neu-induced mouse mammary tumors a degenerate RT-PCR analysis was performed. This procedure has been used to identify novel genes belonging to families which have region(s) of high sequence similarity (Agata *et al.*, 1998; Kusuhara *et al.*, 1999; Leiter *et al.*, 1999) and to detect homologues in different species (Grompe *et al.*, 1992; Osuna *et al.*, 1999). It has also been used to detect gene expression in particular cell types or tissues (Aeschlimann *et al.*, 1998; Wakeman *et al.*, 1998; Jin *et al.*, 1999; O'Donnell *et al.*, 1999). For our purposes, five different pairs of degenerate primers were designed to maximize recovery of all *ets* family members. As well, the high degeneracy of the primers also allowed for the possibility of identifying novel *ets* genes. The spectrum of *ets* genes expressed in normal mouse mammary glands and in mouse mammary tumors was performed by M. Szrabjer and L. Kockeritz respectively. Results

obtained from these analyses led to an examination of the quantitative differences in gene expression using semi-quantitative RT-PCR. Southern blot hybridizations were performed to quantify the results by PhosphorImager analysis. To further substantiate the quantitative differences of *ets* gene expression in normal mouse mammary glands and in HER2/Neu-induced mouse mammary tumors ribonuclease protection analyses were performed.

3.1 Degenerate RT-PCR analysis

To determine which *ets* genes are expressed in mouse mammary tumors a degenerate RT-PCR approach was utilized. All known members of the *ets* gene family were grouped into five different primer pair target groups (A through E) based on high sequence similarity within their ETS domain.

Two N202 tumor samples were used in the degenerate RT-PCR analyses. The first tumor sample analyzed came from a mouse (gift from Lesley MacNeil) that was 8 months of age at the time of sacrifice, had borne the tumor for approximately two months and had no visually detectable lung metastases. RNA from the second tumor analyzed was a gift from Peter Siegel. The mouse was 16 months of age when sacrificed, had carried the tumor for 6 weeks, had no lung metastases and bore two independent tumors in addition to the one analyzed.

3.1.1 Degenerate RT-PCR using group A primers.

The group A degenerate primers targeted the PEA3, ELG, ERF, ERG, and Ets subfamilies of *ets* genes as well as ER71 and FEV *ets* genes. The spectrum of *ets* genes recovered from both tumor samples was very similar (Table 1). Each tumor sample yielded ER81, ERM, GABPα, Ets-1 and Ets-2 clones. ERF, Fli-1 and ERG-3 were recovered from the first tumor but at very low frequency (1/64 clones each). ER81 was the clone isolated with the greatest frequency from the first tumor sample, 24 of 64 clones. ERM and GABPα were recovered with similar frequencies (16/64 and 14/64 clones respectively). Ets-2 was recovered 6 times, while all the other *ets* genes recovered, ERF, Fli-1, ERG-3 and Ets-1 were only recovered once. The second tumor yielded a more limited array of *ets* gene recovery. GABPα, ER81, ERM and Ets-2 clones were recovered 26, 15, 8 and 3 times out of 53 total clones respectively. Ets-1 was the only other clone recovered from the second tumor sample and it was recovered just once.

Surprisingly, PEA3 cDNAs were not recovered from either tumor sample. Previously, Trimble *et al.* had shown PEA3 transcripts are overexpressed in tumors that develop in N202 transgenic mice using Northern hybridization and RNase protection analyses (Trimble *et al.*, 1993). Presumably, the low absolute levels of PEA3 transcripts, compared to those of other *ets* genes accounted for these findings.

Table 1: Results of Degenerate RT-PCR using group A primers.

Numbers in each column represent the number of clones recovered after reverse transcription of RNA using random hexamers, PCR using group A degenerate primers and TA cloning of PCR products of expected size. Analysis of the FVB samples were performed by M. Szrabjer.

Target Group	Subgroup	Gene	FVB 1 8 week virgin	FVB 2 8 week virgin	Tumor 1 8 months old	Tumor 2 16 months old
A	PEA3	ER81	1	0	24	15
		ERM	11	5	16	8
		PEA3	3	0	0	0
	ELG	GABPα	36	35	14	26
	ERF	ERF	0	0	1	0
		H.PE-1	0	0	0	0
	ERG	FLI-1	5	0	1	0
		ERG-1	0	0	0	0
		ERG-2	0	0	0	0
		ERG-3	0	0	1	0
	ETS	ETS-1	11	19	1	1
		ETS-2	15	3	6	3
	other	ER71	0	0	0	0
		H.FEV	0	0	0	0
			82	62	64	53

3.1.2 Degenerate RT-PCR using group B primers.

The group B primers target the ELF subfamily of *ets* genes which consists of ELF, ELF1R, NERF and MEF. ELF was the most frequently recovered *ets* gene in both samples (9 of 10 and 10 of 10 respectively). NERF was recovered once in the first tumor sample (Table 2). Human, but not mouse NERF has been characterized in the literature. The clone recovered in these analyzes has 92% nucleotide sequence identity and 100% predicted amino acid identity with human NERF (Figure 1). Interestingly, several of the recovered ELF clones carried three mutations in the coding sequence, one of which resulted in the production of a stop codon in the middle of the ETS domain (Figure 2). These mutations were recovered in cDNAs from both tumor samples as well as normal and other tumor samples analyzed by others (M. Szrajber and J. Larking data not shown). Because these three mutations occurred together and were recovered in multiple RNA samples, it is unlikely these mutations are artifacts of PCR amplification.

Table 2: Results of Degenerate RT-PCR using group B primers.

Numbers in each column represent the number of clones recovered after reverse transcription of RNA using random hexamers, PCR using group B degenerate primers and TA cloning of PCR products of expected size. Analysis of the FVB samples were performed by M. Szrabjer.

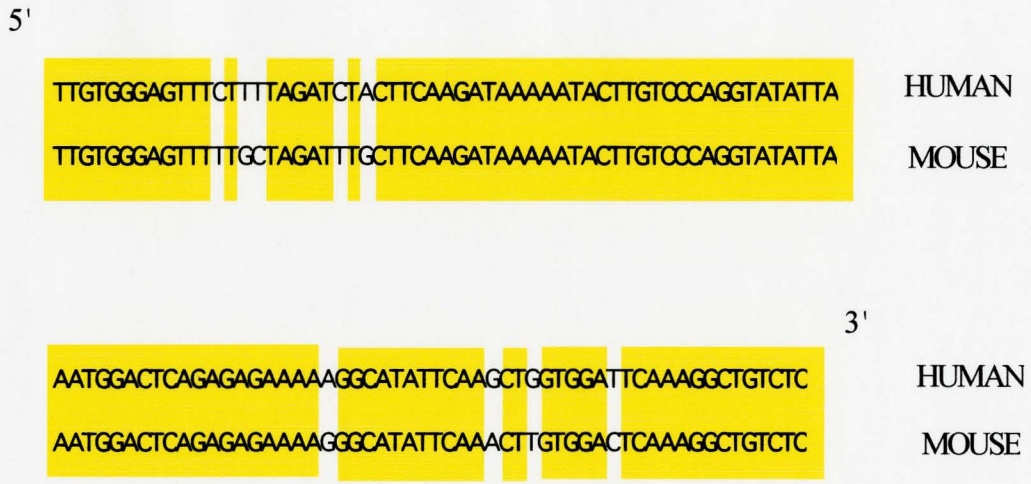
Target Group	Subgroup	Gene	FVB 1 8 week virgin	FVB 2 8 week virgin	Tumor 1 8 months old	Tumor 2 16 months old
B	ELF	ELF	13	10	9	12
		ELF1R	0	0	0	0
		H.NERF	1	0	1	0
		H.MEF	0	1	0	0
			14	11	10	12

Figure 1: Comparison of human NERF and a sequence recovered after using the degenerate RT-PCR technique on mouse mammary gland tumor RNA.

A. cDNA sequence alignment of human NERF and a potential mouse NERF sequence. The two sequences share 92% sequence identity.

B. Predicted amino acid sequence of the putative mouse NERF sequence has 100% amino acid homology to the human NERF sequence.

A


5'
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HUMAN
TTGTGGGAGTTTTTGCTAGATTTGCTTCAAGATAAAAATACTTGTCCCAGGTATATTA
MOUSE
3'
AATGGACTCAGAGAGAAAAAGGCATATTCAAGCTGGTGGATTCAAAGGCTGTCTC
HUMAN
AATGGACTCAGAGAGAAAAGGGCATATTCAAACTTGTGGACTCAAAGGCTGTCTC
MOUSE

B


NH3
COOH
LWEFLLDLLQDKNTCPRYIKWTQREKGIFKLVDSKAV
HUMAN
LWEFLLDLLQDKNTCPRYIKWTQREKGIFKLVDSKAV
MOUSE

Figure 2: Representative sequence of the Elf-1 clones recovered from the degenerate RT-PCR sequence data containing three mutations within the ETS domain.

The middle mutation creates an immature stop codon. One of the sequences recovered contained only the first two mutations.

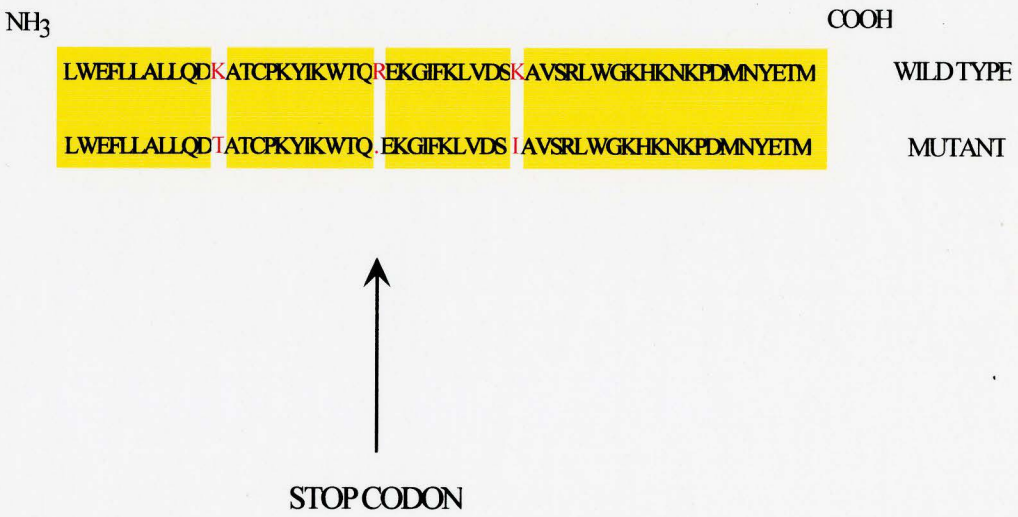
A. DNA sequence alignment of wild type and mutant mouse Elf-1 sequences.

B. Amino acid sequence alignment of wild type and mutant mouse Elf-1.

A


5'
TACCTTTGGGAGTTTTTGCTGGCCCTGCTTCAGGACAAAGCTACCTGTCCTGTCCTAAGTACATCAAGTGG
TACCTTTGGGAGTTTTTGCTGGCCCTGCTTCAGGACACAGCTACCTGTCCTGTCCTAAGTACATCAAGTGG
WILD TYPE
MUTANT
ACCCAGCGAGAAAAGGGCATTTTTAAGCTGGTTGATTCTAAAGCCGTGTCTAGATTGTGGGGGAGCACAA
ACCCAGTGAGAAAAGGGCATTTTTAAGCTGGTTGATTCTATAGCCGTGTCTAGATTGTGGGGGAGCACAA
WILD TYPE
MUTANT
AAACAAACCTGACATGAACTACGAGACCATG
AAACAAACCTGACATGAACTACGAGACCATG
3'
WILD TYPE
MUTANT

B


NH3
COOH
LWEFLLALLQDKATCPKYIKWTQREKGIFKLVDSKAVSRLWGKHKNKPDMNYETM
LWEFLLALLQDTATCPKYIKWTQ.EKGIFKLVDS IAVSRLWGKHKNKPDMNYETM
WILD TYPE
MUTANT
STOP CODON

3.1.3 Degenerate RT-PCR using group C, D and E primers.

The group C primers targeted the ELK subfamily: Sap-1, NET and Elk-1. Both tumor samples recovered Elk-1 with 50% frequency (Table 3). NET was recovered from the remainder of clones isolated from the first tumor sample and Sap-1 was recovered from the second tumor sample. Several *ets* genes targeted by the group A primers were recovered after analysis with the group C primers. The same reverse primer was used in the analyses performed using the group A and C primers. These primers also were highly degenerate. This likely explains the "cross-amplification" that occurred (Tables 4 and 5).

Group D primers, which targeted the Spi subfamily of *ets* genes (PU.1 and Spi-B), were unable to isolate any *ets* sequences from the tumor samples (see Table 3). PU.1 expression is limited to the spleen, bone marrow, interstitial nonhepatocytes of the liver and the interstitial nontubular cells of testis (Galson *et al.*, 1993) and Spi-B is limited to the thymus, spleen and lymph nodes (Su *et al.*, 1996). As the expression of these genes is limited to cells that are most likely present in very small quantities in the tumor samples, their cloning using this technique would be expected to be difficult.

The group E primers targeted TEL and ESE/ESX/Elf-3. Both tumor samples recovered TEL with high frequency (6/7 and 5/7 clones respectively) (see Table 3). EHF, a newly discovered *ets* gene, was recovered once from each tumor sample.

Table 3: Results of Degenerate RT-PCR using group C, D and E primers.

Numbers in each column represent the number of clones recovered after reverse transcription of RNA using random hexamers, PCR using either group C, D or E degenerate primers and TA cloning of PCR products of expected size. Analysis of the FVB samples was performed by M. Szrabjer.

Target Group	Subgroup	Gene	FVB 1 8 week virgin	FVB 2 8 week virgin	Tumor 1 8 months old	Tumor 2 16 months old
C	ELK	SAP-1 NET ELK-1	0 1 0	0 6 0	0 2 2	1 0 1
			1	6	4	2
D	SPI	PU.1 H.SPI-B	2 0	0 0	0 0	0 0
			2	0	0	0
E	YAN other	TEL ESE/ESX EHF	7 0 0	4 0 0	6 0 1	5 0 1
			7	4	7	6

Table 4: Degenerate RT-PCR sequence data from tumor sample 1.

This sample was derived from an N202 transgenic mouse that bore the tumor for 2 months before sacrifice, displayed no visible lung metastases and was 8 months of age at the time of death. Sequence data recovered from each primer pair is illustrated.

Target Group	Subfamily	Gene	Group A Primers	Group B Primers	Group C Primers	Group D Primers	Group E Primers	Total
A	PEA3	ER81	24					24
		ERM	16					16
		PEA3						
	ELG	GABPα	10	1	3			1
	ERF	ERF			1			1
		H.PE-1						
	ERG	FLI-1			1			1
		ERG-1						
		ERG-2						
		ERG-3			1			1
	ETS	ETS-1			1			1
		ETS-2	1	1	4			6
	other	ER71						
		H.FEV						
			51	2	11			64
B	ELF	ELF		9				9
		ELF1R						
		H.NERF		1				1
		H.MEF						
				10				10
C	ELK	SAP-1						
		NET			2			2
		ELK-1			2			2
					4			4
D	SPI	PU.1						
		H.SPI-B						
								0
E	YAN	TEL					6	6
	other	ESE/ESX						
		EHF					1	1
							7	7
non-ets			1	0	0	5	1	7
TOTAL			52	12	15	5	8	92

Table 5: Degenerate RT-PCR sequence data from tumor sample 2.

This sample was derived from an N202 transgenic mouse that bore the tumor for 6 weeks before sacrifice, displayed no visible lung metastases, carried an additional two mammary tumors not analyzed and was 16 months of age at the time of death. Sequence data recovered from each primer pair is illustrated.

Target Group	Subfamily	Gene	Group A Primers	Group B Primers	Group C Primers	Group D Primers	Group E Primers	Total
A	PEA3	ER81	15					15
		ERM	8					8
		PEA3						
	ELG	GABPα	21		5			26
	ERF	ERF						
		H.PE-1						
	ERG	FLI-1						
		ERG-1						
		ERG-2						
		ERG-3						
	ETS	ETS-1	1					1
		ETS-2	1		2			3
	other	ER71						
		H.FEV						
			46		7			53
B	ELF	ELF		12				12
		ELF1R						
		H.NERF						
		H.MEF						
				12				12
C	ELK	SAP-1			1			1
		NET						
		ELK-1			1			1
					2			2
D	SPI	PU.1						
		H.SPI-B						
								0
E	YAN	TEL					5	5
	other	ESE/ESX						
		EHF					1	1
							6	6
non-ets			2	0	3	8	6	19
TOTAL			48	12	12	8	12	92

3.2 Semi-quantitative RT-PCR

Degenerate RT-PCR illustrated several potential differences in *ets* expression between tumor and normal mouse mammary gland tissue. Both ERM and ER81 were recovered more frequently in the two tumor samples than in either of the normal FVB controls. Conversely, Ets-1 was recovered to a lesser extent in the tumor samples than in the FVB control mice. However, due to the nature of the degenerate RT-PCR technique, it was impossible to conclude that these differences reflect a quantitative difference in gene expression. To determine if these differences are indeed real, semi-quantitative RT-PCR was utilized. Gene specific primers for PEA3, ERM, ER81, Ets-1, Ets-2, GABPα, rpL32 and cytokeratin 8 were designed. The *ets* gene specific primers were designed to anneal to regions outside of the ETS domain to prevent cross-amplification due to high sequence similarity in that region. Primers for rpL32 and cytokeratin 8 were designed because detection of these genes' expression would serve as a control for total cellular content and epithelial cell content respectively. Cytokeratin 8 expression is epithelial-specific (reviewed in Lazarides, 1982; reviewed in Steinert and Roop, 1988) and rpL32 is a housekeeping gene (Chung and Perry, 1993) and as such expression should be consistent between cells within a tissue.

The same tumor samples used in the degenerate RT-PCR analyses were used for the semi-quantitative RT-PCR. Virgin FVB mouse mammary glands

were used as a normal control as were the mammary glands from an N202 mouse that was tumor free at the time of sacrifice (TG control). PCR was performed over many cycle numbers for each primer pair with each tissue sample in order to determine the exponential phase of amplification (data not shown). Five consecutive cycles within the exponential phase of amplification were then performed for each primer pair and tissue sample, except for PEA3, which was tested for three non-consecutive cycles within the exponential range of amplification.

3.2.1 The PEA3 subfamily

PEA3 expression was detected after 24 cycles in the two tumor samples and in the TG control (Figure 3). PEA3 expression could not be detected in FVB mouse mammary glands despite many attempts using samples from independent mice. Therefore, quantitation was done in comparison to the TG control. ER81 and ERM expression could be detected much earlier, at least at cycle 17 and 18 respectively (Figures 4, 5). This implies there are higher absolute levels of these transcripts than those of PEA3. Differences in ets gene expression between normal and tumor samples due to differences in total cellular RNA content was corrected by using rpL32 expression levels (Figure 6). In an effort to correct for differences in epithelial cell content between normal and tumor tissue cytokeratin 8 expression levels were also evaluated (Figure 7).

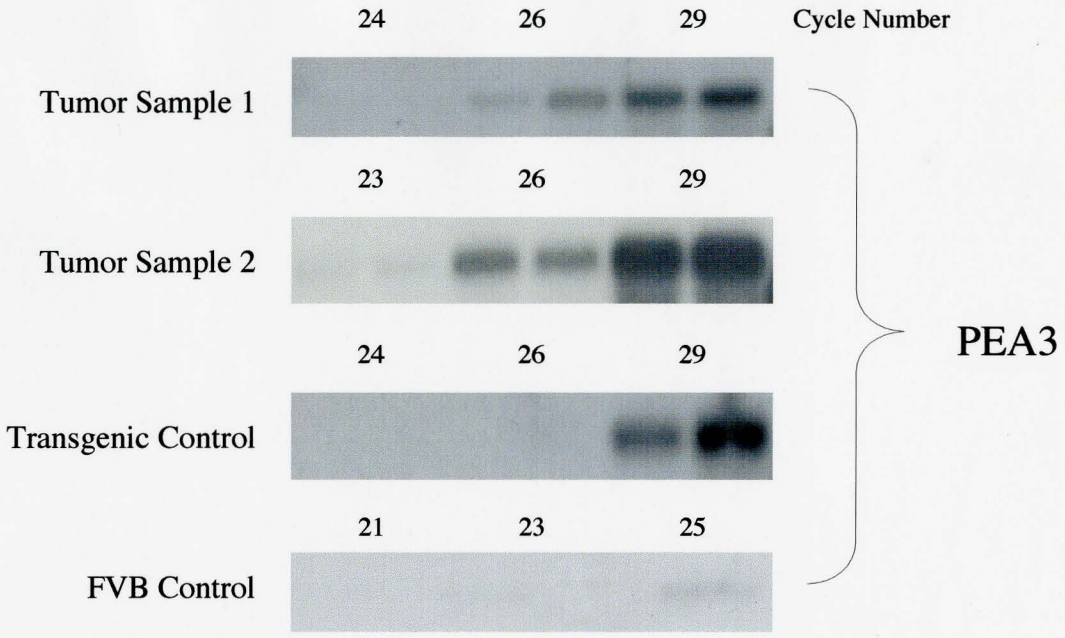
Figure 3: Semi-quantitative RT-PCR analysis of PEA3 expression on four RNA samples.

The two tumor samples were used in the degenerate RT-PCR analyses. The transgenic control sample was derived from RNA from the mammary glands of a transgenic mouse that was sacrificed prior to tumor development and was 14 months of age at the time of death. The FVB control used was 6 months of age.

A. Southern hybridization data after having performed reverse transcription using random hexamers and PCR using gene specific primers in duplication at the noted cycle numbers.

B. Quantification of Southern hybridizations shown above by PhosphorImager analysis. PhosphorImager data was averaged for each cycle number and plotted at the $\log_2$. PEA3 expression in the FVB RNA sample was undetectable by PhosphorImager analysis and could not be quantified.

A


24
26
29
Cycle Number
Tumor Sample 1
23
26
29
Tumor Sample 2
24
26
29
Transgenic Control
21
23
25
FVB Control
PEA3

B

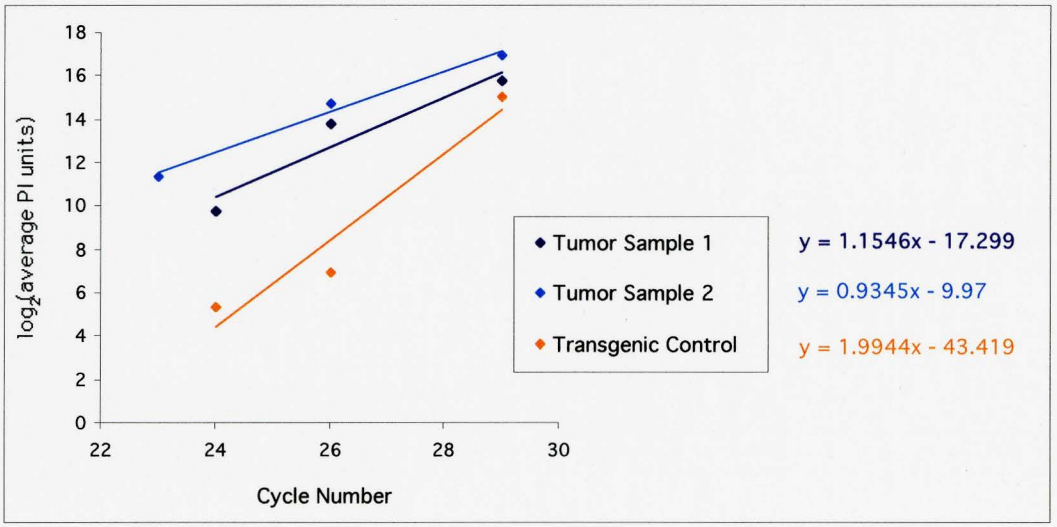

log2(average PI units)
18
16
14
12
10
8
6
4
2
0
22
24
26
28
30
Cycle Number
Tumor Sample 1
Tumor Sample 2
Transgenic Control
y = 1.1546x - 17.299
y = 0.9345x - 9.97
y = 1.9944x - 43.419

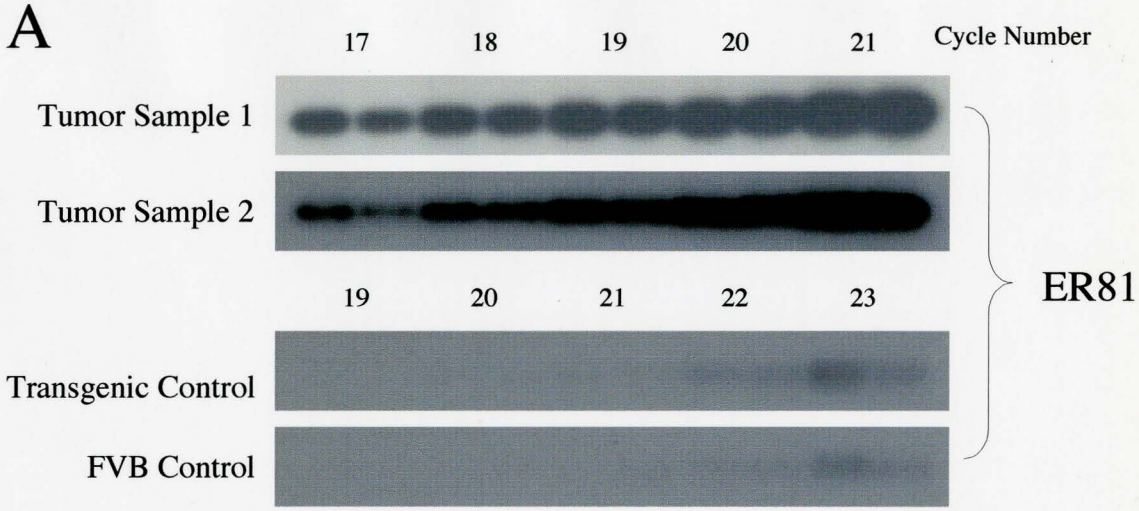
Figure 4: Semi-quantitative RT-PCR analysis of ER81 expression on four RNA samples.

The two tumor samples were used in the degenerate RT-PCR analyses. The transgenic control sample was derived from RNA from the mammary glands of a transgenic mouse that was sacrificed prior to tumor development and was 14 months of age at the time of death. The FVB control used was 6 months of age.

A. Southern hybridization data after having performed reverse transcription using random hexamers and PCR using gene specific primers in duplication at the cycle numbers indicated.

B. Quantification of Southern hybridizations shown above by PhosphorImager analysis. PhosphorImager data was averaged for each cycle number and plotted at the $\log_2$.

A


17
18
19
20
21
Cycle Number
Tumor Sample 1
Tumor Sample 2
19
20
21
22
23
Transgenic Control
FVB Control
ER81

B

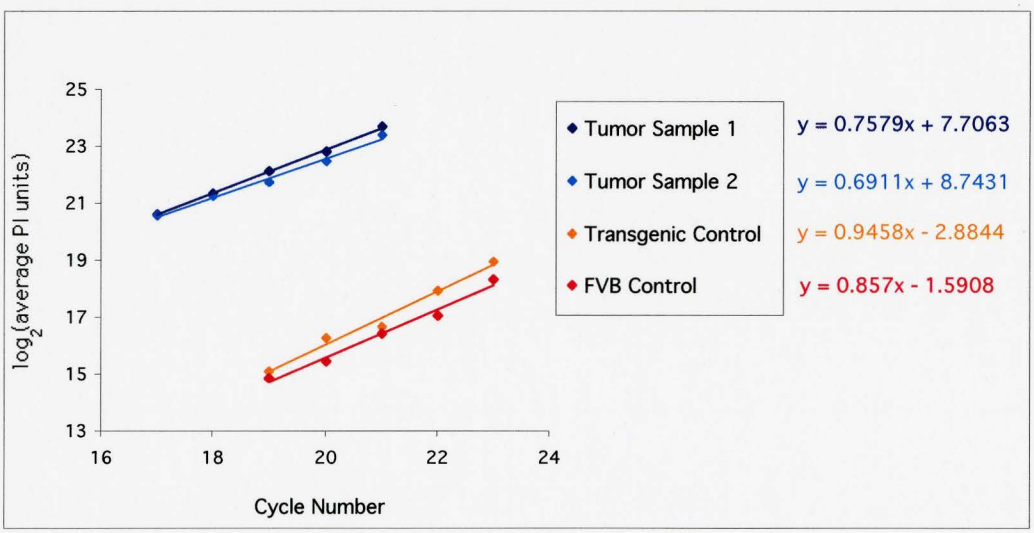

log2(average PI units)
25
23
21
19
17
15
13
16
18
20
22
24
Cycle Number
Tumor Sample 1
Tumor Sample 2
Transgenic Control
FVB Control
y = 0.7579x + 7.7063
y = 0.6911x + 8.7431
y = 0.9458x - 2.8844
y = 0.857x - 1.5908

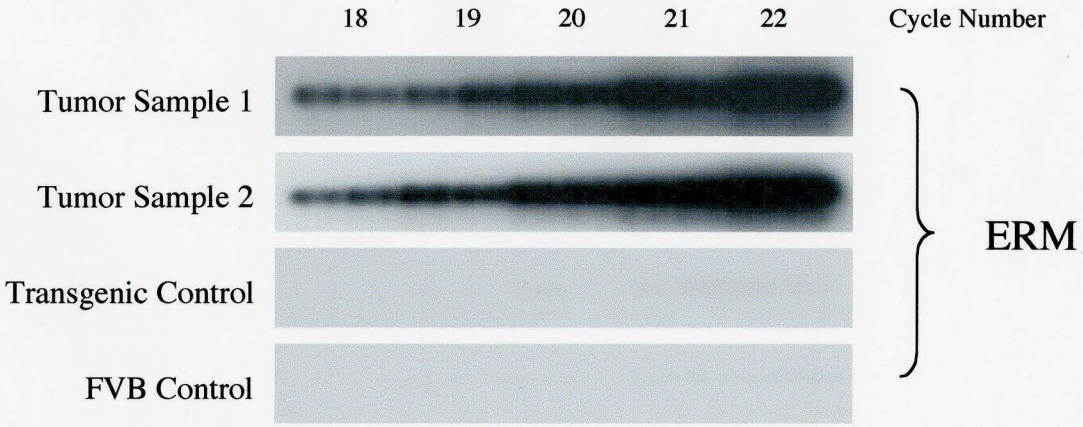
Figure 5: Semi-quantitative RT-PCR analysis of ERM expression on four RNA samples.

The two tumor samples were used in the degenerate RT-PCR analyses. The transgenic control sample was derived from RNA from the mammary glands of a transgenic mouse that was sacrificed prior to tumor development and was 14 months of age at the time of death. The FVB control used was 6 months of age.

A. Southern hybridization data after having performed reverse transcription using random hexamers and PCR using gene specific primers in duplication at the cycle numbers indicated.

B. Quantification of Southern hybridizations shown above by PhosphorImager analysis. PhosphorImager data was averaged for each cycle number and plotted at the $\log_2$.

A


18
19
20
21
22
Cycle Number
Tumor Sample 1
Tumor Sample 2
Transgenic Control
FVB Control
ERM

B

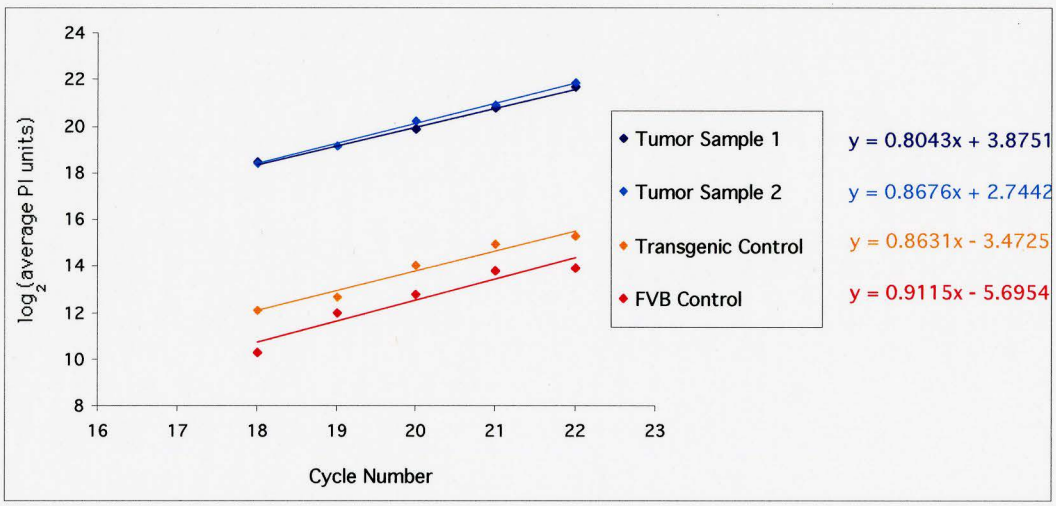

log2 (average PI units)
24
22
20
18
16
14
12
10
8
16
17
18
19
20
21
22
23
Cycle Number
Tumor Sample 1
Tumor Sample 2
Transgenic Control
FVB Control
y = 0.8043x + 3.8751
y = 0.8676x + 2.7442
y = 0.8631x - 3.4725
y = 0.9115x - 5.6954

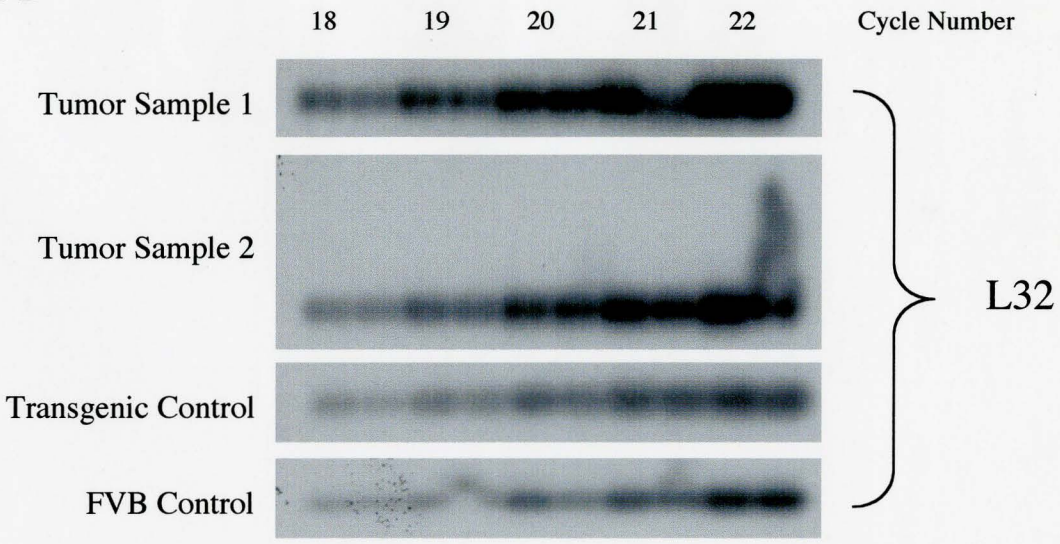
Figure 6: Semi-quantitative RT-PCR analysis of rpL32 expression on four RNA samples.

The two tumor samples were used in the degenerate RT-PCR analyses. The transgenic control sample was derived from RNA from the mammary glands of a transgenic mouse that was sacrificed prior to tumor development and was 14 months of age at the time of death. The FVB control used was 6 months of age.

A. Southern hybridization data after having performed reverse transcription using random hexamers and PCR using gene specific primers in duplication at the cycle numbers indicated.

B. Quantification of Southern hybridizations shown above by PhosphorImager analysis. PhosphorImager data was averaged for each cycle number and plotted at the $\log_2$.

A


18
19
20
21
22
Cycle Number
Tumor Sample 1
Tumor Sample 2
Transgenic Control
FVB Control
L32

B

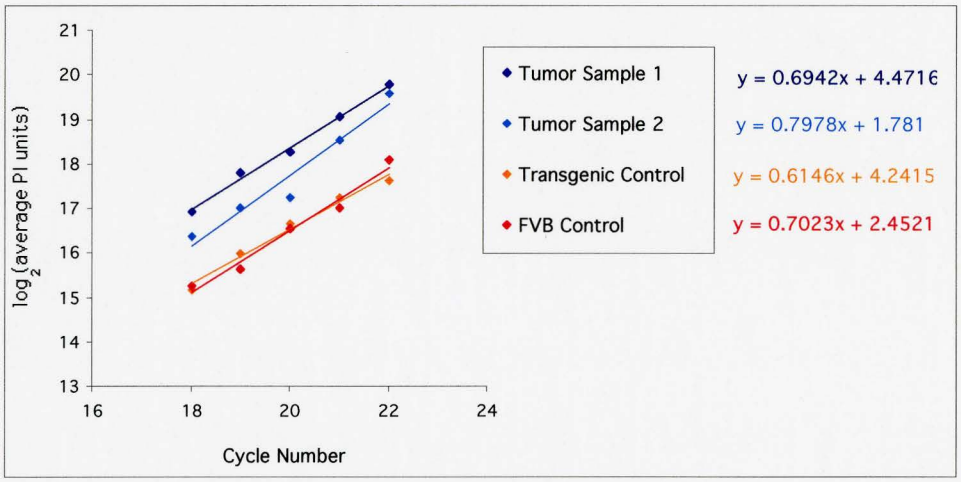

log2 (average PI units)
21
20
19
18
17
16
15
14
13
16
18
20
22
24
Cycle Number
Tumor Sample 1
Tumor Sample 2
Transgenic Control
FVB Control
y = 0.6942x + 4.4716
y = 0.7978x + 1.781
y = 0.6146x + 4.2415
y = 0.7023x + 2.4521

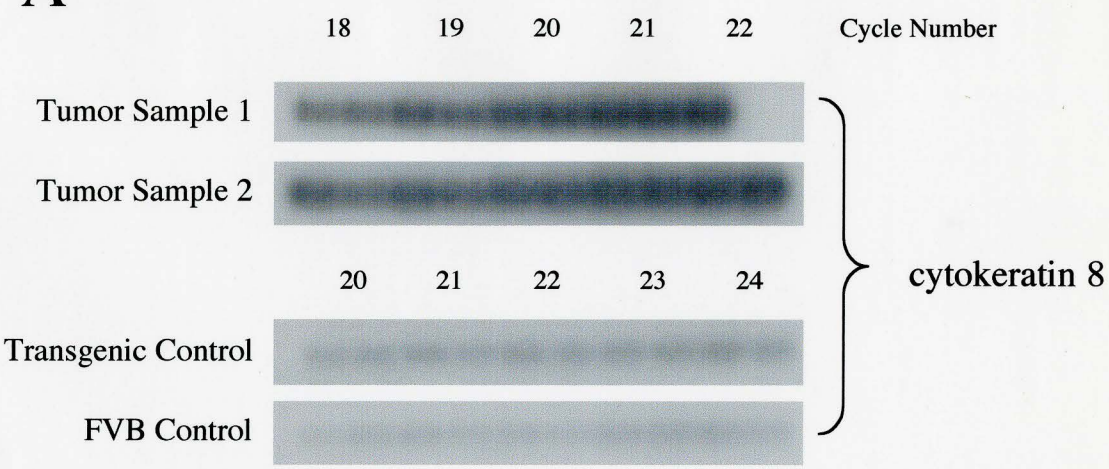
Figure 7: Semi-quantitative RT-PCR analysis of cytokeratin 8 expression on four RNA samples.

The two tumor samples were used in the degenerate RT-PCR analyses. The transgenic control sample was derived from RNA from the mammary glands of a transgenic mouse that was sacrificed prior to tumor development and was 14 months of age at the time of death. The FVB control used was 6 months of age.

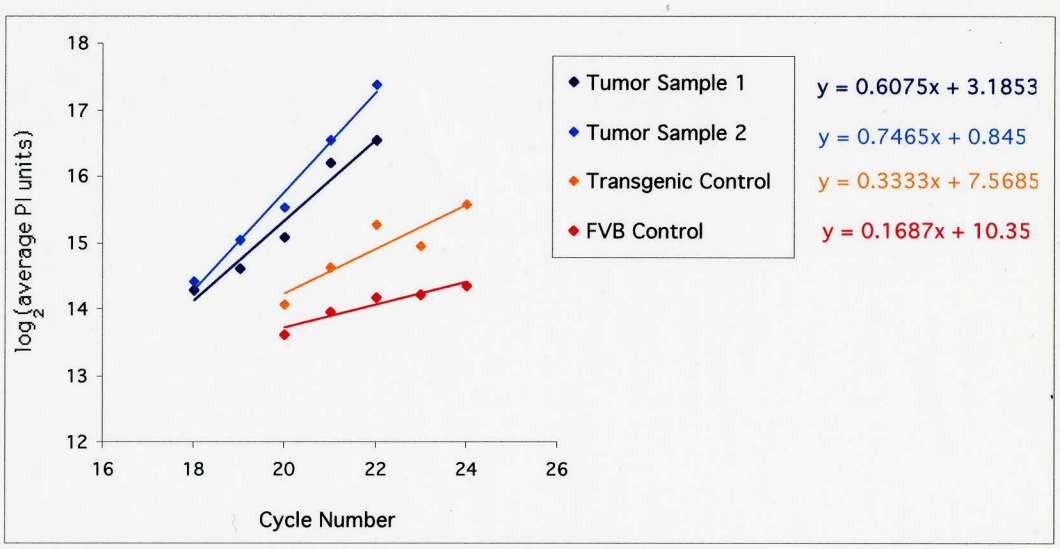
A. Southern hybridization data after having performed reverse transcription using random hexamers and PCR using gene specific primers in duplication at the cycle numbers indicated.

B. Quantification of Southern hybridizations shown above by PhosphorImager analysis. PhosphorImager data was averaged for each cycle number and plotted at the $\log_2$.

A


18 19 20 21 22 Cycle Number
Tumor Sample 1
Tumor Sample 2
20 21 22 23 24
Transgenic Control
FVB Control
cytokeratin 8

B


log2(average PI units)
18
17
16
15
14
13
12
16 18 20 22 24 26
Cycle Number
Tumor Sample 1
Tumor Sample 2
Transgenic Control
FVB Control
y = 0.6075x + 3.1853
y = 0.7465x + 0.845
y = 0.3333x + 7.5685
y = 0.1687x + 10.35

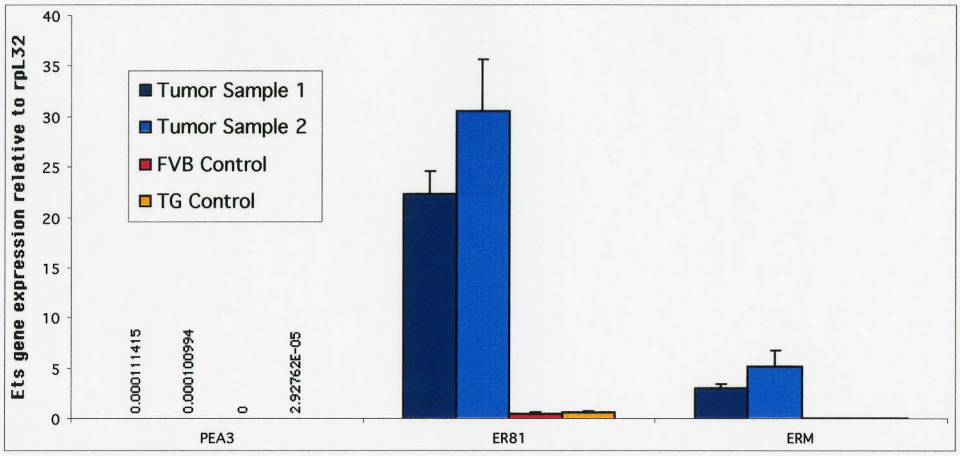
However, this may not be a good marker for epithelial cell content because cytokeratin 8 expression is deregulated in various tumor types itself (Debus *et al.,* 1984; Schaafsma *et al.,* 1990; Trask *et al.,* 1990; Pankov *et al.,* 1994). PEA3 expression was almost 4-fold increased in both tumor samples compared to the TG control after correction for rpL32 expression and equal to expression in the TG control when corrected for cytokeratin 8 expression (Figures 8 and 9 respectively). ER81 expression is 34 and 47-fold increased when corrected for rpL32 and 35 and 23-fold if corrected for cytokeratin 8 expression for each tumor sample (Figures 8 and 9 respectively). ERM levels are 21 and 37-fold and 19 and 15-fold increased in the tumors after rpL32 and cytokeratin 8 corrections respectively (Figures 8 and 9 respectively).

Figure 8: Expression levels of PEA3, ER81 and ERM relative to rpL32 as determined by semi-quantitative RT-PCR

A. The expression level of each *ets* gene was corrected for rpL32 expression at each corresponding cycle number and averaged. The standard deviations are illustrated. The expression levels of PEA3 compared to the other *ets* genes analyzed is very low and is represented numerically.

B. The expression levels of each *ets* gene in each tumor sample and the transgenic control (shown above) corrected for expression in the transgenic control.

A)


Ets gene expression relative to rpL32
40
35
30
25
20
15
10
5
0
Tumor Sample 1
Tumor Sample 2
FVB Control
TG Control
0.000111415
0.000100994
0
2.92762E-05
PEA3
ER81
ERM

B)

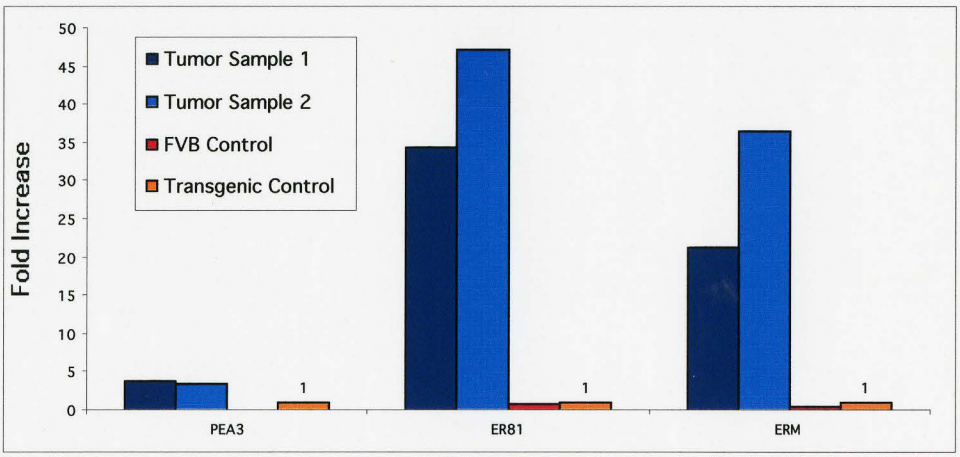
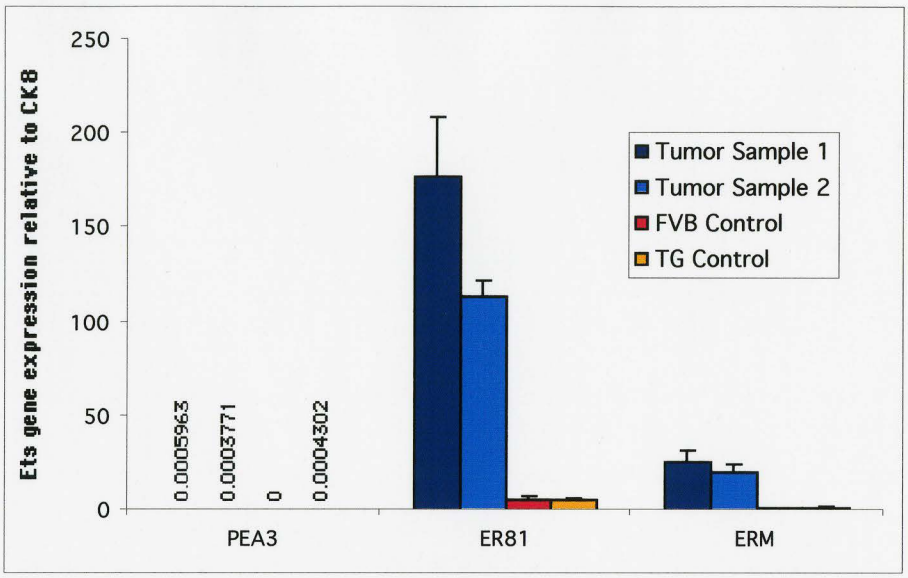

Fold Increase
50
45
40
35
30
25
20
15
10
5
0
Tumor Sample 1
Tumor Sample 2
FVB Control
Transgenic Control
1
1
1
PEA3
ER81
ERM

Figure 9: Expression levels of PEA3, ER81 and ERM relative to cytokeratin 8 as determined by semi-quantitative RT-PCR

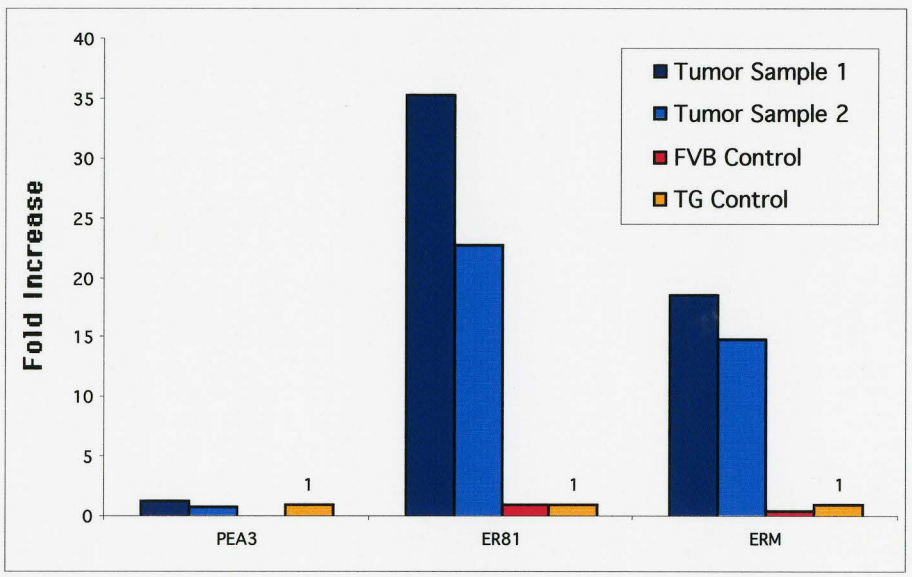
A. The expression level of each *ets* gene was corrected for cytokeratin 8 expression at each corresponding cycle number and averaged. The standard deviations are illustrated. The expression levels of PEA3 compared to the other *ets* genes analyzed is very low and is represented numerically.

B. The expression levels of each *ets* gene in each tumor sample and the transgenic control (shown above) corrected for expression in the transgenic control.

A)


Ets gene expression relative to CK8
250
200
150
100
50
0
0.0005963
0.0003771
0
0.0004302
PEA3
ER81
ERM
Tumor Sample 1
Tumor Sample 2
FVB Control
TG Control

B)


Fold Increase
40
35
30
25
20
15
10
5
0
1
1
1
PEA3
ER81
ERM
Tumor Sample 1
Tumor Sample 2
FVB Control
TG Control

3.2.2 Ets-1, Ets-2 and GABPα

Degenerate RT-PCR recovered 11/82 and 19/62 Ets-1 clones in the two normal mouse mammary glands analyzed but only 1/64 and 1/53 clones in the two tumor samples (see Table 1). This implies that Ets-1 is expressed at lower levels in the two tumor samples. Semi-quantitative RT-PCR performed on these tumor samples and two normal controls using Ets-1 specific primers also illustrates a drop in Ets-1 expression in tumor tissue (Figure 10). Ets-2 and GABPα were also analyzed because the frequency of their recovery was not greatly different in the tumor samples compared to the normal controls. Semi-quantitative RT-PCR also illustrated this (Figures 11 and 12) once corrected for expression of the rpL32 and cytokeratin 8 internal controls. Ets-1 expression, once corrected for rpL32 expression, in the first tumor sample dropped 5-fold compared to the FVB control if corrected for rpL32 expression and 4-fold if corrected for cytokeratin 8 expression (Figures 13 and 14 respectively). The expression level of Ets-1 in the second tumor sample was equal to that of the FVB control if corrected for rpL32 expression and dropped to almost half of normal levels if corrected for epithelial cell content. Ets-2 expression was variable between the two tumors, as the first dropped to 1.7-fold of that in the FVB control, while Ets-2 expression in the second tumor was increased by 2.4-fold, if corrected for rpL32 expression. If corrected for cytokeratin 8 expression, the first tumor sample exhibited half the Ets-2 expression found in the FVB

control and the second tumor and TG control expression levels equal to that of the FVB control. GABPα levels also did not change much compared to the two controls.

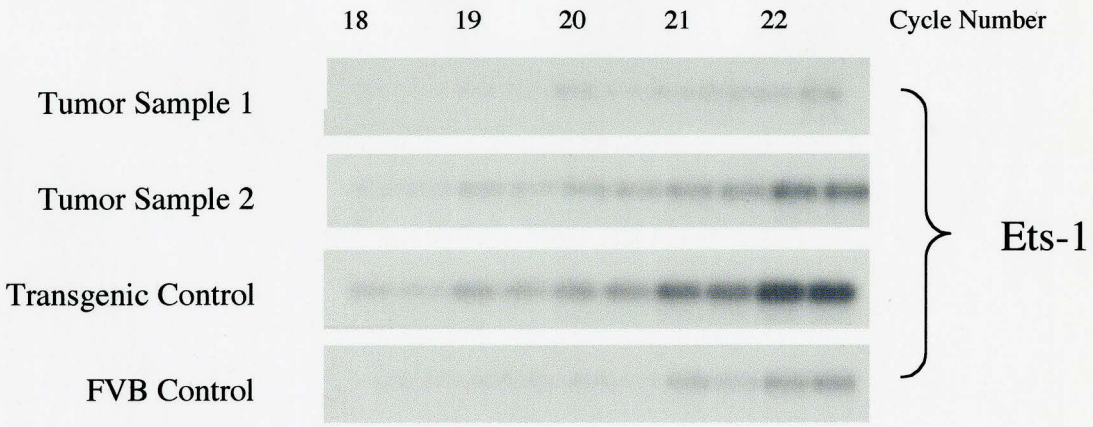
Figure 10: Semi-quantitative RT-PCR analysis of Ets-1 expression on four RNA samples.

The two tumor samples were used in the degenerate RT-PCR analyses. The transgenic control sample was derived from RNA from the mammary glands of a transgenic mouse that was sacrificed prior to tumor development and was 14 months of age at the time of death. The FVB control used was 6 months of age.

A. Southern hybridization data after having performed reverse transcription using random hexamers and PCR using gene specific primers in duplication at the cycle numbers indicated.

B. Quantification of Southern hybridizations shown above by PhosphorImager analysis. PhosphorImager data was averaged for each cycle number and plotted at the $\log_2$.

A


18
19
20
21
22
Cycle Number
Tumor Sample 1
Tumor Sample 2
Transgenic Control
FVB Control
Ets-1

B

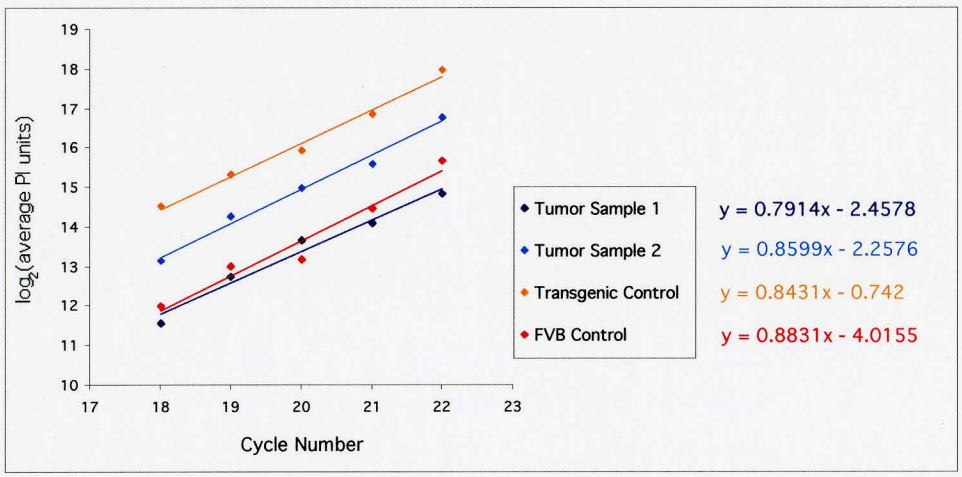

log2(average PI units)
Cycle Number
Tumor Sample 1
Tumor Sample 2
Transgenic Control
FVB Control
y = 0.7914x - 2.4578
y = 0.8599x - 2.2576
y = 0.8431x - 0.742
y = 0.8831x - 4.0155

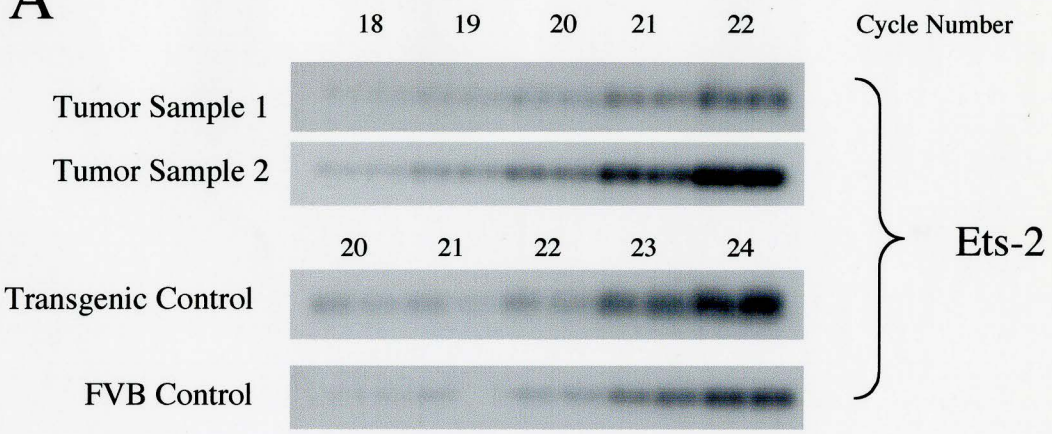
Figure 11: Semi-quantitative RT-PCR analysis of Ets-2 expression on four RNA samples.

The two tumor samples were used in the degenerate RT-PCR analyses. The transgenic control sample was derived from RNA from the mammary glands of a transgenic mouse that was sacrificed prior to tumor development and was 14 months of age at the time of death. The FVB control used was 6 months of age.

A. Southern hybridization data after having performed reverse transcription using random hexamers and PCR using gene specific primers in duplication at the cycle numbers indicated.

B. Quantification of Southern hybridizations shown above by PhosphorImager analysis. PhosphorImager data was averaged for each cycle number and plotted at the $\log_2$.

A


18
19
20
21
22
Cycle Number
Tumor Sample 1
Tumor Sample 2
20
21
22
23
24
Transgenic Control
FVB Control
Ets-2

B

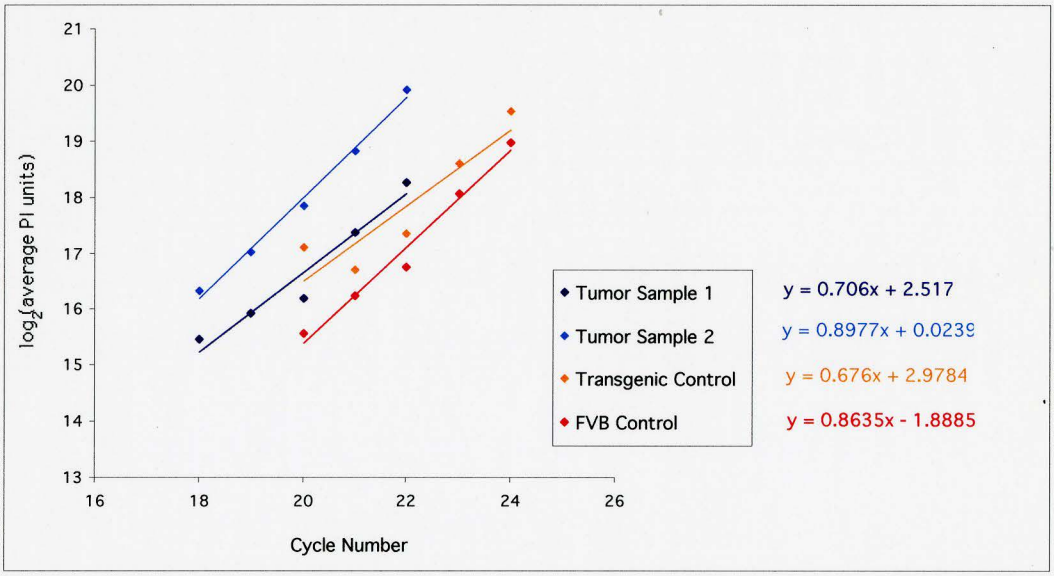

log2(average PI units)
21
20
19
18
17
16
15
14
13
16
18
20
22
24
26
Cycle Number
Tumor Sample 1
Tumor Sample 2
Transgenic Control
FVB Control
y = 0.706x + 2.517
y = 0.8977x + 0.0239
y = 0.676x + 2.9784
y = 0.8635x - 1.8885

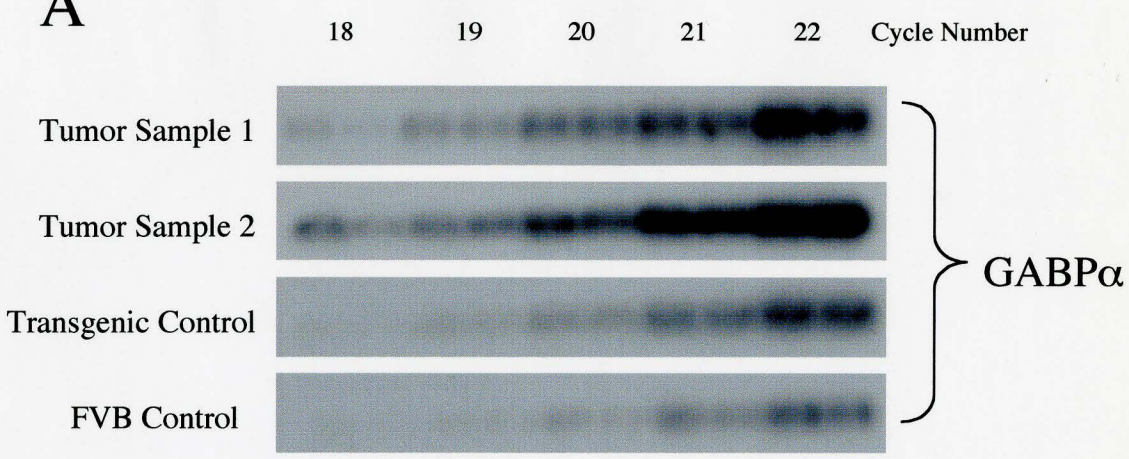
Figure 12: Semi-quantitative RT-PCR analysis of GABPα expression on four RNA samples.

The two tumor samples were used in the degenerate RT-PCR analyses. The transgenic control sample was derived from RNA from the mammary glands of a transgenic mouse that was sacrificed prior to tumor development and was 14 months of age at the time of death. The FVB control used was 6 months of age.

A. Southern hybridization data after having performed reverse transcription using random hexamers and PCR using gene specific primers in duplication at the cycle numbers indicated.

B. Quantification of Southern hybridizations shown above by PhosphorImager analysis. PhosphorImager data was averaged for each cycle number and plotted at the $\log_2$.

A


18
19
20
21
22
Cycle Number
Tumor Sample 1
Tumor Sample 2
Transgenic Control
FVB Control
GABPα

B

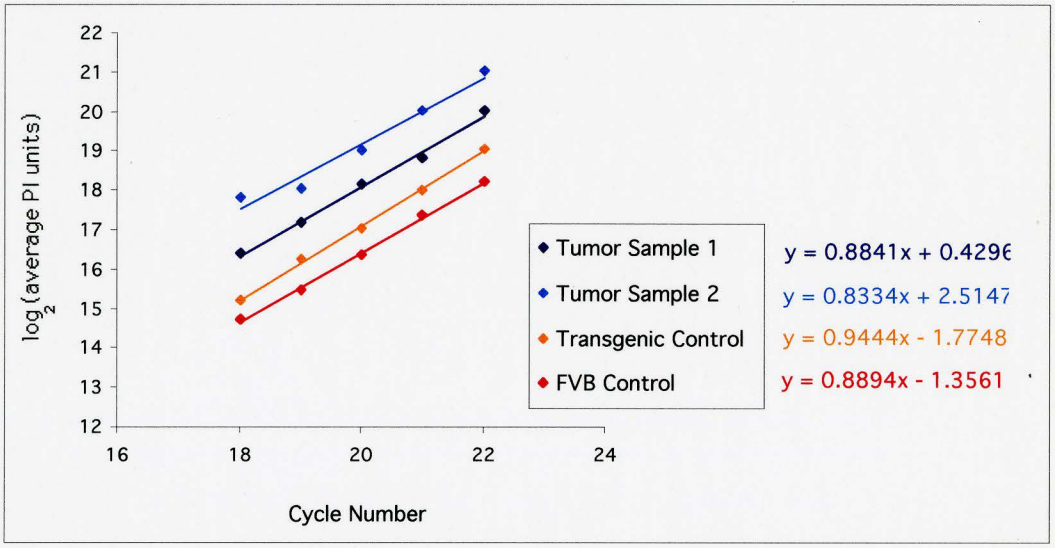
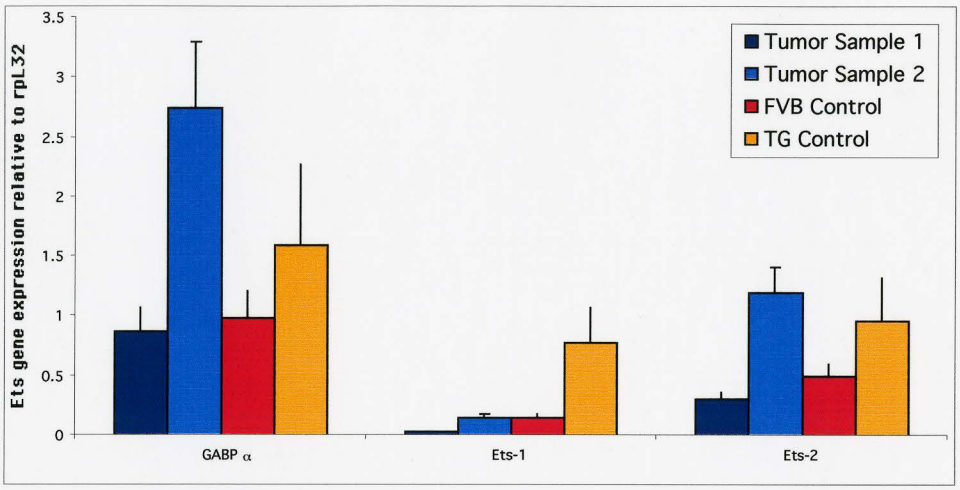

log2 (average PI units)
22
21
20
19
18
17
16
15
14
13
12
16
18
20
22
24
Cycle Number
Tumor Sample 1
Tumor Sample 2
Transgenic Control
FVB Control
y = 0.8841x + 0.429
y = 0.8334x + 2.5147
y = 0.9444x - 1.7748
y = 0.8894x - 1.3561

Figure 13: Expression levels of Ets-1, Ets-2 and GABPα relative to rpL32 as determined by semi-quantitative RT-PCR

A. The expression level of each *ets* gene was corrected for rpL32 expression at each corresponding cycle number and averaged. The standard deviations are illustrated. The expression levels of PEA3 compared to the other *ets* genes analyzed is very low and is represented numerically.

B. The expression levels of each *ets* gene in each tumor sample and the transgenic control (shown above) corrected for expression in the transgenic control.

A)


Ets gene expression relative to rpL32
3.5
3
2.5
2
1.5
1
0.5
0
Tumor Sample 1
Tumor Sample 2
FVB Control
TG Control
GABP α
Ets-1
Ets-2

B)

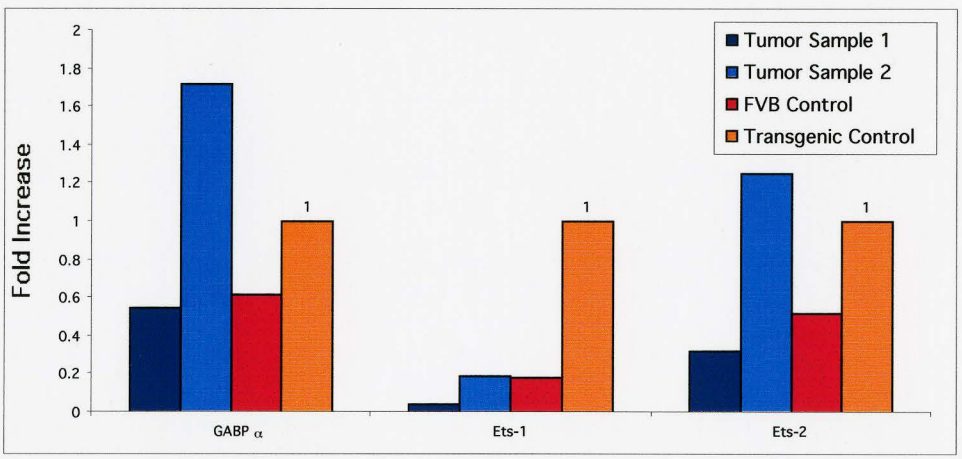
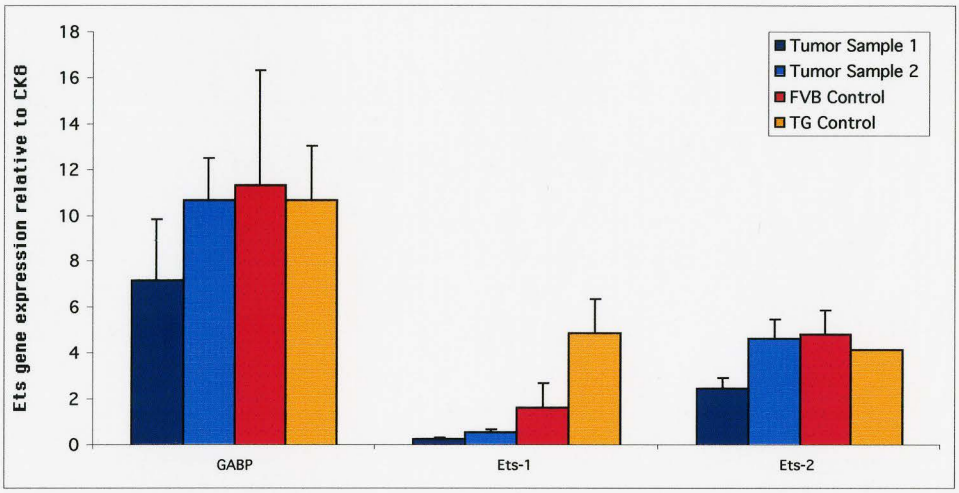

Fold Increase
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Tumor Sample 1
Tumor Sample 2
FVB Control
Transgenic Control
1
1
1
GABP α
Ets-1
Ets-2

Figure 14: Expression levels of Ets-1, Ets-2 and GABPα relative to cytokeratin 8 as determined by semi-quantitative RT-PCR

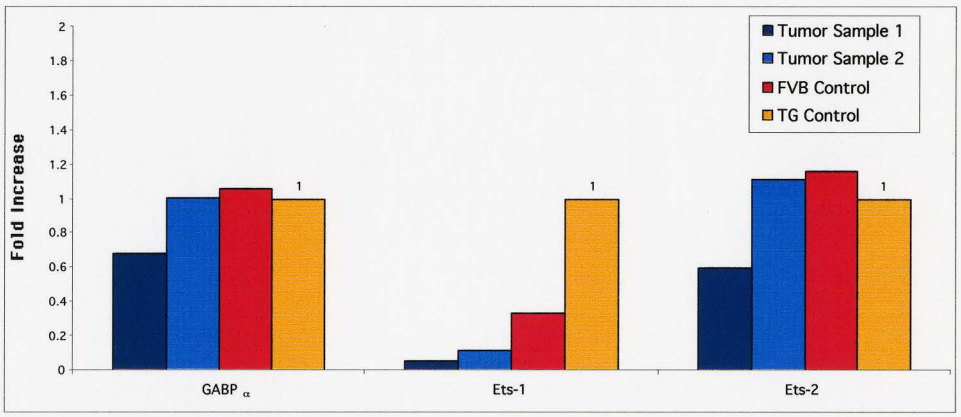
A. The expression level of each *ets* gene was corrected for cytokeratin 8 expression at each corresponding cycle number and averaged. The standard deviations are illustrated. The expression levels of PEA3 compared to the other *ets* genes analyzed is very low and is represented numerically.

B. The expression levels of each *ets* gene in each tumor sample and the transgenic control (shown above) corrected for expression in the transgenic control.

A)


Ets gene expression relative to CK8
18
16
14
12
10
8
6
4
2
0
GABP
Ets-1
Ets-2
Tumor Sample 1
Tumor Sample 2
FVB Control
TG Control

B)


Fold Increase
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
1
1
GABP α
Ets-1
Ets-2
Tumor Sample 1
Tumor Sample 2
FVB Control
TG Control

3.3 Ribonuclease Protection Analyses

3.3.1 PEA3 subfamily

To confirm the semi-quantitative RT-PCR data, ribonuclease protection analyses were performed. The initial experiment was performed by T. Shepherd (Figure 15), with subsequent analyses performed by L. Kockeritz (Figures 16 to 18). PEA3, ERM and ER81 were expressed at very low levels in the FVB normal controls and the MMTV/HER2/Neu transgenic mouse control. By contrast, all three PEA3 family members were expressed at much higher levels in the tumor samples. Whereas the magnitude of the increase in PEA3 subfamily expression varied among the tumors (Figure 19), the relative expression level of each subfamily member within a tumor was remarkable similar. ER81 was overexpressed to the greatest extent, whereas ERM tends to be overexpressed more than PEA3. These findings suggest that the PEA3 subfamily of *ets* genes is coordinately overexpressed in HER2/Neu-induced mammary tumors.

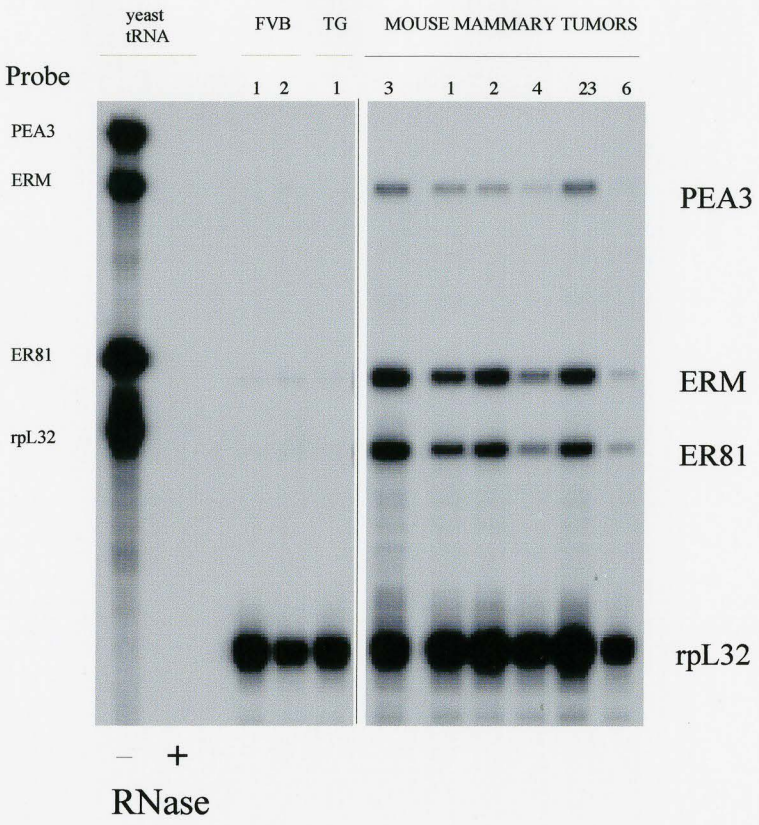
The characteristics of the mice tested in the ribonuclease protection assays including age at autopsy, age at tumor detection, presence of lung metastases and tumor size are listed in Table 6. The only correlation that exists between extent of PEA3 subgroup overexpression and tumor characteristic is that within one mouse, the larger tumors tend to have lower levels of PEA3 subfamily overexpression.

Figure 15: Quantitative analysis of PEA3 subfamily overexpression in N202 mouse mammary tumors.

A. Ribonuclease protection analyses performed on tumor RNA from various N202 transgenic mice using PEA3, ERM, ER81 and rpL32 riboprobes. 10 ug of total RNA was hybridized with 50 000 cpm of each probe. This particular experiment was performed by T. Shepherd. Each number represents a different N202 mouse.

B. Quantification of above RNase protection by PhosphorImager analysis. Fold-increase of expression is based on setting average expression levels of each gene in the FVB controls to 1 after correction for rpL32 expression levels.

A


yeast tRNA
FVB
TG
MOUSE MAMMARY TUMORS
Probe
1 2 1 3 1 2 4 23 6
PEA3
ERM
ER81
rpL32
PEA3
ERM
ER81
rpL32
– +
RNase

B

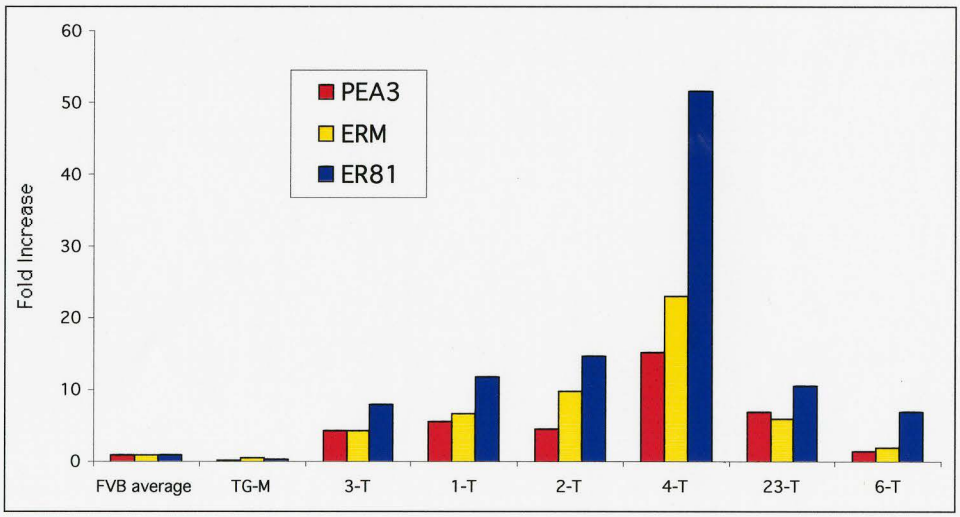
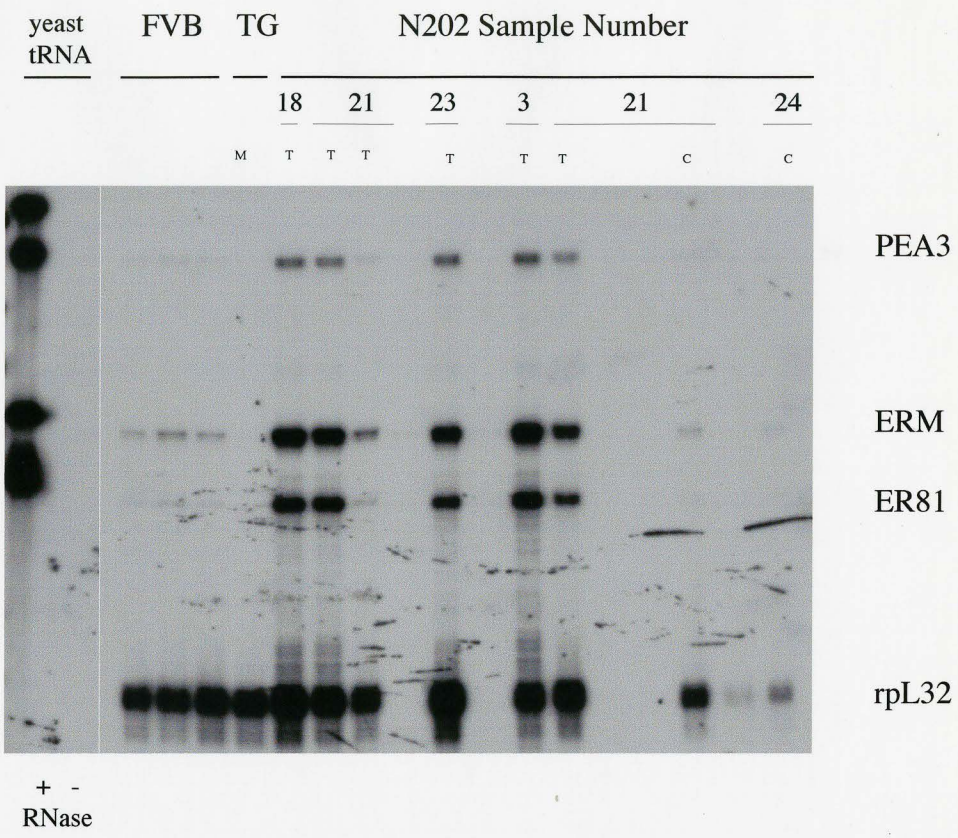

60
50
40
30
20
10
0
Fold Increase
PEA3
ERM
ER81
FVB average
TG-M
3-T
1-T
2-T
4-T
23-T
6-T

Figure 16: Quantitative analysis of PEA3 subfamily overexpression in additional N202 mouse mammary tumors.

A. Ribonuclease protection analyses performed on tumor RNA from various N202 transgenic mice using PEA3, ERM, ER81 and rpL32 riboprobes. 10 ug of total RNA was hybridized with 50 000 cpm of each probe. Each number represents a different N202 mouse. Symbols: (T) mammary tumor; (C) contralateral mammary gland, non-tumor bearing mammary gland from a tumor bearing mouse.

B. Quantification of above RNase protection by PhosphorImager analysis. Fold-increase of expression is based on setting average expression levels of each gene in the FVB controls to 1 after correction for rpL32 expression levels.

A


yeast tRNA
FVB
TG
N202 Sample Number
18
21
23
3
21
24
M
T
T
T
T
T
T
C
C
PEA3
ERM
ER81
rpL32
+
-
RNase

B

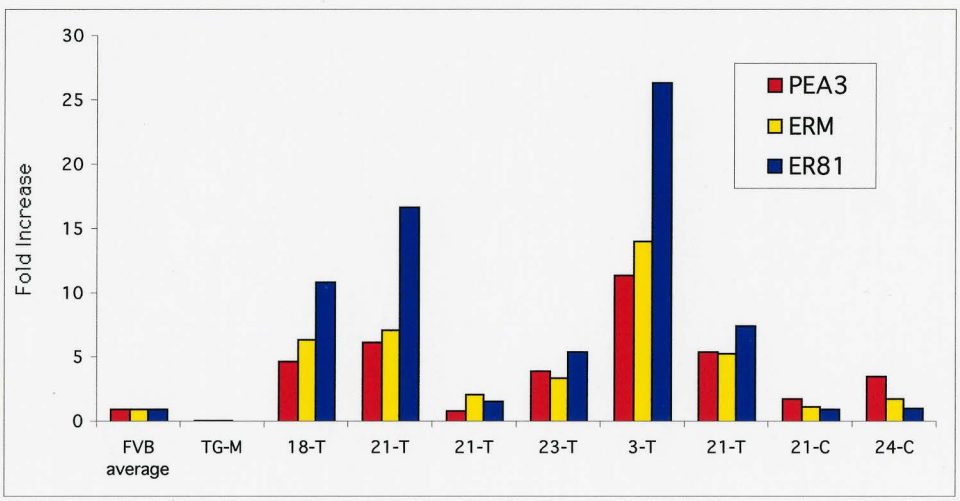
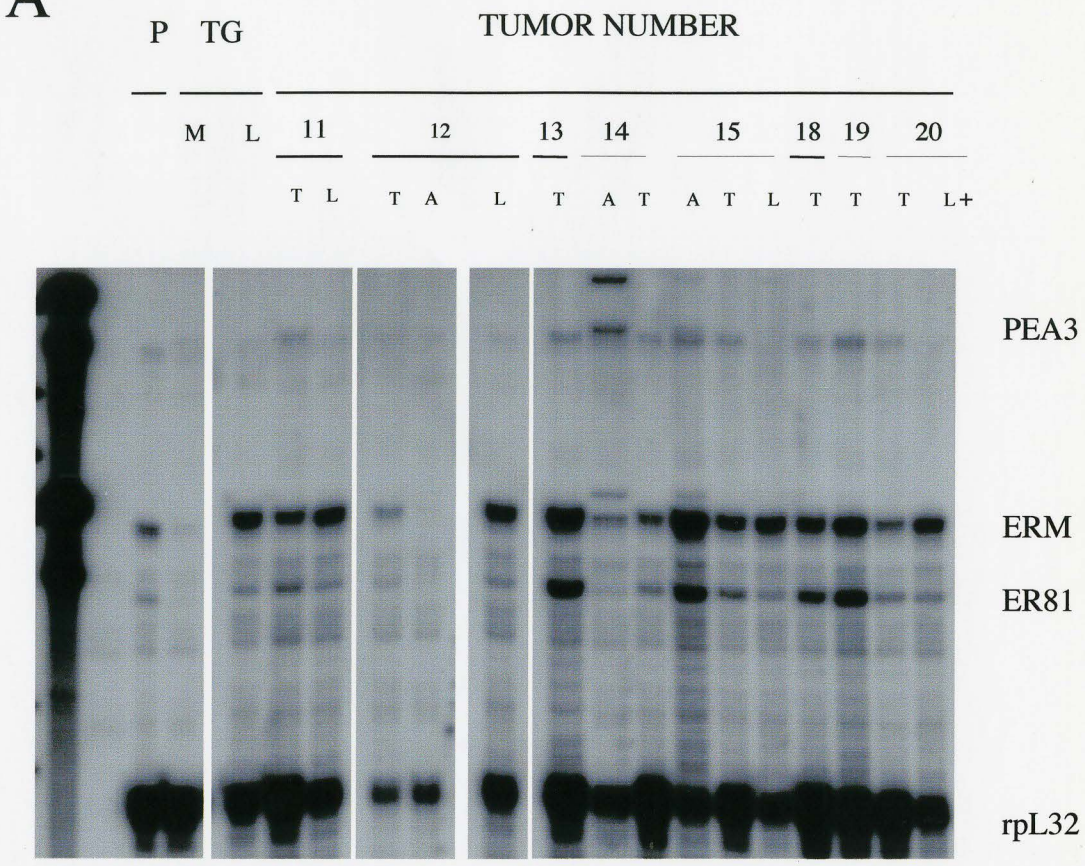

Fold Increase
30
25
20
15
10
5
0
PEA3
ERM
ER81
FVB average
TG-M
18-T
21-T
21-T
23-T
3-T
21-T
21-C
24-C

Figure 17: Quantitative analysis of PEA3 subfamily overexpression in additional N202 mouse mammary tumors.

A. Ribonuclease protection analyses performed on tumor RNA from various N202 transgenic mice using PEA3, ERM, ER81 and rpL32 riboprobes. 10 ug of total RNA was hybridized with 50 000 cpm of each probe. Each number represents a different N202 mouse. Symbols: (M) normal mammary gland; (T) mammary tumor; (L) lung tissue; (L+) metastatic lung tissue; (A) normal mammary gland adjacent to the mammary tumor.

B. Quantification of above RNase protection by PhosphorImager analysis. Fold-increase of expression is based on setting average expression levels of each gene in the FVB controls to 1 after correction for rpL32 expression levels. Quantitative data not available for the two 12-A samples due to incomplete digestion resulting in high amounts of background bands.

A


P
TG
TUMOR NUMBER
M
L
11
12
13
14
15
18
19
20
T L
T A
L
T
A T
A T
L
T
T
T
L+
PEA3
ERM
ER81
rpL32

B

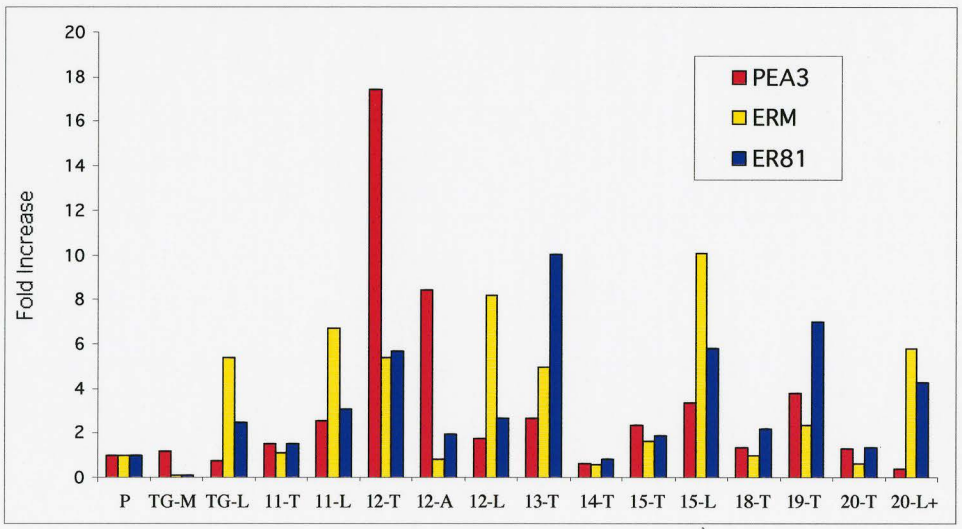
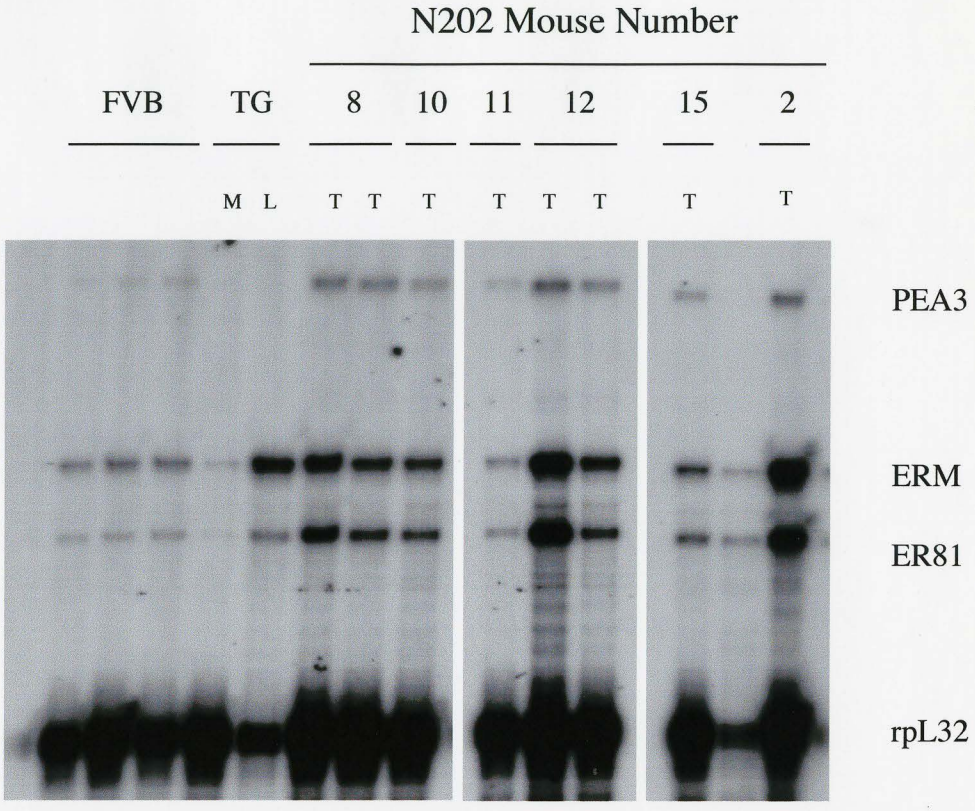

Fold Increase
0
2
4
6
8
10
12
14
16
18
20
PEA3
ERM
ER81
P
TG-M
TG-L
11-T
11-L
12-T
12-A
12-L
13-T
14-T
15-T
15-L
18-T
19-T
20-T
20-L+

Figure 18: Quantitative analysis of PEA3 subfamily overexpression in additional N202 mouse mammary tumors.

A. Ribonuclease protection analyses performed on tumor RNA from various N202 transgenic mice using PEA3, ERM, ER81 and rpL32 riboprobes. 10 ug of total RNA was hybridized with 50 000 cpm of each probe. Each number represents a different N202 mouse. Symbols: (M) normal mammary gland; (L) lung tissue.

B. Quantification of above RNase protection by PhosphorImager analysis. Fold-increase of expression is based on setting average expression levels of each gene in the FVB controls to 1 after correction for rpL32 expression levels.

A


N202 Mouse Number
FVB
TG
8
10
11
12
15
2
M L
T T T T T T T
PEA3
ERM
ER81
rpL32

B

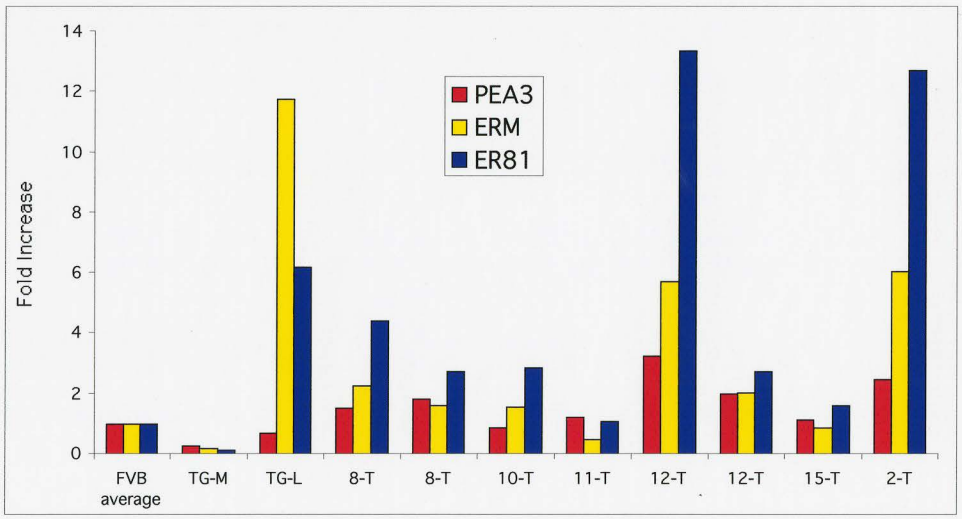

Fold Increase
0
2
4
6
8
10
12
14
PEA3
ERM
ER81
FVB average
TG-M
TG-L
8-T
8-T
10-T
11-T
12-T
12-T
15-T
2-T

Figure 19: Compilation of RNase protection analyses performed on N202 mouse mammary tumors using PEA3 subfamily riboprobes.

The overexpression of the PEA3 subfamily of *ets* genes in each N202 tumor sample analyzed is depicted in comparison to expression levels in FVB normal mouse mammary glands. The expression levels within tumors that were analyzed twice were averaged and the standard deviations are illustrated.

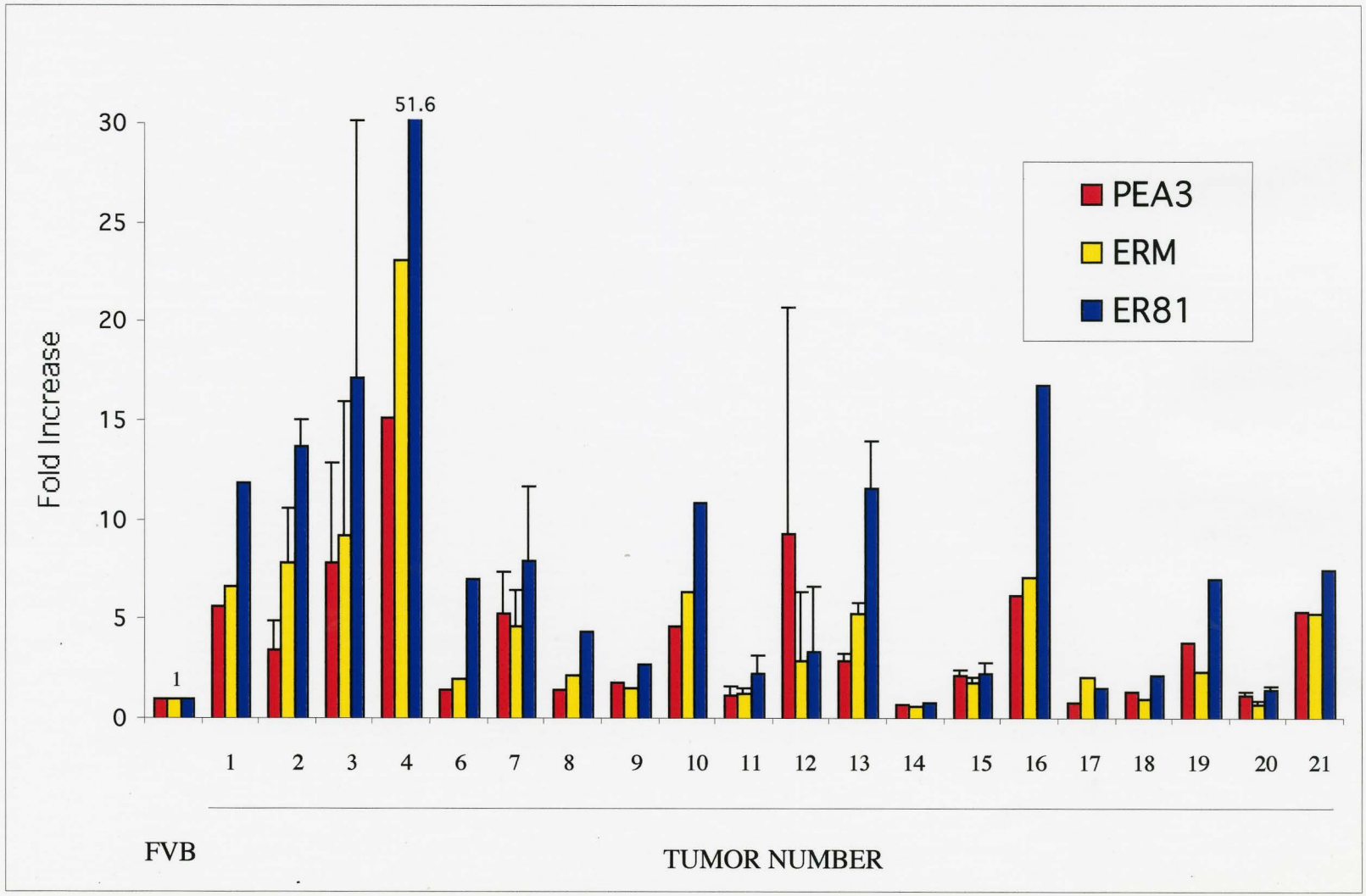

51.6
30
25
20
15
10
5
0
Fold Increase
PEA3
ERM
ER81
1
1
2
3
4
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
FVB
TUMOR NUMBER

Table 6: Compilation of the characteristics of N202 mice from whom tumors were extracted and analyzed for PEA3 subfamily overexpression.

Mice were sacrificed by CO_2 asphyxiation, tumors removed for RNA extraction and lungs were examined visually for the presence of lung metastases. The overexpression of each PEA3 subfamily member in comparison to normal FVB mouse mammary glands are noted.

*These tumors were analyzed twice and their overexpression from each experiment was averaged.

Mouse Number	Tumor Number	Age at Autopsy (months)	Tumor Borne (weeks)	Lung Metastases (Y/N)	size (cm2)	PEA3	ERM	ER81	notes
1	1	8	4	N	N/A	5.7	6.7	11.9	same sample used in degenerate and semi-quantitative RT-PCR
2	1*	16	6	N	N/A	3.5	7.9	13.7	same sample used in degenerate and semi-quantitative RT-PCR
3	1*	13	3	N	N/A	7.85	9.2	17.2	
4	1	16	3	N	N/A	15.2	23.1	51.6	
5	1	13	N/A	N	N/A	N/A	N/A	N/A	
6	1	12	4	N	N/A	1.5	2	7	
7	1*	14	3	Y	N/A	5.35	4.7	8	two tumors present
8	1	9	6	N	N/A	1.5	2.2	4.4	tumors were within the same mammary gland
	2	"	"	"		1.8	1.6	2.7	"
9	1	6	3	N	0.5	4.7	6.4	10.9	two tumors present
10	1*	8	3	N	1.5	1.2	1.3	2.25	
11	1*	8	5	N	2.89	9.3	2.95	3.4	
12	1*	9	5	N	0.36	2.95	5.35	11.65	tumor derived from mammary gland no. 2
	2	"	"	"	1.95	0.7	0.6	0.8	tumor derived from mammary gland no. 3
	3*	"	"	"	1.44	2.2	1.85	2.3	tumor derived from mammary gland no. 4
13	1	21	3	N	0.35	6.2	7.1	16.8	tumor derived from mammary gland no. 2
	2	"	"	"	1.5	0.8	2.1	1.6	tumor derived from mammary gland no. 3
14	1	9	9	Y	1.18	1.4	1	2.2	tumors were within the same mammary gland
	2				0.13	3.8	2.4	7	
	3*				1.84	1.2	0.725	1.45	
15	1	8	8	N	N/A	5.4	5.3	7.5	mated mouse

3.3.2 Ets-1, Ets-2 and GABPα are not overexpressed in mouse mammary tumors.

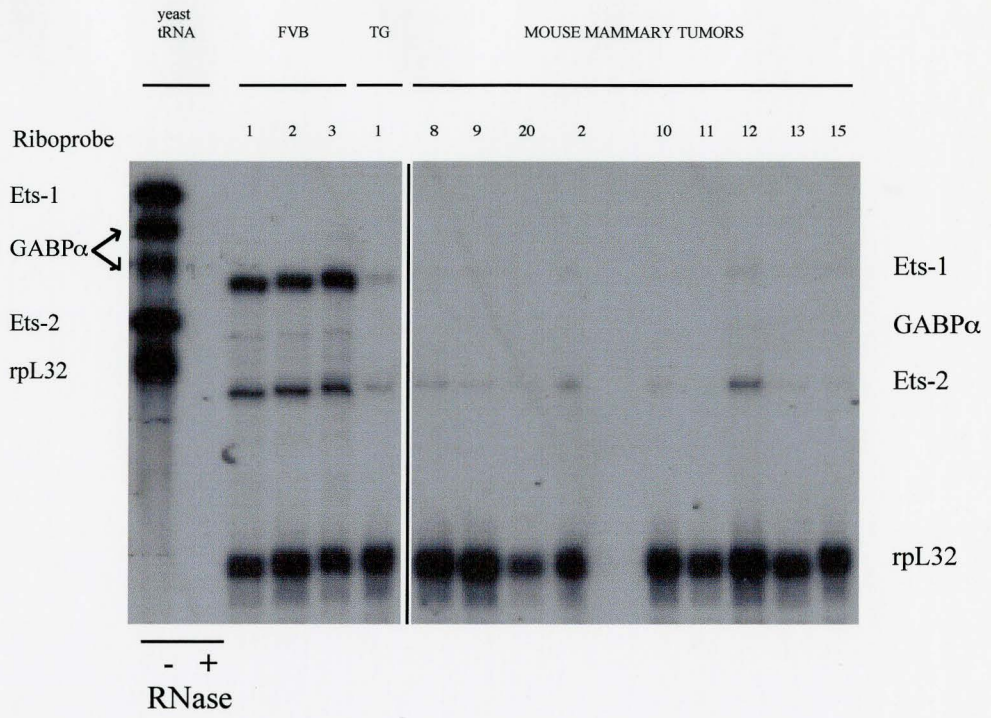
Using ribonuclease protection analyses the expression levels of Ets-1, Ets-2 and GABPα dropped in comparison to the FVB normal control, and ranged from undetectable levels to 0.10, 0.43 and 0.26-fold expression respectively (Figures 21 and 22). Interestingly, the levels of expression of these three genes also dropped in the transgenic mouse control, to levels similar to those detected in the tumor samples. This dramatic drop in expression, compared to the FVB controls may simply be due to mouse-to-mouse variation.

Figure 20: Quantitative analysis of *ets* genes GABPα, Ets-1 and Ets-2 expression in N202 mouse mammary tumors.

A. Ribonuclease protection analyses performed on 10ug of RNA derived from tumors from various N202 transgenic mice using Ets-1, Ets-2, GABPα and rpL32 riboprobes

B. Quantification of above RNase protection by PhosphorImager analysis. Fold-increase of expression is based on setting average expression levels of each gene in the FVB controls to 1 after correction for rpL32 expression levels.

A


yeast tRNA
FVB
TG
MOUSE MAMMARY TUMORS
Riboprobe
1 2 3 1 8 9 20 2 10 11 12 13 15
Ets-1
GABPα
Ets-2
rpL32
Ets-1
GABPα
Ets-2
rpL32
- +
RNase

B

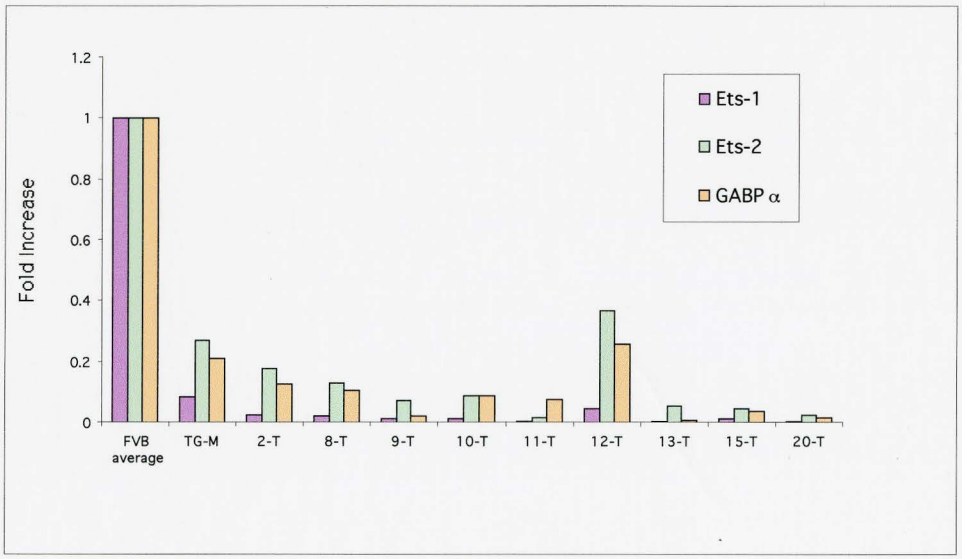
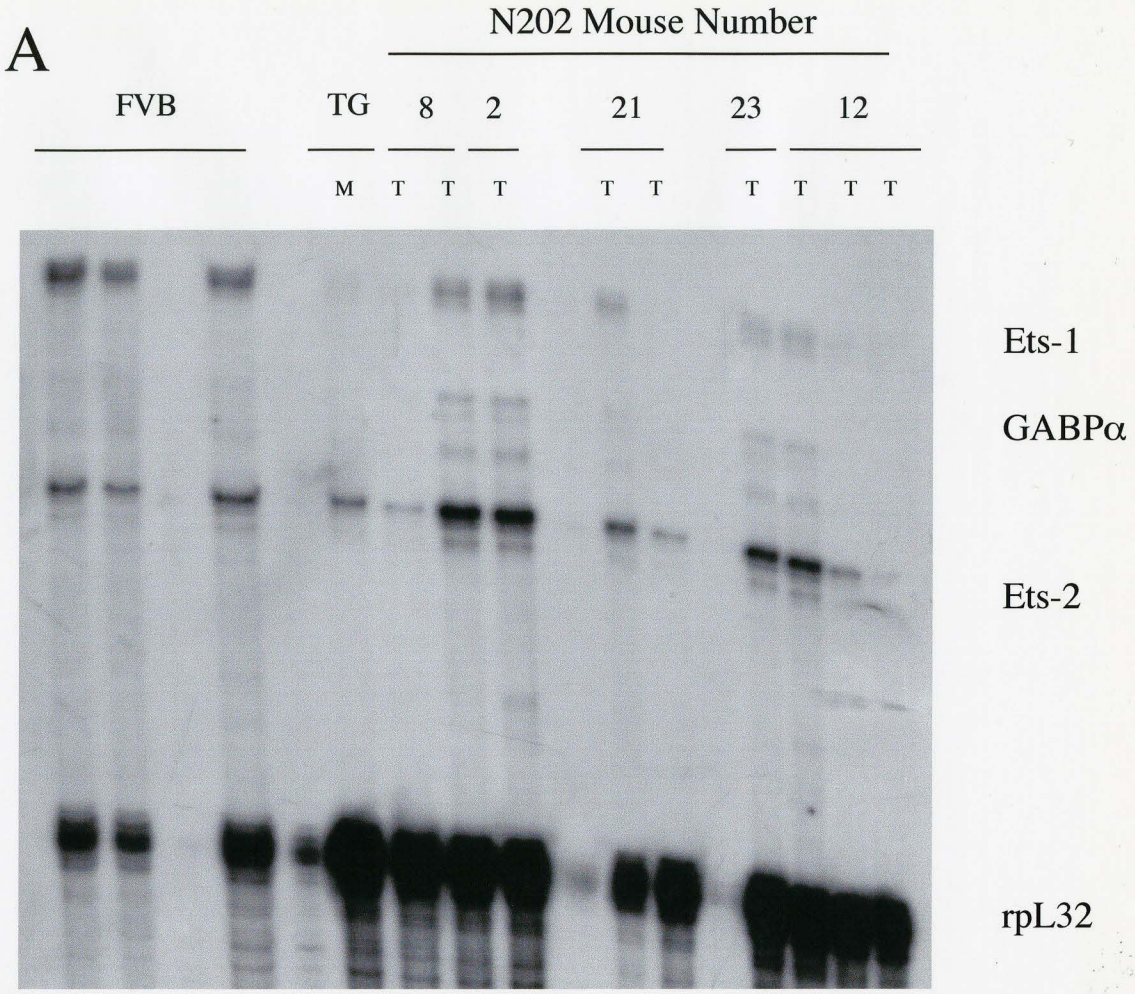

Fold Increase
1.2
1
0.8
0.6
0.4
0.2
0
Ets-1
Ets-2
GABP α
FVB average
TG-M
2-T
8-T
9-T
10-T
11-T
12-T
13-T
15-T
20-T

Figure 21: Quantitative analysis of *ets* genes GABPα, Ets-1 and Ets-2 expression in additional N202 mouse mammary tumors.

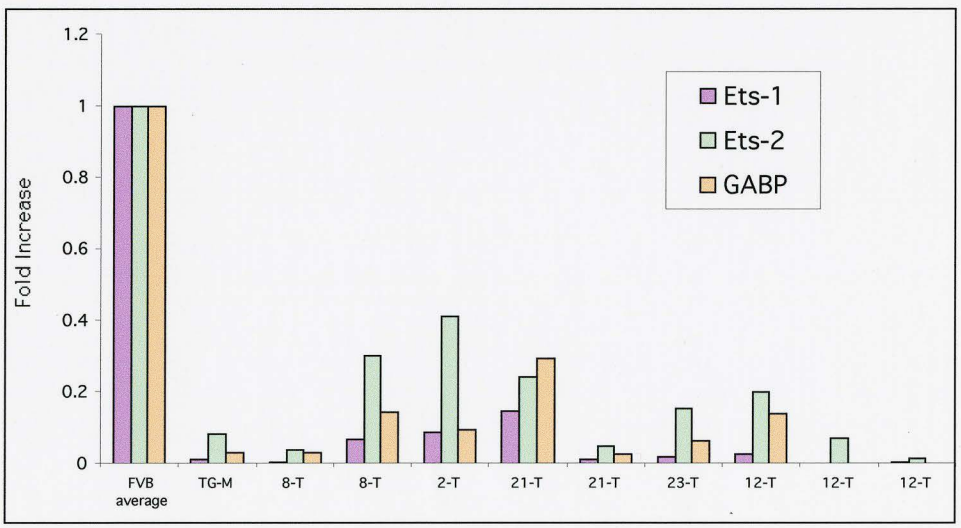
A. Ribonuclease protection analyses performed on 10ug of RNA derived from tumors from various N202 transgenic mice using Ets-1, Ets-2, GABPα and rpL32 riboprobes.

B. Quantification of above RNase protection by PhosphorImager analysis. Fold-increase of expression is based on setting average expression levels of each gene in the FVB controls to 1 after correction for rpL32 expression levels.

A


N202 Mouse Number
FVB
TG
8
2
21
23
12
M T T T T T T T T T
Ets-1
GABPα
Ets-2
rpL32

B


Fold Increase
1.2
1
0.8
0.6
0.4
0.2
0
Ets-1
Ets-2
GABP
FVB average
TG-M
8-T
8-T
2-T
21-T
21-T
23-T
12-T
12-T
12-T

Discussion

Knowing which genes are influential in the development and growth of a cancer is integral to the development of methods of treatment. Identification of genes that are deregulated in cancers has been the focus of much cancer-related research. The identification of such genes leads to the discovery of their role in cancer and would open the door to the development of treatments based on the function of that gene.

HER2/Neu is a gene that is amplified in approximately 17-28% of all human breast cancers (Zhou *et al.*, 1987; Berger *et al.*, 1988; Lacroix *et al.*, 1989; Slamon *et al.*, 1989; Parkes *et al.*, 1990). This gene product is a receptor tyrosine kinase that signals through the Ras pathway, a pathway commonly involved in human cancers. Previous work has shown that PEA3, founding member of the PEA3 subfamily of *ets* genes is upregulated in 93% of HER2/Neu positive human breast tumors and in 76% of all total human breast cancers (Benz *et al.*, 1997). Several *ets* genes, through either translocation or deregulation of expression, are implicated in several human cancers. The identification of deregulation of *ets* genes other than PEA3 in HER2/Neu-induced breast cancer would broaden our current knowledge of how these tumors work and shed light onto possible therapeutic strategies.

A degenerate RT-PCR approach was utilized to quickly screen for expression of all the members of the *ets* gene family in tumors derived from a mouse model of HER2/Neu-induced mammary tumorigenesis. The PEA3 subgroup, Ets-1, Ets-2 and GABPα were chosen for quantitative studies. Semi-quantitative RT-PCR and ribonuclease protection analyses were performed to identify potential quantitative differences in expression of these genes in tumor tissue compared to normal mouse mammary glands.

The degenerate RT-PCR analysis identified several *ets* clones in both tumor samples analyzed. The spectrum of *ets* gene expression in HER2/Neu-induced mouse mammary tumors was found to be very broad. At least one member of each subfamily except the Spi subfamily was represented in both or one of the tumor samples analyzed. The Spi subfamily of *ets* genes is expressed specifically in hematopoietic cell types (Ray *et al.*, 1992; Galson *et al.*, 1993; Moreau-Gachelin *et al.*, 1996; Su *et al.*, 1996). Therefore, their expression would be overshadowed by genes expressed in the epithelial component of the tumor and would then be difficult to clone.

The degenerate RT-PCR strategy is flawed however, due to the nature of the random selection of clones identified for sequence analysis. Genes expressed at low levels may not have been detected. For example, no PEA3 clones were isolated from either tumor sample, even though prior analyses by M. Trimble have shown that PEA3 is overexpressed in tumors of this transgenic line

of mice (Trimble *et al.,* 1993). Subsequent semi-quantitative RT-PCR and ribonuclease protection analyses showed that PEA3 is expressed in these tumors but at low levels compared to other *ets* genes. Initially however, the lack of PEA3 clones recovered using degenerate RT-PCR led us to think that the degenerate primers were preferentially amplifying sequences other than PEA3 and that the PEA3 specific primers within the degenerate primer mix were non-functional. However, upon inspection of ERM, ER81 and GABPα sequences recovered, it was noted that a variety of degenerate primers were used in PCR amplification. Also, ERM, ER81 and GABPα were amplified with primers that were not 100% identical in sequence, illustrating the flexibility of these primers to amplify sequences not perfectly identical to themselves. Finally, even PEA3 specific primers were able to amplify ERM/ER81/GABPα. Therefore, there is no physical reason for the degenerate primers to be unable to amplify PEA3 specifically. Indeed, the degenerate primers were able to amplify PEA3 cDNA (data not shown).

Both semi-quantitative RT-PCR and ribonuclease protection assays illustrate the overexpression of the PEA3 subfamily of *ets* genes. Ribonuclease protection analyses directly quantify mRNA levels by hybridization of a riboprobe to sample total RNA followed by RNase digestion of single stranded RNA species. The semi-quantitative RT-PCR analyses indirectly measure mRNA levels through reverse transcription of total RNA and PCR amplification using

gene specific primers. Quantification of the PCR analysis was possible by PhosphorImager analysis of Southern hybridizations of the PCR products. There are several inherent weaknesses in such a protocol to detect quantitative differences in gene expression. However, both analyses were able to detect comparable levels of expression of various *ets* genes.

Differences in calculated fold-increase that exist between the two methods are most likely due to the nature of the semi-quantitative RT-PCR analysis. Although steps were taken to maximize the efficiency of each PCR reaction ($MgCl_2$ concentration, annealing temperature during amplification) the slopes of each reaction were not always equal, although at most times were at least comparable. PhosphorImager data was plotted as the $\log_2$ because amplification within the exponential phase would be indicated by a slope equal to 1. In some cases, especially with cytokeratin 8, detection of gene expression in the TG and FVB controls by Southern hybridization was extremely difficult and as such, many PCR cycles were required to detect a signal on the PhosphorImager. In this case the slope of the lines indicate that these analyses were most likely during saturation of the PCR amplification process. However, in cases where there is clear overexpression (ER81 and ERM), minor differences in slope are most likely inconsequential.

It is interesting to note that the degenerate RT-PCR data recovered many more clones of ERM and ER81 in the tumor samples, than in the FVB controls.

This mirrors very nicely, the results of the two quantitative analyses. However, if the degenerate RT-PCR data is to be believed, because GABPα was recovered very frequently in both tissues tested, one would expect to see high levels of GABPα expression in the semi-quantitative RT-PCR and RNase protection analyses. Unfortunately, this is not the case. Therefore, although the degenerate RT-PCR data is able to detect qualitative differences in gene expression (on or off), it is unable to "rank" expression levels of clones that are recovered with some frequency.

Both quantitative analyses detected differences in gene expression of PEA3, ER81 and ERM in mouse mammary tumors compared to two normal controls, an FVB control and a transgenic mouse control. Neither method was able to detect great differences of expression of other *ets* genes in these samples including Ets-1, Ets-2 and GABPα.

It is important to note that these experiments attempt to compare expression profiles of two tissues that differ in cell-type composition. These transgenic mice bear the HER2/Neu gene behind the MMTV LTR, which directs expression to the ductal epithelium of the mammary gland (Choi *et al.*, 1987). Therefore, the tumor sample is primarily composed of cells that arose from the luminal epithelium of the normal mammary gland and the normal mammary gland is a heterogeneous mixture of differentiated epithelial cells, fibroblasts, adipocytes and endothelial cells. Difference in expression levels may be explained by difference in cell type

composition. Interestingly, PEA3 expression has been localized to the cap cells and myoepithelium of the normal mammary gland (MacNeil *et al.,* in preparation). The detection of high levels of PEA3 mRNA in these mouse mammary tumors therefore reflect a deregulation of PEA3 expression in a cell type PEA3 is not normally expressed in.

The expression levels of Ets-1, Ets-2 and GABP α were lower in the tumor samples than in the FVB normal controls. Although localization of these genes in normal mouse mammary glands has yet to be determined, this may simply be an artifact from comparing two samples of differing cellular composition. For example, Ets-1 is expressed in hematopoietic cells; any loss in mRNA expression may be due simply to a loss of specific cells that Ets-1 is normally expressed in. Alternatively, it has been proposed that Ets-1 is expressed in stromal fibroblasts surrounding neoplastic cells, but not in epithelial tumor cells (Wernert *et al.*, 1994). This would also explain the loss of expression in the tumors. However, the expression levels of these genes did not change in comparison to the non-tumor bearing HER2/Neu transgenic mouse control. This might be explained by potential differences in mammary tissue that displays transgene expression and those that do not or mouse-to-mouse variation. An interesting alternative explanation is that early down-regulation of these genes is important in the early development of these tumors.

The coordinate overexpression of the PEA3 illustrates the importance of their target genes in mouse mammary tumorigenesis. Several matrix metalloproteinases have already been identified as PEA3 target genes. Matrix metalloproteinase activity is necessary for the breakdown of the extracellular matrix responsible for the development of metastatic lesions (reviewed in Westermarck and Kahari, 1999). PEA3 can bind to and transactivate MMP-1, -3 and –9 promoters (Higashino *et al*, 1995). PEA3 expression in the non-invasive human breast cancer cell line MCF-7 can induce invasive characteristics in these cells and the expression of MMP-9 (Kaya *et al.*, 1996). Due to high homology within the ETS domain of each member of the PEA3 subgroup it is conceivable that ERM and ER81 activate the transcription of PEA3 target genes as well. Conversely, the divergence that does exist may reflect the diversification of target genes this subfamily targets. Regardless, the overexpression of this subfamily could have serious consequences in the deregulation of numerous target genes resulting in the progression of tumor formation and metastasis.

ERM and ER81 have been described to be downstream targets of signaling cascades. ERM-mediated transactivation through both the Ras/Raf-1/MAPK and PKA pathways has been illustrated, as well as its phosphorylation by activated ERK2 and activated PKA (Janknecht *et al.,* 1996). ER81 can be phosphorylated by ERK1 and ER81 transcriptional activity is also regulated by the Ras/Raf-1/MAPK pathway (Janknecht, 1996).

Interestingly, PEA3 can bind to and regulate the HER2/Neu promoter (Benz *et al.*, 1997). As both PEA3 and HER2/Neu are overexpressed in breast carcinomas, an autostimulatory feedback loop involving PEA3 and HER2/Neu would deregulate the expression of HER2/Neu downstream target genes contributing to in breast tumorigenesis.

Previous work in our laboratory has demonstrated the overexpression of PEA3 transcripts in 73% of all human breast tumors. Our present results illustrate that ER81 is overexpressed in HER2/Neu-induced mouse mammary tumors to a greater extent than PEA3. It is conceivable that ER81 overexpression will be detected in human breast tumors more frequently than PEA3.

In mice with multiple tumors, there is a trend for PEA3 subfamily expression to be greater in tumors of smaller size. Although these observations are based on three mice and a total of eight tumors only, the overexpression of the PEA3 subfamily, and ER81 in particular, might serve as a useful prognostic factor for early stages of mammary tumorigenesis. In fact, PEA3 subfamily expression can be detected in the normal adjacent epithelium derived from tumors of transgenic mice bearing an activated allele of HER2/Neu (observation made by L. MacNeil).

Interestingly, there is a trend for the PEA3 subfamily to be coordinately upregulated in the HER2/Neu-induced mouse mammary tumors. Typically, ER81 is overexpressed to a greater extent than both ERM and PEA3. ERM

overexpression tends to be either greater than or equal to that of PEA3. There could be a mechanism of regulating the expression of all three subfamily members together. As the PEA3 subfamily is overexpressed in the majority of HER2/Neu-induced mouse mammary tumors and PEA3 is overexpressed in the majority of all human breast tumors, it will be interesting to see if the subfamily is also coordinately overexpressed in the majority of human breast tumors. If this is the case, this subfamily could be the target of therapeutic strategies for human breast cancer. In particular, if there is one mechanism of PEA3 subfamily regulation, this would offer a realistic target to block deregulation of PEA3 subfamily target genes.

CONCLUSION

Knowing the mechanisms behind cellular transformation is an important aspect of cancer research. Identifying which genes are involved in the initiation, progression and metastatic potential of specific cancers is integral to developing therapeutic strategies. It has been shown that PEA3, the founding member of the PEA3 subfamily of the Ets family of transcription factors, is upregulated in the majority of human primary breast tumors. PEA3 target genes are believed to be involved in the breakdown of the basement membrane, leading to invasion and metastases. In an effort to identify if other *ets* genes play a role in mammary tumorigenesis, a mouse model of human breast tumorigenesis was screened for *ets* expression using degenerate RT-PCR. Almost each subfamily of *ets* subfamilies is expressed in these mouse mammary tumors, however of those tested, only the PEA3 subfamily is differentially expressed in tumor tissue compared to normal controls. PEA3, ERM and ER81 are coordinately overexpressed in the N202 line of transgenic mice, implicating them and their downstream target genes in this model of mammary tumorigenesis. If this subfamily is regulated by a common mechanism, this would be an important point of interest in developing therapeutic strategies for HER2/Neu positive breast tumors. Since PEA3 is also overexpressed in the majority of all human

breast cancers, regardless of HER2/Neu status, it is conceivable that so are ERM and ER81 and this subfamily could then be considered a therapeutic target for all human breast carcinomas.

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