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# Changes in the Expression of Many Ets Family Transcription Factors and of Potential Target Genes in Normal Mammary Tissue and Tumors*

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**Interfering with Ets transcription factor function reverses multiple aspects of the transformed phenotype of mouse or human tumor cells. However, the unknown number of individual Ets factors expressed in any cellular context and the similar DNA binding specificities of Ets family members complicates the identification of those that mediate transformation. By utilizing quantitative PCR assays for 25 mouse Ets factors, we analyzed the expression of essentially the entire Ets family in normal mammary tissue, mammary-related cell lines, and mammary tumors. In normal mammary tissue, 24 Ets factors were expressed. Even clonal derived cell lines expressed 14–20 Ets members. The most abundant Ets factor mRNAs measured in normal mammary tissue were Elk4, Elf1, and Ets2. Subtractive analysis of mammary tissue identified which Ets factors were predominantly expressed in the myeloid/lymphoid or epithelial cell compartments. Comparison of Ets factor expression in normal mammary tissue and mammary tumors identified significantly elevated expression of Pse/PDEF, Ese2/Elf5, Ese3/Ehf, TEL/Etv6, and Elf2/NERF in mammary tumors and confirmed previously reported alterations in expression of Ese1/Elf3 and the PEA3 subfamily. Expression of 13 Ets target genes, implicated in various aspects of tumor progression, was also analyzed. Altered expression of particular Ets target genes was significantly correlated with particular Ets factors (*e.g.* maspin and Ese2), suggesting specific *in vivo* regulatory roles. Together, this comprehensive analysis revealed unexpectedly diverse Ets family gene expression, characterized novel Ets factor changes in mammary tumors, and implicated specific Ets factors in the regulation of multiple genes involved in mammary tumor progression.**

The Ets family of transcription factors in mouse or humans is comprised of at least 26 unique family members that contain an evolutionarily conserved DNA binding domain called the Ets domain. The Ets family includes both transcriptional activators and repressors. These factors have been implicated as critical mediators of a wide range of cellular processes, including development, differentiation, growth, and transformation (reviewed in Ref. 1). Ets family members bind to similar sites containing a GGA(A/T) core sequence. The regulatory specificity of individual Ets family members comes from their pattern of expression, their modifications by signaling pathways, and interactions with partner proteins (1, 2). Although there has been substantial progress in understanding signaling pathways to individual Ets factors and their interactions with other proteins, information about the expression pattern of the whole Ets family is fragmentary. Studies of the expression pattern of a number of Ets factors have suggested that their expression is quite tissue-specific. However, despite potentially overlapping Ets factor function, even the broadest studies have characterized the expression of less than half of the known Ets family members in any single cell type or tissue (3, 4).

There are multiple lines of evidence indicating that Ets factors are important mediators of cellular transformation and tumor formation. This evidence includes association of Ets factor misregulation or fusion proteins with human cancers, the effects of Ets dominant negative constructs, and correlative expression studies in tumors (5–7). Experiments with various dominant negative constructs that inhibit Ets activity have shown that interfering with Ets factor function can reverse the transformed phenotype of a variety of mouse and human cell types (8–11). Such Ets dominant negative studies have also demonstrated the importance of Ets factors in the transformed phenotype of breast cancer cell lines (12, 13) and in a transgenic mouse mammary tumor model (14). However, because Ets dominant negative constructs can broadly interfere with Ets family function (15), these studies do not identify which of the ~26 Ets factors are key in transformation. Gene disruption analysis of individual Ets factors in tumors has been complicated by embryonic lethality (reviewed in Ref. 16). However, decreasing Ets2 by even half was shown to delay tumor formation in a mouse mammary tumor model (17).

A number of significant changes in Ets family gene expression have been observed between normal mammary tissue and mammary tumors. There is increased expression in mammary tumors of the three members of the Ets factor PEA3 subfamily, PEA3, ERM, and ER81 (14, 18), suggesting the importance of the PEA3 subfamily in breast cancer (19, 20). Ese1, an Ets factor largely expressed in epithelial cells, is also expressed at elevated levels in mouse and human mammary tumors (21, 22). Other Ets factors whose increased expression is associated with human mammary tumor progression include Ets1 (23) and Pse (24, 25).

Interfering with Ets factor function can reverse multiple aspects of breast tumor cell transformation, including anchor-

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age-independent growth, *in vitro* motility and invasiveness, resistance to apoptotic stimuli, and tumorigenicity (12, 13).[1] These observations imply there are a number of biologically important Ets target genes in breast cancers. Over 200 genes, whose products are involved in every aspect of cellular growth and metabolism, have been identified as Ets targets by various criteria (reviewed in Ref. 26). Many of these studies demonstrate that overexpression of one or more Ets factors can regulate the expression of a reporter gene. Due to the similarity of Ets DNA-binding sites as well as the uncharacterized expression of most Ets family members, such experiments primarily provide evidence of potential function (2, 27). Indeed, experimental overexpression of at least eight different Ets factors stimulates the expression of ErbB2/HER2/Neu (28, 29), an Ets target gene that has been a focus for therapeutic approaches to breast cancer. Ets factors also regulate the expression of several genes involved in tumor angiogenesis (30) and of many matrix metalloproteinases (MMPs)[2] associated with invasiveness (31).

Because of the strong potential for overlapping function of Ets family members, a necessary step in understanding the role of individual Ets family members in cancers is to determine which Ets factors are present in the relevant cells and tissues. We have now measured the mRNA expression of essentially all the Ets gene family members in normal mammary tissue (mamma), several cellular compartments of normal mammary tissue, representative cell lines, and in mammary tumors arising in four different transgenic mouse mammary tumor models. We discovered that there was an unexpected large number of Ets factors expressed in all contexts, with 24 Ets factors expressed in whole mammary tissue and 14 or more Ets factors expressed even in clonal cell lines. Quantitative analysis revealed both anticipated and novel differences in the expression levels of multiple Ets factors between normal mammary tissue and mammary tumors. When the expression of Ets factors *in vivo* was correlated with a variety of endogenous Ets target genes, several unexpected links between specific Ets members and targets such as maspin, ErbB2, and osteopontin were revealed. Overall, these studies provide new qualitative and quantitative insights into the expression pattern of the Ets family members and the changes that take place in the Ets family and their target genes in breast cancers.

## EXPERIMENTAL PROCEDURES

*Cell Lines, Tissues, and Tumors*—The normal mouse mammary epithelial cell lines used in this study were NMuNg (32) and HC11 (33). Mouse embryo fibroblasts from FVB/N mice were prepared as described (34). The MT1.2 cell line was a gift from Dr. Helene Baribault, who isolated this line by explanting an FVB/N-PyMT tumor into Dulbecco's modified Eagle's culture medium containing 15% fetal bovine serum, 5 $\mu$g/ml insulin, 40 ng/ml dexamethasone, $10^{-4}$ M 2-mercaptoethanol. The established cells were cloned, and a subclone resistant to puromycin was isolated by transfection with PGK-puro vector and puromycin selection. Antibody staining and RNase protection analysis revealed this line uniformly expressed simple epithelial keratins 8 and 18 but no longer expressed detectable levels of PyMT antigen RNA. The normal mamma, with or without the associated visible lymph node, were isolated from FVB/N 12-week-old virgin female mice. Surgically cleared number four mammary fat pads were prepared as described (35), and where indicated, the visible lymph node was removed. Mammary tumors were isolated from four previously described transgenic mice lines. MT mice express the polyoma middle T antigen (PyMT) under the control of the mouse mammary tumor virus (MMTV) enhancer and rapidly develop multifocal mammary tumors (36). The PyMTdb mice are similar, except that they express PyMT with the Y315F/Y322F mutations, which disrupts PyMT signaling to phosphatidylinositol 3-kinase. These mice exhibit extended mammary tissue hyperplasia, and then form tumors despite increased apoptosis (37). The MMTV-cNeu line, originally called N#202, is an MMTV-driven cNeu transgenic line (38), and the NeuNT KI line contains an activated Neu allele inserted by homologous recombination into the normal ErbB2 locus (39). Both of these transgenic lines form metastatic mammary tumors after an extended latency period.

*Isolation of RNA*—Cell lines were grown to 80% confluency on 10-cm dishes. The cells were washed twice with phosphate-buffered saline and then lysed directly on the dishes with 1 ml of TRIzol reagent (Invitrogen). Total RNA was extracted from the cell line and precipitated as recommended by the manufacturer. RNA from normal mammary tissue was prepared tissue dissected from FVB/N mice. The mammary tissues were homogenized with a Polytron homogenizer in 1 ml of TRIzol, and RNA was prepared as with the cell lines. RNA from mammary tumors was prepared from dissected tumor pieces by the method of Chirgwin *et al.* (40) with the modifications described previously (37). The concentration of total RNA was determined spectrophotometrically, or the purity and yield were estimated by ethidium bromide staining intensity of the 28 S RNA relative to standards, following electrophoresis on a 1% agarose TAE gel.

*cDNA Preparation*—Residual genomic DNA in the RNA samples was degraded by DNase treatment, and the DNase was subsequently removed using the DNA-free kit (Ambion) as recommended by the manufacturer. Following DNase treatment of the RNA, it was confirmed that significant Q-PCR signals were not obtained with RNA that had not been reverse-transcribed to cDNA. To make cDNA, 4 $\mu$g of DNase-treated total RNA was reverse-transcribed using oligo$(dT)_{18}$ primer, SuperScript II enzyme, and other reagents in the SuperScript II First-Strand Synthesis System kit (Invitrogen) in a final volume of 20 $\mu$l. The cDNA yield was analyzed by real time PCR analysis of the abundance of four different housekeeping genes, as described below. To establish that signals did not result from residual genomic DNA, it was confirmed that RNA samples that were not reverse-transcribed did not yield significant signals in the standard Q-PCR. The amount of cDNA used in a typical Q-PCR was made from 10 ng of whole RNA, which represents 500 cells, assuming 20 pg of RNA/cell.

*Primers and Primer Design*—Standard unmodified primers of ~20 nucleotides in length were obtained from either Genbase (San Diego) or Operon/Qiagen (Valencia, CA). Primers were designed using Primer 3 (41), a web-based primer design tool (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) using the default parameters, except the following: $T_m$, minimum 54, optimal 56, maximum 59; maximum 3′ self-complimentarity: 2; maximum poly($X$), 4; GC clamp, 1. Primers were typically designed to amplify unique sequences within 1,000 bp of the 3′ end of the mRNA and, where feasible, to cross intron/exon boundaries. The primer sequences (5′ to 3′) of the sense ("L") and the antisense ("R") primers are shown for the Ets family, control genes, and Ets target genes in Table I. The only characterized mouse Ets factor omitted from our analysis was the B lymphocyte-specific Spi-B, due to sequence ambiguity and subsequent technical difficulty of PCR product specificity.

*Q-PCRs*—The Q-PCRs were performed using the LightCycler SYBR green DNA master mix from Roche Applied Science. To achieve hot start PCR, prior to assembling the Q-PCRs, each microliter of the master mix was incubated on ice for >5 min with 0.07 $\mu$l of TaqStart antibody (Clontech). A 10-$\mu$l Q-PCR contained 1 $\mu$l of cDNA (typically a 1:20 dilution of the cDNA stock) and 1 $\mu$l of antibody-inactivated SYBR green DNA master mix. The reaction also contained a final concentration of 4 mM $MgCl_2$ and 0.5 mM of each primer and was brought to 10 $\mu$l with distilled water. The reaction mixtures were placed into capillaries and analyzed on a Roche Applied Science LightCycler real time PCR instrument. The amplification protocol consisted of an initial denaturation/antibody inactivation step at 95 °C for 70s, followed by 42 amplification cycles of 95 °C for 0 s, 56 °C for 7 s, and 72 °C for 20 s. Fluorescence was measured once at the end of each extension step. Melting analysis was performed after a final melting step at 95 °C for 0 s, followed by a 15 s annealing at 68 °C, and then increasing the temperature to 97° at 0.1°/s with continuous fluorescence measurements.

*Verification of Q-PCR Specificity*—Three criteria were used to establish that the observed SYBR green I fluorescence measured in the LightCycler resulted from the specific amplification of the cDNA from each Ets family member. These were analysis of the Q-PCR product melting characteristics, length, and DNA sequence. For this analysis,

[1] G. Foos and C. A. Hauser, manuscript in preparation.

[2] The abbreviations used are: MMPs, matrix metalloproteinases; PyMT, polyoma middle T antigen; MMTV, mouse mammary tumor virus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Cph, cyclophilin A; VEGF, vascular endothelial growth factor; Q-PCR, quantitative PCR; Opn, osteopontin.

20-μl Q-PCR were performed with each of the primer pairs on a cDNA mixture derived from mouse lung, thymus, prostate, and testis RNA. A characteristic sharp melting peak of d(fluorescence)/d$T$ at a specific temperature was determined for each specific PCR product, using the Roche Applied Science LightCycler software (version 3.3). For quantitation, the PCR products were then recovered from the capillaries and purified using the QIAquick PCR purification kit (Qiagen). Following gel electrophoresis on a 2% NuSieve agarose 1% standard agarose TAE gel, the size and purity of each PCR product were analyzed. The fluorescence intensity of the ethidium bromide-stained gel band was used to determine the DNA concentration of the PCR product, using the AlphaImager 1200 CCD imaging system (Alpha Innotech, San Leandro, CA) and a standard curve generated from Low Mass markers (Invitrogen). Primer pairs that failed to yield a product with a single sharp melting peak or a single gel band of the anticipated size were discarded, and a new primer pair for that gene was designed and tested. Finally, the DNA sequences of the purified PCR products that met these two criteria were determined, using the appropriate L primer and standard automated fluorescent sequencing in the Burnham Institute Gene Analysis Core Facility. Each DNA sequence was subjected to BLAST analysis (www.ncbi.nlm.nih.gov/blast/) to confirm that the PCR product represented the correct Ets family gene. Following this three-way validation, subsequent Q-PCR specificity was monitored by melting curve analysis of every Q-PCR product.

*Q-PCR Relative Expression Analysis*—Initial analysis and normalization of the data were performed using LightCycler Analysis software version 3.3. In brief, after setting a noise band to exclude the non-linear portion of the amplification curves, this software determines a crossing point for each Q-PCR. This crossing point, similar to the crossing threshold ($C_T$) of other software, can then be used in combination with a standard curve to calculate the concentration of each cDNA. For the relative analysis of gene expression, calculated concentration was based upon a standard curve relating GAPDH cDNA concentration to the $C_T$, which was found to be linear over 5 orders of magnitude (Pearson correlation coefficient $r > 0.99$). A standard curve was generated for each experiment by analyzing serial dilutions of the cDNA sample with GAPDH primers. This relative $C_T$-based analysis assumes equivalent PCR efficiency for other primer pairs in the log-linear phase of amplification, which subsequent analysis with gene-specific standard curves revealed to be quite accurate. Consistent with previous observations (42), we observed that expression of peptidylprolyl isomerase A/cyclophilin A (Cph) was significantly more constant between samples from different tissues and cultured cells than GAPDH expression (data not shown). In addition, GAPDH is not highly suitable for expression normalization in tumor studies, as it is a well characterized hypoxia-responsive gene (43, 44) with hypoxia-induced expression in cell lines and tumors (45). Thus, Cph expression was chosen as a constant internal reference standard to control for differences in RNA preparation and cDNA yield throughout this work. Relative expression data for the different tissues or cell lines were normalized by expressing the calculated concentration of each cDNA as a percentage of the amount of the calculated Cph cDNA from the same cDNA sample analyzed in the same experiment. Results in Table II are the average of at least three separate experiments, and standard deviations of representative samples are shown by error bars in the figures.

*Determining cDNA Molecule Concentration for Each Ets Factor Using Gene-specific Standard Curves*—The cDNA samples used for Q-PCR were derived from 10 ng of total RNA, which represents ~500 cells. To minimize sequence-specific differences in converting mRNA to cDNA, primers were designed to amplify sequences within 1,000 bp of poly(A). Gene-specific standard curves were generated for each Ets factor, using quantitated purified PCR products isolated as described above. A known amount of each purified Ets factor PCR product (*e.g.* 2 pg of 172-bp Ets2 PCR fragment = $10^7$ molecules) was serially diluted and analyzed using standard Q-PCRs. The data from each run were plotted as the number of molecules ($x$ axis, log scale) *versus* $C_T$ ($y$ axis, linear scale). A pooled whole mammary tissue cDNA sample and the cNeu mammary tumor 1 cDNA sample were analyzed with the same primers in the same experiment, and least square fit analysis of the standard curve (Microsoft Excel) was used to determine the number of molecules in the tissue cDNA samples. The number of molecules in the normal mammary tissue and cNeu tumor sample was normalized to the Cph levels, which resulted in a 2-fold increase in the calculated amount of cNeu tumor cDNAs. To confirm that the results obtained using purified PCR products reflected the quantitation obtained in the more complex cDNA mixtures, mixing experiments were performed where the Q-PCR contained both $1.0 \times 10^5$ molecules of purified PCR product and a standard amount of mammary tissue cDNA. The results revealed that the other components present in cDNA did not interfere with Ets factor quantitation (data not shown).

*Correlation Analysis*—The values for correlation constants across the 12 tissue and cell types examined, correlating changes in expression of each Ets factor relative to changes in the Ets target genes, were determined using Microsoft Excel. The significance of the correlation constant $r$ for this sample size ($n = 12$) was determined using a web-based calculator (department.obg.cuhk.edu.hk/researchsupport/Correlation_coeff.asp), using the formula $t = \sqrt{n-2/1-r^2}$. In our analysis, a correlation coefficient with an absolute value of >0.576 was significant ($p < 0.05$).

## RESULTS

*Specificity of Ets Family Q-PCR Analysis*—The PCR primer pairs (Table I) were designed to amplify unique regions of each mouse Ets factor cDNA. Where possible, the cDNA representing the 3′-untranslated portion of the mRNA was chosen as a PCR target. This increased the probability of obtaining unique sequences and also allowed for similar efficiency of cDNA synthesis by using oligo(dT) priming. The grouping of Ets family members in Table I is based on the molecular phylogeny of their Ets domains (46). The names used for the Ets factors in this work were selected on the basis of common usage or to emphasize membership in subfamilies. Alternative names of these Ets factors and their human homologs are also listed in Table I. Three complementary approaches were used to confirm the specificity of the SYBR green-based Q-PCR for each Ets family member. The purity of Q-PCR products from a mixed tissue sample was analyzed by melting curves, agarose gel electrophoresis, and DNA sequencing. The observed melting temperature peak and the anticipated size of each PCR product is shown in Table I, with the agarose gel electrophoresis analysis shown in Fig. 1. Finally, the identity of each Ets family PCR product was verified by DNA sequence analysis (data not shown). Overall, this analysis confirmed that reverse transcription of mixed tissue mRNA followed by Q-PCR could sensitively and specifically detect the expression of each of the 25 Ets family members listed in Table I. This is all but one of the characterized Ets factors, Spi-B. Bioinformatic analysis of the mouse genome sequence (47) suggests that few additional Ets factors still remain to be discovered.

*Almost All Ets Family Members Are Expressed in Normal Mammary Tissue*—We first determined how many different Ets family member mRNAs were expressed in a normal mammary tissue. Total RNA was isolated from young adult number 4 mammae with their associated lymph nodes, primed with oligo(dT), and reverse-transcribed into cDNA. Unexpectedly, Q-PCR analysis of this cDNA revealed that all of the Ets factors, except Pet-1, were expressed in mammary tissue. Pet-1 expression was detected in a sample of mixed tissues, but it was not substantially expressed in any of the mammary tissues or cell lines examined. The average $C_T$ values for each Ets factor primer pair, obtained from cDNA samples prepared from three different whole mammary tissue samples, are shown in Table I. For the subsequent relative comparison of gene expression in mammary-related tissues and cell lines (Table II), the abundance of each of the 24 Ets factors found expressed in normal mammary tissue was used as the basis for comparison, and mammary expression for each was defined as 100%. Similarly, the expression level in normal mammary tissue for each of the 13 Ets target genes analyzed (see below) was also defined as 100%.

*Ets Family Expression in Mammary Lymph Node and Epithelial Compartments*—The mamma consists of a variety of cell types, including fat, endothelia, pericyte, luminal epithelia, myoepithelia, and lymphoid cells. To better understand the cell type expression of the Ets factors in the mammary tissues, we employed a subtractive approach. To reduce the contribution of

TABLE I
*Primers used for PCR analysis*

| | Gene[a] | Alternative Names[b] | Accession #[c] | L-primer | R-primer | Prod. length[d] | Prod. Tm[e] | $C_t$ mam. cDNA[f] |
|---|---|---|---|---|---|---|---|---|
| 1 | Ets2 | | AK014602 | CAGAGGCCTAATCCTCAGTC | GGCCAAATTACAAAACCTTC | 172 | 82.7 | 24.4 |
| 2 | Ets1 | | BC013089 | ACAGCTTTGTTGTCCATCTG | AGATCTGTCCATCCTTCCTG | 166 | 84.8 | 22.2 |
| 3 | ER71 | ETSRP71, (h)ETV2 | NM_007959 | TACACCACCACGTGGAATAC | CGAGTCAATGTGGCTCTATC | 122 | 86.3 | 34.2 |
| 4 | Gabpa | | BC013562 | TGGCAAGTTCTGTCATTTTC | CAGTCACAGGTTTGTTTTGC | 124 | 77.9 | 34.8 |
| 5 | PEA3 | Etv4, (h)E1AF | NM_008815 | TCTGGGGTATCCAGAAGAAC | ACGTTGATTATCTGGGAAGG | 173 | 88.1 | 28.5 |
| 6 | ER81 | Etv1, EtsRP81 | NM_007960 | GGCGATGAACTATGACAAAC | GTGTCCTCTTCGTTGATGTG | 195 | 85.6 | 29.6 |
| 7 | ERM | Etv5 | BC034680 | TGTACAGATTGGGTTTGGAG | AGTCAGCTCTGTGTCCTGTC | 137 | 87.5 | 25.9 |
| 8 | Fli1 | (h)ERGB | NM_008026 | CTCCTACATGCCTTCCTACC | GTTTGGATAGATCCCAGCAG | 145 | 85.2 | 28.4 |
| 9 | Erg | | AB073080 | GACCTTGCTTGACCTACCAG | CTTCTTTGGTTTTGACATGG | 135 | 81.9 | 31.0 |
| 10 | Erf | (h)PE-2 | TC985870 | CTGCCTCTTACCTTGTCCTC | GTATAAGAAACCCAGGCACTC | 106 | 88.7 | 35.7 |
| 11 | PE1 | Etv3, METS | NM_012051 | TCCCTCTCGGATTAAGGTAG | AGTGCTAATGGACACAGAGG | 184 | 87.7 | 30.4 |
| 12 | Elk1 | | NM_007922 | CCTGAACTCTCCAGTTAGCC | CACCATTGAAGTAGGAGACG | 191 | 82.7 | 27.8 |
| 13 | Elk3 | Net, Sap-2, Erp | NM_013508 | CGAACACACTTTTCCAGTTC | ATCATCCGTCATCTGTTCTG | 175 | 90.0 | 25.3 |
| 14 | Elk4 | Sap1a | BC004798 | GGAGACCTGCAAAGTATTCC | TTGAGATGACCATTCTGTCC | 126 | 79.0 | 23.7 |
| 15 | Elf1 | | NM_007920 | GAAGCAATGGGAGTCCTG | AACTCTACTTCGGAAACTTGG | 145 | 79.7 | 25.3 |
| 16 | Elf2 | (h)NERF | NM_023502 | AGATGCTGACAGAAAACTGC | GCATCAAGAAATGCTCTTTG | 198 | 82.5 | 28.3 |
| 17 | Elf4 | MEF, ELFR | NM_019680 | CTAGGTCAGCCACAGAGATG | CCCATTGAAGTTCTCCTACC | 175 | 83.4 | 29.9 |
| 18 | Ese1 | Elf3, ESX, jen, Ert | NM_007921 | GGCCAGAAGAAGAAGAACAG | CCTTAATTCCGACTCTCTCC | 173 | 86.4 | 27.7 |
| 19 | Ese2 | Elf5 | NM_010125 | AAATTTCTGCATGTCTCAGC | ATAACACTGGCTCCCTTAGC | 175 | 84.3 | 28.1 |
| 20 | Ese3 | Ehf | NM_007914 | GGACACAAACCAAAACACAC | TCCCGTCAGAACTAGATTTTC | 129 | 78.9 | 28.1 |
| 21 | Pse | (h)PDEF | NM_013891 | TGAAAGAACTGCTGCTCAAG | TCTTGTAATACTGGCGGATG | 180 | 88.7 | 34.0 |
| 22 | TEL | Etv6 | TC552323 | GCTAGTTAGAGCTGCTGTGC | GTTTAGTGTTGATGCGGATG | 180 | 87.2 | 24.8 |
| 23 | PU.1 | Spi-1, Sfpi1 | NM_011355 | GTCTGATGGAGAAGCTGATG | AGGTACCTTTGTCCTTGTCC | 161 | 88.4 | 26.9 |
| 24 | SpiC | Spi-C, Prf | NM_011461 | TGTCTGTCAGAAAAGCCAAG | TCAAACATGTTCTGTGTCTCTC | 127 | 81.2 | 28.0 |
| 25 | Pet1 | Fev, (h)HSRNAFEV | AY049085 | GAGGTCTTCCTTGAACATCAG | ATAGGGGTCATAACGAGGTG | 163 | 85.3 | 37.8 |
| 26 | K8 | Krt2-8, Keratin 8 | NM_031170 | GATGCAGAACATGAGCATTC | CTCGGGTTTCAATCTTCTTC | 197 | 88.9 | 24.4 |
| 27 | K18 | Krt1-18, Keratin 18 | NM_010664 | AGGAATATGAAGCCCTCTTG | TAGTCTCGGACACCACTCTG | 186 | 86.7 | 23.0 |
| 28 | CPH | Cyclophilin A ,PPIA | NM_008907 | AGACCAGCAAGAAGATCACC | GGAAAATATGGAACCCAAAG | 177 | 83.6 | 19.6 |
| 29 | ARP | PRLP0 , P0; RPP0 | NM_001002 | CGACCTGGAAGTCCAACTAC | ATCTGCTGCATCTGCTTG | 109 | 81.6 | 21.9 |
| 30 | GAPDH | (GAD5' primer) | NM_008084 | CCAGTATGACTCCACTCACG | GACTCCACGACATACTCAGC | 153 | 86.5 | 20.2 |
| 31 | GAPDH | (GAPD primer) | NM_008084 | CATGACCACAGTCCATGC | AGTGAGCTTCCCGTTCAG | 166 | 88.6 | 26.4 |
| 32 | Thbs1 | Thrombospondin 1 | NM_011580 | ACCTCATTTGTTGTGTGACTG | AAGCAACGGTGTAAAAGATG | 219 | 80.0 | 26.1 |
| 33 | TenC | Tenascin C,Tnc | NM_011607 | GAACTGTCATCGTGTCAACC | TGATGCTTTGGTAAAAATCC | 234 | 85.3 | 29.2 |
| 34 | Mmp3 | Stromelysin-1 | NM_010809 | TGACCCACATATTGAAGAGC | ACTTGACGTTGACTGGTGTC | 132 | 88.4 | 26.1 |
| 35 | Mmp9 | GelatinaseB | NM_013599 | ACTCACACGACATCTTCCAG | AGAAGGAGCCCTAGTTCAAG | 176 | 86.8 | 28.0 |
| 36 | Mmp7 | Matrilysin | NM_010810 | CCCGGTACTGTGATGTACCC | AATGGAGGACCCAGTGAGTG | 164 | 83.3 | 34.9 |
| 37 | Vegfa | VEGF alpha | NM_009505 | GAAGAAGAGGCCTGGTAATG | GGAAGATGAGGAAGGGTAAG | 172 | 87.1 | 26.6 |
| 38 | Vim | Vimentin | NM_011701 | TCACTTCCTCTGGTTGACAC | TTTCCTTCTTGCTGGTACTG | 161 | 84.1 | 20.2 |
| 39 | uPA | Plau, urokinase type.... | NM_008873 | AGACACTAACGGCTTGAGG | GGGAAATACCTAGACTTGCAG | 159 | 82.4 | 29.6 |
| 40 | Masp | Maspin, Serpinb5, | NM_009257 | AAGACCCTAAGGGAAAGCTC | GCCACAAAACTCTTAGCATC | 184 | 83.5 | 29.2 |
| 41 | ErbB2 | Erbb2, HER2, Neu | NM_010152 | AAGGCCTAACTTGTGGTCTC | ACTCACGATTGAAGCATCG | 132 | 88.4 | 25.6 |
| 42 | BclXL | Bcl2l , Bcl2-like | NM_009743 | GACGTGATCATTTCCCATC | GAGGTTCTGGTCCTTGTCTC | 149 | 85.4 | 25.7 |
| 43 | CycD1 | Cyclin D1, Ccnd1 | NM_007631 | AGGTAATTTGCACACCTCTG | ACAAAGCAATGAGAATCTGG | 200 | 87.2 | 23.3 |
| 44 | Opn | Osteopontin, Spp1 | NM_009263 | CAAGCAGACACTTTCACTCC | TTCATGTGAGAGGTGAGGTC | 158 | 85.5 | 23.3 |

[a] Gene names used in this work. The first 25 genes listed are the mouse Ets family members analyzed. Underline indicates current LocusLink symbol (www.ncbi.nlm.nih.gov/LocusLink/).
[b] Alternative names for the same gene. Names primarily used for human Ets family orthologs are designated by (h).
[c] GenBank accession number for the mRNA sequence containing the amplified region. Designations starting with TC are 3′ mRNA sequences identified in the TIGR mouse gene index (www.tigr.org/tdb/tgi/mgi/).
[d] Length of PCR product in base pairs.
[e] Melting temperature of the PCR product (°C).
[f] Average threshold cycle ($C_t$) of product from whole mammary tissue cDNA sample derived from 10 ng of total RNA.

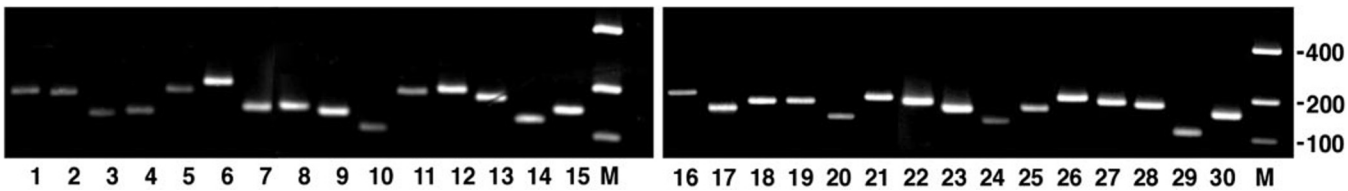



FIG. 1. **PCR products for the Ets family members.** Loading dye was added to samples recovered after real time PCR analyses, which were then subjected to 2% agarose gel electrophoresis analysis and stained with ethidium bromide. *Lanes 1–30* correspond to primer pairs 1–30 in Table I. *M* indicates markers, whose length in base pairs is indicated.

the myeloid/lymphoid compartment, the associated lymph node was surgically removed before RNA preparation. Similarly, to strongly reduce the contribution of the epithelial compartment, Ets family expression was analyzed in mammary fat pads surgically cleared of epithelial tissue 2 weeks prior to harvest. Ets expression was also analyzed in cleared fat pads without the associated lymph node. For the relative expression analysis in Table II, the average Cph normalized expression value for each Ets factor in the whole mammary tissue cDNA of three individuals was defined as 100% and was used as the basis of comparison. The data reveal that upon removal of the lymph node, expression of PU.1, SpiC, PE1, Elf4, Ets1, ER71, and Elk1 were all reduced to below 20% of their expression level in whole mammary tissue (highlighted by *dark green fill* in Table II). Data from the two types of cleared fat pad mammary tissue samples showed reduction to below 20% of normal mammary

TABLE II
*Relative expression of Ets factors and Ets target genes in mammary-related tissues and cell lines*

| Ets Factor[a] | mam -LN[b] | mam Clr | mam Clr-LN | MEF cells | HC11 line | NMuNg line | MT1.2 line | PyMT tumors | cNeu tumors | NeuNT tumors | PyMTdb tumors |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Fli | 10 | 21 | 36 | 20 | 2 | 0 | 0 | 31 | 18 | 10 | 7 |
| PU.1 | 9 | 9 | 6 | 8 | 0 | 0 | 0 | 51 | 9 | 49 | 12 |
| Ets1 | 8 | 10 | 16 | 28 | 33 | 49 | 24 | 18 | 14 | 9 | 6 |
| SpiC | 14 | 41 | 10 | 0 | 0 | 16 | 0 | 12 | 21 | 3 | 1 |
| PE1 | 0 | 90 | 2 | 97 | 183 | 0 | 55 | 32 | 112 | 25 | 8 |
| Elf4 | 2 | 33 | 1 | 37 | 107 | 42 | 91 | 19 | 55 | 12 | 3 |
| ER71 | 6 | 1,359 | 0 | 50 | 954 | 0 | 236 | 59 | 147 | 72 | 0 |
| Elk1 | 10 | 58 | 4 | 102 | 220 | 79 | 146 | 105 | 93 | 143 | 39 |
| Elk3 | 22 | 23 | 41 | 105 | 104 | 39 | 132 | 59 | 95 | 44 | 19 |
| ERG | 65 | 411 | 84 | 24 | 36 | 7 | 21 | 57 | 165 | 18 | 7 |
| Ets2 | 80 | 61 | 167 | 25 | 203 | 329 | 58 | 150 | 66 | 130 | 112 |
| Elk4 | 51 | 69 | 85 | 48 | 79 | 91 | 41 | 65 | 65 | 45 | 40 |
| Elf1 | 38 | 32 | 41 | 19 | 92 | 61 | 104 | 43 | 76 | 59 | 46 |
| GABPa | 55 | 95 | 373 | 238 | 1,877 | 197 | 299 | 158 | 522 | 78 | 42 |
| ERF | 58 | 72 | 24 | 156 | 627 | 203 | 466 | 85 | 296 | 68 | 57 |
| Elf2 | 68 | 69 | 163 | 172 | 325 | 362 | 408 | 387 | 434 | 255 | 156 |
| TEL | 75 | 32 | 30 | 88 | 106 | 139 | 74 | 200 | 210 | 231 | 128 |
| ER81 | 57 | 56 | 150 | 52 | 25 | 0 | 1,412 | 1,695 | 10,890 | 3,063 | 2,102 |
| PEA3 | 21 | 116 | 4 | 501 | 811 | 0 | 1,247 | 372 | 2,537 | 2,618 | 105 |
| ERM | 24 | 33 | 8 | 80 | 20 | 94 | 208 | 394 | 1,074 | 1,453 | 157 |
| PSE | 97 | 0 | 0 | 0 | 178 | 0 | 0 | 3,614 | 2,149 | 251 | 613 |
| Ese2 | 113 | 11 | 1 | 0 | 38 | 11 | 3 | 1,309 | 489 | 331 | 313 |
| Ese1 | 68 | 20 | 1 | 0 | 207 | 209 | 18 | 312 | 417 | 412 | 118 |
| Ese3 | 114 | 51 | 8 | 21 | 355 | 331 | 36 | 597 | 743 | 471 | 416 |
| *K8* | 63 | 4 | 1 | 5 | 27 | 1,016 | 514 | 744 | 468 | 476 | 182 |
| *K18* | 158 | 3 | 2 | 0 | 12 | 550 | 218 | 455 | 1,071 | 1,095 | 307 |
| *GAPDH* | 79 | 38 | 40 | 205 | 112 | 91 | 339 | 165 | 427 | 330 | 54 |
| **Ets Target Genes** | | | | | | | | | | | |
| Thbs1 | 111 | 48 | 37 | 6,719 | 396 | 24 | 719 | 692 | 135 | 295 | 93 |
| TenC | 28 | 60 | 47 | 7,987 | 308 | 72 | 16 | 174 | 62 | 144 | 81 |
| MMP3 | 158 | 68 | 67 | 355 | 23 | 1 | 8 | 64 | 51 | 69 | 33 |
| MMP9 | 34 | 68 | 103 | 20 | 1 | 1 | 4 | 29 | 7 | 43 | 6 |
| MMP7 | 21 | 297 | 0 | 0 | 5 | 50 | 0 | 252 | 20 | 15 | 7 |
| VEGF | 21 | 159 | 46 | 65 | 15 | 56 | 98 | 50 | 48 | 115 | 10 |
| Vim | 132 | 85 | 66 | 265 | 12 | 95 | 404 | 45 | 35 | 128 | 22 |
| UPA | 14 | 137 | 422 | 81 | 92 | 14 | 206 | 157 | 43 | 309 | 22 |
| Masp | 13 | 19 | 2 | 0 | 84 | 2 | 7 | 223 | 29 | 20 | 25 |
| ErbB2 | 58 | 42 | 51 | 82 | 58 | 42 | 64 | 122 | 67 | 286 | 37 |
| BclXL | 50 | 88 | 104 | 197 | 40 | 120 | 463 | 138 | 58 | 339 | 28 |
| CycD1 | 101 | 30 | 41 | 165 | 19 | 127 | 312 | 136 | 116 | 271 | 60 |
| OPN | 5 | 7 | 1 | 462 | 420 | 224 | 485 | 610 | 606 | 948 | 978 |

[a] Genes assayed are listed in the left column, with Ets factors in black, reference genes in red, and Ets target genes in blue.

[b] Cell lines or tissues analyzed are indicated across the top. Tissue abbreviations: mam, mammary; -LN, without associated lymph node; Clr, cleared mammary fat pad.

[c] Values shown are the average percent expression of each gene in the indicated tissue, relative to the expression of the same gene in normal whole mammary tissue.

| % normal mammary[c] |
|---|
| >1,000% |
| >500% |
| >300% |
| >200% |
| 50-199% |
| <50% |
| <30% |
| <10% |

tissue in the expression of the Ese subfamily (Ese1, Ese2, and to a lesser degree Ese3), 2 of 3 members of the PEA3 subfamily (PEA3 and ERM, not ER81), Pse, and the simple epithelium marker genes keratin 8 and 18. Even in a mammary fat pad essentially lacking the lymphoid and epithelial compartments (cleared fat pad, no lymph node), there was still significant expression of 11 different Ets factors (Table II). Together, these data confirm and extend previous observations of which Ets factors are predominantly lymphoid or epithelially expressed and are also important for interpreting the differences in Ets factor expression seen between normal mammary tissue and mammary tumors described below.

*Unexpected Diversity of Ets Factor Expression in Cell Lines*—To test whether the expression of the entire Ets family in the whole mammary tissue was simply a consequence of the many cell types in this organ, each expressing several Ets factors, relevant cell lines were assayed. Ets family expression in clonal cell lines derived from normal mouse mammary epithelium (NMuNg and HC11) and a carcinoma cell line derived from a PyMT-induced mouse mammary tumor (MT1.2) were analyzed. In addition, mouse embryo fibroblasts were analyzed as a representative of a stromal cell type. To highlight meaningful levels of expression, a cut-off of 20% of expression in whole mammary tissue was again applied for each gene. Table II shows an average of 17 Ets factors were expressed above this 20% level in these cell lines, with a high of 20 different Ets factors in HC11 cells, to a low of 14 Ets factors in NMuNg cells. The pattern of Ets factor expression in the tissue subtraction analysis and in these cell lines also demonstrated the specificity and appropriate sensitivity of this Ets family Q-PCR analysis. For example, the well characterized myeloid/lymphoid Ets factors such as PU.1, SpiC, and Fli1 were not significantly expressed in mammary tissue without the associated lymph node or in any of the cell lines. Similarly, epithelial cell-associated Ets factors (*e.g.* the Ese/Pse subfamily) were not significantly expressed in cleared fat pad or in the mouse embryo

fibroblasts. Overall, analysis of cell lines revealed that there is an unexpected and previously unappreciated broad expression of Ets family members, not only in tissues but even in individual cell lines.

*Multiple Differences in Ets Factor Expression between Normal Mammary Tissue and Mammary Tumors*—To analyze differences in Ets factor expression between normal mammary tissue and mammary tumors, four types of previously characterized transgenic mouse mammary tumor models were employed (see "Experimental Procedures"). Ets family expression data from cDNA derived from two different tumors from each transgenic mouse line were averaged, and the percent expression of each Ets factor in each tumor type relative to its expression in normal mammary tissue is shown in Table II. The Ets family pattern of expression in tumors from the PyMT, MMTV-cNeu, and NeuNT-KI mice were quite similar. In these tumors, at least 13 of the Ets family members exhibited over a 2-fold difference in expression relative to that seen in normal mammary tissues. The Ets factors listed in the 1st column of Table II were ordered to cluster these observed tumor/normal differences. As expected, a number of the Ets factors we identified as predominantly lymph node-associated (Fli, PU.1, Ets1, SpiC, Elf4, and PE1) were expressed at a reduced level in the tumor samples relative to normal mammary tissues. The largest magnitude of tumor/normal increased expression was observed in the PEA3 subfamily (PEA3, ER81, and ERM), whose increased expression has been correlated previously (14) with mammary tumor development. Also consistent with previous observations (21), Ese1 expression was elevated in three of the tumor models. In addition to these changes characterized previously, we discovered that there was significantly increased mammary tumor expression of five other Ets factors as follows: Pse, Ese2, Ese3, TEL, and Elf2. The dramatically elevated Pse levels in the mouse PyMT and c-Neu tumors (36- and 21-fold, respectively) parallels the increased expression of the Pse human ortholog (PDEF) reported recently (24, 25) in human breast tumors. We found that the expression of Ese2 and Ese3, whose expression has not been correlated previously with mammary tumor formation, increased significantly (3–13-fold) in all four mouse tumor models. Finally, two other Ets factors, Elf2 and TEL, exhibited modest (2–4-fold) but consistent tumor/normal expression increases in three mammary tumor models. Overall, the significant increase observed in mRNA expression of nine different Ets factors in mammary tumors represents an important level of Ets family regulation.

*Ets Factor Expression in Cell Lines Representing Normal Mammary Tissue and Mammary Tumor*—Quantitation of Ets factor expression in cell lines representing normal mammary epithelium (HC11 and NMuNg) or a PyMT-induced mammary tumor (MT1.2) revealed similarities and differences with their tissue counterparts. In contrast to the NMuNg cell line, the HC11 line we analyzed expressed little K8 or K18 epithelial marker genes (Table II), suggesting it may no longer represent simple epithelium. Both of these normal mammary cell lines expressed low levels of most PEA3 subfamily members, as well as Pse and Ese2. However, these cell lines expressed elevated levels of Ese1 and Ese3, relative to normal mammary tissue. Fig. 2*A* shows a graphical comparison of the expression of selected Ets genes in the NMuNg and MT1.2 cell lines and in PyMT- or c-Neu-induced mammary tumors. As expected, PEA3 (and the related ER81, not shown) was expressed at low level in normal NMuNg cells, and expression was substantially elevated in the MT1.2 tumor line and in tumors. However, in contrast to the tumors, the MT1.2 cell line expressed the Ese subfamily at low levels, with NMuNg cells exhibiting variable Ese subfamily expression. Pse, which was highly expressed in the tumors, was not significantly expressed in either NMuNg or MT1.2 cell lines (Fig. 2*A*). The observed differences in Ets factor expression in these cell lines and the tissue samples may reflect a combination of different cellular composition and changes that may have occurred in cell culture, highlighting the importance of analyzing Ets expression in tumors.

*Effects of Mammary Tumor Signaling Pathways on Ets Family Expression*—Of the four types of mouse mammary tumor models analyzed, the PyMTdb tumors exhibited a substantially different pattern of Ets factor expression than the other three. The PyMT(Y315F/Y322F) oncogene expressed in the PyMTdb mice has mutations in tyrosine residues required for PyMT association with phosphotidylinositol 3-kinase and is defective in phosphatidylinositol 3-kinase signaling. Mice expressing this oncogene exhibit an extended period of mammary hyperplasia, followed by development of tumors with apoptotic centers (37). One clear difference when comparing the PyMTdb tumors and the other tumors was a larger reduction of predominantly lymphoid Ets factor expression relative to normal mammary tissue (Table II), suggestive of a reduced inflammatory response. The graphical comparison (Fig. 2*B*) of selected Ets gene expression in the four tumor types shows that in contrast to the other three tumor models, the PyMTdb tumors did not exhibit significantly increased expression of PEA3, ERM, Ese1, and TEL. Thus, the altered oncogenic signaling in PyMTdb mice led to a substantially different pattern of Ets factor expression in the tumors that arose in these mice, relative to three other mammary tumor models.

*Quantitation of Ets Factor cDNA Levels Using Gene-specific Standard Curves*—In addition to changes in Ets factor expression between normal mammary tissue and tumors, it is important to understand the abundance of each Ets factor. The analysis shown in Table II reveals quantitative changes in the mRNA expression of each Ets factor but does not allow comparison of the abundance of different Ets factor mRNAs due to differences in the initial amplification efficiency with different primer pairs. To quantitate the cDNA levels of each Ets factor, gene-specific standard curves were generated for analysis of cDNA samples from pooled normal mammary tissue or from the c-Neu mammary tumor. This allowed the calculation of the number of cDNA molecules for each Ets factor in the pooled normal mammae and cNeu mammary tumor samples. An example of why gene-specific standard curves are required to compare results with different primer pairs is shown in Fig. 3*A*. Both primer pairs used in this analysis detect cDNA synthesized from the same GAPDH housekeeping gene, with the GAD5′ primers recognizing a more 5′ sequence of the cDNA than the GAPD primers. By using the appropriate standard curve for each primer pair, the calculated number of GAPDH cDNA molecules was within 2-fold of each other (Fig. 3*A*). In contrast, the inappropriate use of the GAD5′ primer curve for the GAPD primers would lead to an approximate 100-fold error in the calculated cDNA concentration, based on the 6 cycle $C_T$ difference between the two primer pairs. The 40,000 GAPDH cDNA molecules measured in the normal mammary tissue cDNA sample represent ~80 cDNA molecules per cell. This amount is 7% of the 1200 GAPDH mRNA molecules per cell reported in a human cell line (48), a yield similar to the modest reported efficiency of converting oligo(dT)-primed mRNA to cDNA (49, 50). However, this low absolute efficiency of conversion of mRNA to cDNA does not compromise estimation of mRNA abundance as long as the conversion is representative and is not biased.

*Ets Factor mRNA Levels in Normal Mammary Tissue and Mammary Tumors*—Gene-specific standard curve analysis of

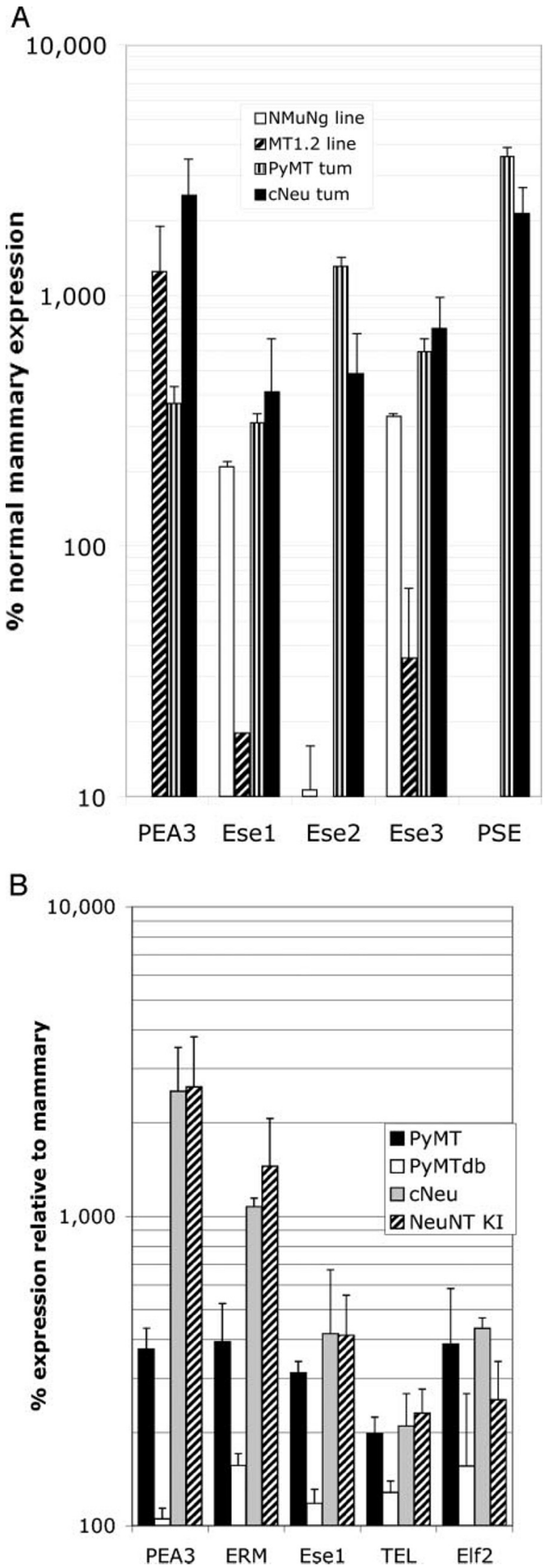
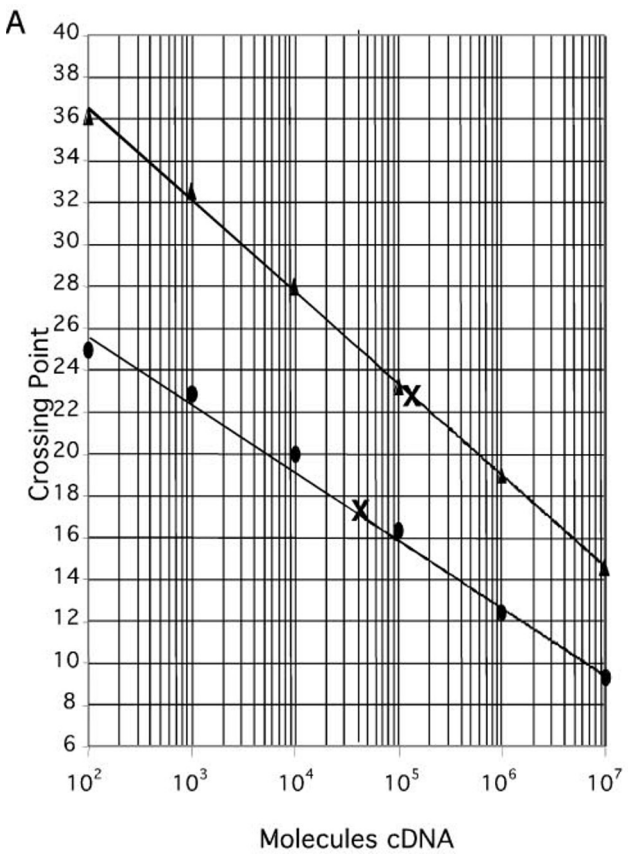


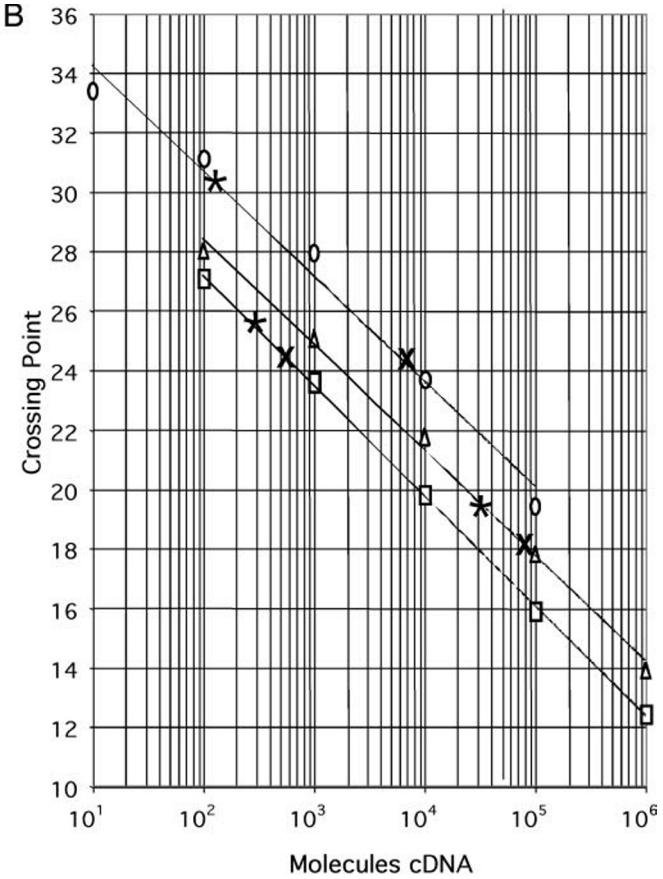

FIG. 2. **Altered expression of Ets factors in mammary cell lines and tumors.** *A*, expression of the indicated Ets genes was quantitated by real time PCR as described in the text, in a normal mammary epithelial cell line (NMuNg), in a mammary tumor cell line (MT1.2), and in two types of mammary tumor models (PyMT and cNeu). Relative expression indicated is the percent expression of the same gene measured in normal whole mammary tissue. The average and standard deviation shown are from three separate assays of gene expression in the cell lines or two assays of two different tumors of each genotype. *B*, differences in the relative expression of five Ets factors in four mammary tumor models, where mammary tumor formation was initiated by distinct signaling events in the indicated transgenic strains. Relative expression and data analysis are as in *A*.

cDNA abundance for normal mammary tissue and the cNeu1 mammary tumor sample were performed for each of the Ets family members. Representative gene-specific standard curves for the cDNAs from the Ets factors Elf2 and PEA3, as well as the Cph standard, are shown in Fig. 3*B*. The data points for the normal mammary tissue or an MMTV-cNeu mammary tumor 1 cDNA are plotted on the standard curves. After normalizing the data for the 2-fold different Cph levels, there were ~250 Elf2 cDNA molecules in both samples. In contrast, there were 126 molecules of PEA3 cDNA in the normal mammary tissue sample, whereas there were 2893 cDNA molecules from PEA3 in the mammary tumor sample (Fig. 3*B*). The results of similar analysis for the entire Ets family are summarized in Fig. 3*C*.

The number of cDNA molecules obtained from reverse transcription of whole RNA (Fig. 3*C*) represents an estimate of the relative mRNA levels of each Ets factor in normal mammary tissue and in a mammary tumor. Whereas this mRNA estimation is subject to sequence-specific differences in oligo(dT) primed cDNA synthesis, the PCR primers were designed to minimize such variation. These data revealed that some Ets factor mRNAs were expressed at high abundance but did not vary greatly between normal mammary tissues and tumors (*e.g.* Elk4, 0.4-fold tumor/normal difference but 5655 cDNA molecules in tumor sample). Three other highly abundant Ets factor mRNAs whose importance was not highlighted by the tumor/normal difference analysis are Elf1, Ets2, and Ets1. The potentially high abundance of these Ets factors could have a strong influence on Ets-dependent gene expression in the mammary tissue. Not only are the mRNAs for these four Ets factors estimated to be the most abundant in normal mammary tissue but even in cNeu tumors, Elk4 and Elf1 expression rank 3rd and 4th in abundance (Fig. 3*C*). Conversely, some Ets factors that exhibited dramatic tumor/normal differences are expressed at quite low levels. Examples shown in Fig. 3*D* include the high level but fairly constant expression of Elk4 and Elf1, the 10-fold increase in ERM expression in tumors leading to its high abundance, increases in Ese3 and PEA3 expression in tumors changing their abundance from low to moderate, and finally, the 16.7-fold increase in Pse expression in tumors still resulting in a very low level of expression. Overall, this quantitation of cDNA molecules complements the tumor/normal relative results by also providing a first level estimate of Ets factor abundance in normal mammary tissue and mammary tumors.

*Similar Results from Analysis of Ets Factor Relative Expression or cDNA Molecules*—In addition to providing an estimate of the mRNA abundance for each Ets factor, the cDNA molecule data allowed assessment of the accuracy of the relative expression analysis for each Ets factor between normal mammary tissues and cNeu-induced tumors (see the data in Table II). The results were very similar for these distinct approaches, one with the relative difference calculations all based on a GAPDH standard curve *versus* cDNA molecule determination using gene-specific standard curves (Fig. 3*C*, *two rightmost columns*). The calculated fold difference in expression between normal mammary tissue and c-Neu tumor samples was comparable both for Ets factors with low tumor/normal expression (*e.g.* Fli1, PU.1, Ets1, and SpiC) and high tumor/normal expression (*e.g.* the PEA3 and Ese/Pse subfamilies). Thus, in contrast to comparing expression of different genes to each other, it was quite accurate to use a GAPDH standard curve to measure




C

| Ets factor | cDNA molec. norm mam | cDNA molec. cNeu tum | cDNA molec. tum/norm | relative expression tum/norm |
|---|---|---|---|---|
| Fli | 287 | 56 | 0.2 | 0.1 |
| PU.1 | 174 | 47 | 0.3 | 0.1 |
| Ets1 | 3,607 | 1,061 | 0.3 | 0.2 |
| SpiC | 197 | 13 | 0.1 | 0.2 |
| PE1 | 31 | 24 | 0.8 | 0.7 |
| Elf4 | 12 | 4 | 0.4 | 0.5 |
| ER71 | 17 | 3 | 0.2 | 2.9 |
| Elk1 | 75 | 60 | 0.8 | 0.4 |
| Elk3 | 824 | 629 | 0.8 | 1.0 |
| ERG | 99 | 22 | 0.2 | 1.3 |
| GABPa | 400 | 312 | 0.8 | 2.1 |
| ERF | 18 | 31 | 1.7 | 2.5 |
| Elf2 | 259 | 257 | 1.0 | 4.1 |
| TEL | 668 | 1,448 | 2.2 | 2.5 |
| Ets2 | 4,572 | 720 | 0.2 | 0.5 |
| Elk4 | 15,627 | 5,655 | 0.4 | 0.6 |
| Elf1 | 5,510 | 2,946 | 0.5 | 0.7 |
| ER81 | 436 | 96,983 | 222.3 | 124.2 |
| PEA3 | 126 | 2,893 | 23.0 | 18.5 |
| ERM | 1,646 | 16,463 | 10.0 | 10.2 |
| PSE | 3 | 43 | 16.7 | 17.6 |
| Ese2 | 25 | 111 | 4.4 | 3.3 |
| Ese1 | 910 | 429 | 0.5 | 2.4 |
| Ese3 | 330 | 1,735 | 5.3 | 5.7 |

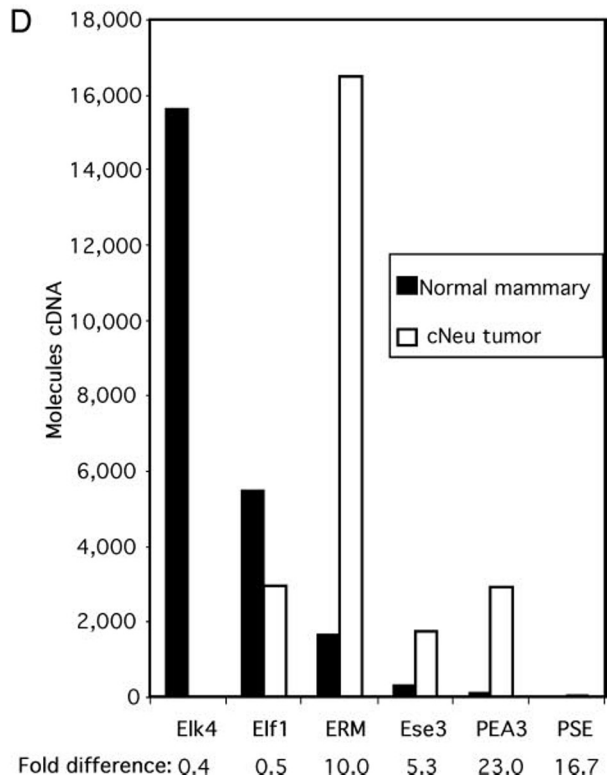



FIG. 3. **Quantitation of Ets factor cDNA molecules.** *A*, requirement for standard curves. Serial dilutions of purified and quantitated PCR products were then analyzed under the standard Q-PCR conditions, and the $C_T$ value was determined. *Circles* indicate data from the GAD5′ primer pair, and *triangles* indicate the GADP primer pair. Standard curves were generated using linear regression. The $C_T$ value of a cDNA sample from whole mammary tissue was analyzed in the same Q-PCR experiment with the same primers, is indicated with an × on each standard curve. *B*, quantitating Ets factor cDNA. Standard curves were generated as in *A* for PEA3 (*circles*), Cph (*triangles*), or Elf2 (*squares*). The $C_T$ values from

TABLE III

*Expression correlation of Ets target genes with each Ets factor*

Correlation constants ($r$) for the expression of the indicated 13 Ets target genes with the expression of 24 indicated Ets factors, across the 12 tissues, cell lines, and tumors in Table II. Statistically significant coefficients ($p < 0.05$) are highlighted in dark gray, with additional positive correlation coefficients ($r > 0.33$) lightly shaded to highlight trends.

| | Thbs1 | TenC | MMP3 | MMP9 | MMP7 | VEGF | Vim | UPA | Masp | BclXL | CycD1 | ErbB2 | OPN |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fli | 0.14 | 0.15 | 0.30 | 0.75 | 0.42 | 0.10 | -0.27 | 0.49 | 0.32 | -0.23 | -0.30 | 0.04 | -0.25 |
| PU.1 | -0.06 | -0.10 | 0.02 | 0.17 | 0.38 | 0.20 | -0.20 | 0.30 | 0.61 | 0.21 | 0.32 | 0.80 | 0.51 |
| Ets1 | 0.22 | 0.22 | -0.08 | -0.43 | -0.16 | -0.14 | 0.16 | -0.22 | -0.02 | 0.09 | 0.06 | -0.25 | -0.20 |
| SpiC | -0.34 | -0.30 | -0.15 | 0.39 | 0.73 | 0.49 | -0.31 | -0.13 | -0.01 | -0.37 | -0.41 | -0.26 | -0.54 |
| PE1 | 0.26 | 0.26 | 0.10 | -0.30 | 0.03 | 0.04 | -0.06 | -0.20 | 0.14 | -0.12 | -0.21 | -0.12 | 0.04 |
| Elf4 | -0.16 | -0.14 | -0.18 | 0.13 | 0.52 | 0.46 | -0.16 | -0.05 | 0.05 | -0.17 | -0.40 | -0.21 | -0.31 |
| ER71 | -0.16 | -0.14 | -0.18 | 0.13 | 0.52 | 0.46 | -0.16 | -0.05 | 0.05 | -0.17 | -0.40 | -0.21 | -0.31 |
| Elk1 | 0.16 | 0.11 | -0.16 | -0.58 | -0.08 | 0.02 | 0.17 | -0.10 | 0.32 | 0.30 | 0.24 | 0.16 | 0.32 |
| Elk3 | 0.43 | 0.37 | 0.13 | -0.46 | -0.33 | -0.01 | 0.54 | 0.01 | 0.06 | 0.48 | 0.44 | -0.02 | 0.17 |
| ERG | -0.20 | -0.17 | -0.05 | 0.45 | 0.69 | 0.60 | -0.21 | 0.01 | -0.06 | -0.27 | -0.40 | -0.23 | -0.45 |
| Ets2 | -0.41 | -0.38 | -0.52 | -0.12 | -0.05 | -0.29 | -0.42 | -0.01 | 0.15 | -0.22 | -0.23 | -0.05 | -0.11 |
| Elk4 | -0.30 | -0.25 | -0.32 | 0.26 | 0.21 | -0.10 | -0.50 | 0.10 | 0.12 | -0.45 | -0.57 | -0.34 | -0.57 |
| Elf1 | -0.40 | -0.46 | -0.65 | -0.51 | -0.34 | -0.08 | 0.19 | 0.02 | 0.02 | 0.37 | 0.34 | 0.01 | 0.23 |
| GABPa | 0.08 | 0.07 | -0.06 | 0.00 | -0.29 | -0.10 | 0.10 | 0.20 | -0.12 | 0.06 | 0.06 | -0.22 | -0.12 |
| ERF | 0.00 | -0.04 | -0.28 | -0.60 | -0.30 | -0.16 | 0.21 | -0.19 | 0.06 | 0.18 | 0.11 | -0.20 | 0.07 |
| Elf2 | -0.13 | -0.19 | -0.44 | -0.56 | -0.12 | -0.11 | 0.09 | -0.04 | 0.37 | 0.33 | 0.42 | 0.14 | 0.38 |
| TEL | -0.12 | -0.14 | -0.19 | -0.43 | -0.03 | -0.10 | -0.28 | -0.13 | 0.42 | 0.10 | 0.37 | 0.64 | 0.74 |
| ER81 | -0.18 | -0.18 | -0.18 | -0.25 | -0.13 | -0.05 | -0.20 | -0.13 | 0.03 | -0.05 | 0.16 | 0.17 | 0.44 |
| PEA3 | -0.05 | -0.08 | -0.13 | -0.23 | -0.27 | 0.25 | 0.11 | 0.18 | -0.05 | 0.43 | 0.55 | 0.65 | 0.56 |
| ERM | -0.15 | -0.16 | -0.14 | -0.08 | -0.11 | 0.26 | -0.08 | 0.22 | 0.06 | 0.33 | 0.50 | 0.80 | 0.62 |
| PSE | -0.12 | -0.17 | -0.14 | -0.15 | 0.43 | -0.20 | -0.37 | -0.11 | 0.83 | -0.18 | -0.01 | 0.15 | 0.35 |
| Ese2 | -0.14 | -0.19 | -0.13 | -0.10 | 0.46 | -0.15 | -0.35 | -0.02 | 0.87 | -0.09 | 0.08 | 0.33 | 0.44 |
| Ese1 | -0.31 | -0.32 | -0.34 | -0.32 | 0.03 | -0.07 | -0.44 | -0.08 | 0.41 | -0.03 | 0.20 | 0.59 | 0.57 |
| Ese3 | -0.32 | -0.33 | -0.37 | -0.43 | 0.07 | -0.27 | -0.56 | -0.26 | 0.51 | -0.23 | 0.01 | 0.33 | 0.63 |

changes in the expression of individual Ets genes with a given primer pair.

*Regulation of Ets Target Gene Expression in Normal Mammary Cells, Tissues, and Mammary Tumors*—There are many cellular genes that have been described as targets of Ets transcription factors (26). Thirteen reported Ets target genes, whose products have been implicated as playing important roles in diverse tumor functions such as growth, survival, vascularization, or invasiveness, were selected for analysis. Expression of each of these target genes was assayed in the same mammary tissues, cell lines, and tumors as for the Ets family. These Ets target genes, with alternative gene names and primer information listed in the lower part of Table I, were Thbs1 (51), TenC (52), MMP3 (53), MMP9 (54), MMP7 (55), VEGF (23), Vim (56), uPA (13), Maspin (57), ErbB-2 (29), $BclX_L$ (58), CycD1 (59), and Opn (60). Analysis of the expression of these endogenous Ets target genes, relative to the level in normal mammary tissues, was performed using Q-PCR analysis in the same manner as for the Ets factors, and the results are shown in the lower portion of Table II. Osteopontin (Opn) was the only Ets target gene whose expression increased in all of the mammary tumor types, whereas expression of several of the Ets target genes (Thbs1, uPA, Vim, $BclX_L$, and CycD1) was increased in the MT1.2 tumor cell line and at least one of the tumor types. The highest expression of Thbs1, TenC, and MMP3 was seen in the mouse embryo fibroblasts, suggesting their combined expression might provide an estimate of fibroblast contribution to the tumor sample. Overall, a complex pattern of Ets target gene expression was observed in these tissues and cell lines, with few simple relationships evident.

*Correlating Ets Factor Expression with Altered Ets Target Gene Expression*—Correlation between expression of an Ets target gene with a particular Ets factor could identify an important *in vivo* regulatory linkage and potential direct targets. Thus, we performed correlation analysis of the mRNA expression pattern of the 13 Ets target genes with the expression of 24 Ets factors across all of the same mammary-related tissues (see data in Table II). The 18 correlation coefficients found to be statistically significant ($|r| > 0.576$, $p < 0.05$) are indicated in Table III by *dark gray shading*, with other positive correlations suggesting trends ($r > 0.33$) *lightly shaded*. The expression of Opn significantly correlated with the expression of TEL, ERM, and Ese3, with additional positive correlation with all of the nine Ets factors up-regulated in tumors (Elf2, TEL, and the seven members of the PEA3 and Ese/Pse subfamilies). ErbB2 expression exhibited highly significant correlation with ERM expression ($p = 0.002$) and additionally showed significant correlation with expression of TEL, PEA3, and Ese1 and the low expression of PU.1. Maspin expression was strongly linked to expression of Pse ($p = 0.0008$) and of Ese2 ($p = 0.0002$). Maspin expression also significantly correlated with PU.1 expression and positively correlated with expression of Ets factors up-regulated in tumors, except for the PEA3 subfamily. Conversely, although none of the Ets factor correlations with CycD1 or $BclX_L$ expression reached the level of $p < 0.05$, there were multiple positive correlations observed in the expression

simultaneously analyzed cDNA samples from normal mammary tissue or cNeu-induced tumor 1 are indicated by a × or *, respectively. *C*, Ets factor cDNA molecules. The number of cDNA molecules for each Ets factor (derived from a 10-ng RNA sample) from pooled normal mammary tissue or a cNeu tumor was determined from gene-specific standard curves. The tumor/normal ratio of each Ets factor cDNA from this analysis is shown (*cDNA molec. tum/norm*), with the adjoining column displaying the tumor/normal ratio determined from the average relative expression analysis shown in Table II. *D*, overlaying cDNA abundance and tumor-specific changes. Comparison of the number of cDNA molecules for the indicated Ets family members in samples derived from pooled normal mammary tissue or a Neu tumor. Shown *below* each Ets factor is the fold difference observed in the tumor/normal mammary cDNA molecules.

of these genes with Ets factors up-regulated in tumors, except for the Ese/Pse subfamily. Expression of both MMP7 and VEGFa were found to significantly correlate with ERG expression. An interesting significant negative correlation was observed between the expression of MMP9 and the Ets family repressor ERF. Together, these correlation analyses identify candidate Ets factors or subgroups of Ets factors that regulate many Ets target genes, either directly or indirectly.

## DISCUSSION

The expression status of the majority of the Ets factors had not been characterized previously in any cellular context, and this information is essential as a starting point to determine which Ets factors may be functionally important in tumor formation. Here we have analyzed the mRNA expression of essentially the entire Ets family and a variety of Ets target genes in mammary tissue, relevant cell lines, and several types of mammary tumors. The mouse mammary system was chosen to analyze Ets family expression because of the extensive *in vitro* and *in vivo* evidence implicating the Ets family in breast cancers. In addition, this mouse model system allowed analysis of Ets family expression in normal mammary tissue and in tumors from four distinct transgenic mammary tumor models all in a common genetic background. Our findings should have relevance to human breast cancers, as previous studies (22, 61) with these transgenic mouse mammary tumor models have revealed multiple parallels with the development of human breast cancers. To analyze gene expression of the entire Ets family, real time PCR was chosen over a microarray-based approach because of the following: (i) it is more quantitative; (ii) it provides better specificity in analyzing related family members; and (iii) it is sufficiently sensitive to detect low abundance transcription factor mRNAs.

The discovery of the expression of 24 of the 25 Ets factors in a whole mammary tissue indicates the unexpected broad expression of the Ets family. This was reinforced by the expression of 14–20 different Ets factor mRNAs in cell lines. This broad expression likely generates a complex cellular mixture of Ets factors that compete for Ets-binding sites based on their abundance, affinity, and presence of co-factors. Whereas reverse transcriptase-PCR-based analysis is very sensitive, two findings argue that meaningful levels of Ets gene expression were detected. First, we observed appropriate tissue-specific expression of Ets factors that have been found previously to be tightly regulated (*e.g.* lymphoid-specific expression of PU.1 and SpiC, and the epithelial specific expression of the Ese/Pse subfamily). Second, estimation of mRNA abundance in normal mammary tissue by quantitation of cDNA molecules (Fig. 3*C*) showed that only a few of the Ets factors are expressed at very low levels.

The subtractive analysis of Ets expression in mammary cellular compartments revealed which Ets factors are predominantly expressed in the lymphoid and epithelial compartments. There are substantial changes in the cellular composition during tumor development, as normal mammary tissue is a complex mixture of cell-types including a lymph node, whereas tumors are predominantly composed of epithelial cells. We used keratin 8 and 18 expression as molecular markers to monitor epithelial cell content, and they reflected a 4–10-fold increase in epithelial cell content between normal mammary tissues and tumors. These results helped to distinguish changes caused by tumor signaling from those resulting from altered cellular composition.

In addition to the reported increased expression of the PEA3 subfamily (14) and Ese1 (21) in mammary tumors relative to normal mammary tissue, we identified five other Ets factors whose expression was increased in mouse mammary tumors. These appear to be *bona fide* tumor-specific changes in Ets family expression, as they were observed in different transgenic mouse tumor models and were consistent in tumors that arose in two different transgenic mice of each genotype. Increased expression of nine Ets factors was observed in both Neu-induced mouse tumor models (Table II), including tumors resulting from an amplification of an activated NeuNT allele driven by the endogenous ErbB-2 promoter, an event similar to that seen in many human breast cancers. Expression of Ese2 and Ese3 were elevated an average of 6- and 5.5-fold, respectively, across all four transgenic mouse mammary tumor models tested. Ese2 and Ese3 are expressed in glandular epithelium (53, 62), but increased expression in breast tumors has not been reported previously. The *Ese3* gene was postulated to suppress mammary tumor development, based on the observation that it is deleted in some mammary tumors (53), along with its ability to act as a context-dependent negative regulator (63). However, in our mouse models, the observed increase in Ese3 expression observed in tumors is not consistent with a tumor suppressor role. Overall, the comparable increase in expression of Ese1, Ese2, and Ese3, with that of the K8/K18 epithelial cell markers, suggests that the expression of the Ese subfamily may remain quite constant in epithelial cells during their transition from glandular epithelium to carcinoma. Expression of Pse was strongly increased (21–36-fold) in two of the tumor models, a level well above their increase in epithelial cell content. The observed Pse expression levels are interesting in light of recent reports that the human ortholog, PDEF, is a potential tumor progression marker in breast cancers (24, 25) whose expression is reduced in tumor cell lines (64). Expression of two other Ets factors not associated with epithelial cells, Elf2 and TEL, were modestly elevated (2–4-fold) in three of the mammary tumor models. Expression of the human Elf2 ortholog NERF is induced by hypoxia (65). Evidence of elevated hypoxic signaling in tumors was observed in several contexts, as reflected by substantially increased GAPDH expression (Table II) and parallel increases in Pgk1 and Glut-1 expression.[3] However, the pattern of hypoxic target expression was distinct from the elevation of Elf2 in the tissues and cell lines, suggesting additional influences on Elf2 expression.

The changes in Ets factor abundance in mammary tumors may reflect both the cellular makeup of tumors (*e.g.* increased epithelial cell content) and the sensitivity of Ets gene promoter elements to oncogene signaling. An example of the latter is the finding that activated Neu signaling stimulates PEA3 transcription (66). However, even very large increases in Ets factor expression do not necessarily reveal a causal role in tumorigenesis, as demonstrated by the failure of high level transgenic expression of ER81 in mammary epithelium to stimulate tumor formation (67). A complementary approach to identifying Ets factors that may mediate breast cancer signaling was to determine which Ets factor mRNAs are abundant in all mammary tissue contexts, using cDNA molecule quantitation from gene-specific standard curves (Fig. 3*C*). This analysis identified several relatively abundant Ets factors whose possible importance was not highlighted by differences in expression between normal and tumor tissue. An example of this was Ets2, whose mRNA expression was quite high and fairly constant in nearly all tissues examined, with the apparent reduction of Ets2 expression in the single tumor sample assayed in Fig. 3*C* not reflected in averaged values. Despite its constant expression, Ets2 is currently the only Ets factor functionally implicated in the development of mammary tumors (17, 68) Thus, abun-

[3] C. Galang and C. Hauser, unpublished data.

dantly expressed Ets factors in normal mammary tissues such as Ets1, Ets2, Elk4, and Elf1, are also important candidates for mediating tumor signaling.

Due to their similar DNA binding specificities, it is difficult to infer from overexpression studies which of the 25 Ets factors regulate the expression of most Ets family target genes *in vivo*. We have correlated *in vivo* changes observed in Ets factor gene expression and expression of endogenous Ets target genes. The similar results of correlation analysis (Table III) and covariance analysis (data not shown) revealed both expected and novel associations of Ets factors with target gene expression. An extraordinary level of correlation was seen for maspin expression with the expression of two Ets factors, Pse and Ese2. The potential functional significance of the correlation of maspin and Pse/PDEF expression ($p = 0.0008$) is supported by the recent finding that PDEF specifically stimulates the expression of a maspin reporter gene (64). The even stronger correlation of Ese2 and maspin expression ($p = 0.0002$) suggests a novel regulatory relationship. For ErbB-2 expression, the significant correlations with expression of PEA3, ERM, and Ese1 are supported by overexpression studies showing that these are among several Ets factors that can regulate *ErbB-2* reporter gene expression (28, 29). The significant positive correlation of both ErbB-2 and Opn expression with the expression of TEL was unexpected, but oncogenic signaling can prevent repression by TEL (69). Another unexpected correlation was the expression of ERG with both MMP7 and VEGF. Although there is no previous link between ERG and VEGF expression, it is interesting that ERG activity was recently linked to endothelial cell differentiation (51). Overall, such correlation analysis can reveal the potential importance of a subset of transcriptionally modulated Ets factors in Ets target gene regulation.

Although the expression analysis experiments were designed to minimize the impact of sequence-specific differences in efficiency of cDNA synthesis, such differences can impact the estimates of mRNA molecules. In addition, there are a number of post-transcriptional mechanisms, including protein stability, that will clearly impact the cellular abundance and activity of Ets factors. Translational regulation of Ets factors is not a widely observed phenomenon but was reported for Pse (70) and isoforms of Fli1 (71). Post-translational modifications also regulate the transcriptional activity of many Ets family members, particularly through phosphorylation (reviewed in Refs. 7 and 72). Interactions with a variety of partner proteins further influence Ets factor activity (1, 2, 73). In the future, proteomic, DNA binding, and/or activity strategies combined with loss-of-function applications will be needed to complement this RNA expression profile of the Ets family.

Overall, this work represents a genomics analysis of a transcription factor gene family, in contrast to the traditional focus on only a few family members at a time. The studies described here have revealed for the first time which of the Ets factors are expressed in various mammary cell types. Insights from these studies included new observations revealing how broadly Ets factors are expressed, as well as the tissue-specific regulation, mRNA abundance, and tumor-specific changes in expression of each of the 25 Ets factors studied. In addition, correlation analysis of Ets factor expression and expression of endogenous Ets target genes has implicated specific Ets factors in the regulation of distinct Ets target genes whose products are involved in tumor progression. These correlations have generated testable predictions for conditional loss-of-function analysis (*e.g.* utilizing small interfering RNA) of the specific role of individual Ets factors in mediating breast cancer signaling. Together, our findings have provided a substantial increase in the understanding of Ets factor gene expression in normal breast tissue and tumors and form the basis for a more comprehensive understanding of the function of Ets family transcription factors.

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