Ets Gene PEA3 Cooperates with β-Catenin-Lef-1 and c-Jun in Regulation of Osteopontin Transcription*

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Osteopontin (OPN) is a multifunctional protein implicated in mammary development, neoplastic change, and metastasis. OPN is a target gene for β-catenin-T cell factor signaling, which is commonly disturbed during mammary oncogenesis, but the understanding of OPN regulation is incomplete. Data base-assisted bioinformatic analysis of the OPN promoter region has revealed the presence of T cell factor-, Ets-, and AP-1-binding motifs. Here we report that β-catenin, Lef-1, Ets transcription factors, and the AP-1 protein c-Jun each weakly enhanced luciferase expression from a OPN promoter-luciferase reporter construct, transiently transduced into a rat mammary cell line. OPN promoter responsiveness to β-catenin and Lef-1, however, was considerably enhanced by Ets transcription factors including Ets-1, Ets-2, ERM, and particularly PEA3. PEA3 also enhanced promoter responsiveness to the AP-1 protein c-Jun. Co-transfection of cells with β-catenin, Lef-1, PEA3, and c-Jun in combination increased luciferase expression by up to 280-fold and induced expression of endogenous rat OPN. In six human breast cell lines, those that highly expressed OPN also expressed PEA3 and Ets-1. Moreover, there was a significant association of immunocytochemical staining for OPN and one of β-catenin, Ets-1, Ets-2, PEA3, or c-Jun, in the 29 human breast carcinomas tested. This study shows that β-catenin/Lef-1, Ets, and AP-1 transcription factors can cooperate in a rat mammary cell line in stimulating transcription of OPN and that their independent presence is associated with that of OPN in a group of human breast cancers. These results suggest that the presence of these transcription factors in human breast cancer is responsible in part for the overexpression of OPN that, in turn, is implicated in mammary neoplastic progression and metastasis.

Osteopontin (OPN) is an acidic hydrophilic glycoprophosphoprotein that binds to cell surface integrins and may activate growth factor receptors (1). OPN transcription may be activated by the Ras oncogene (2) and plays a key role in neoplastic transformation, metastasis (3), and cancer progression (4). OPN is usually absent or expressed at a low level in normal tissues but is up-regulated in certain neoplastic and neoplastic epithelia (4–7), including that of the breast (8). Transfection of an expression vector for OPN induces malignant transformation and induction of metastasis in a benign rat mammary epithelial cell line (9), whereas transfection of OPN antisense cDNA inhibits these processes in a cell line already overexpressing osteopontin (10, 11). These results suggest that OPN overexpression may represent a key molecular event in tumor progression and metastasis, particularly that of the breast. Unlike many proto-oncogenes activated by a gain of function mutation, OPN is not typically mutated during stepwise tumorigenesis (3). Instead various responsive elements in its promoter regulate OPN expression for its diverse physiological roles (12–14), and it is presumably these elements that allow the overexpression of OPN in certain cancers.

β-Catenin is a component of the Wnt signal transduction pathway, implicated inter alia in initiation and progression of breast cancer (15, 16). β-Catenin translocates to the nucleus, where it binds to the Tcf/Lef family to initiate transcription of responsive genes (17–19). β-Catenin and OPN overexpression may coincide (20), and activated β-catenin may induce OPN expression in migrating cells (21). OPN may be a transcriptional target of the β-catenin-Tcf complex (22). Lef/Tcf factors are sequence-specific DNA-binding proteins that have a single high mobility group domain, located in the middle or near their C terminus (23). The ability of Lef-1 to regulate transcription may involve its association with different DNA-binding proteins (24–26). The OPN promoter also contains Tcf responses (27, and Ets transcription factor-binding domains (12). Mutagenesis of AP-1 or Ets sites separately impede OPN transcription in several cell lines and reporter-promoter systems, suggesting their involvement at this promoter (27). In a data base-assisted bioinformatics analysis, we identified three Lef-1/Tcf-binding sites (CAAGA) in addition to one AP-1-binding domain (TGAGTCA) and three Ets-binding motifs (AGGAAR) within the rat OPN promoter. Although Ets proteins bind to DNA recognition sites bearing a 5’-AGGA(A)3′ central core, flanking sequences influence their binding specificity (28). AP-1 and Ets proteins participate in transcriptional regulation of metastasis-associated matrix metalloproteinases (29) and synergize with β-catenin-Lef-1 in regulation of tumor-associated matrixins (30). Activation of transcription factors AP-1 and members of the Ets family may therefore represent key events in cell transformation (31). To test the hypothesis that β-catenin-Lef-1 synergizes with AP-1 and Ets in regulation of OPN expression, we have transfected Rama 37 rat mammary
epithelial cells with an OPN promoter-luciferase reporter construct and cDNA expression vectors for \(\beta\)-catenin, Lef-1, the AP-1 protein c-Jun, and for the Ets family of transcription factors, alone and in combination. PEAs3 (polyomavirus enhancer activator protein 3), an Ets family member, rendered the OPN promoter strongly responsive to transactivation by \(\beta\)-catenin/Lef-1 or c-Jun. Ets family members PEA3 and Ets-1 were overexpressed in human breast cancer cell lines, which highly expressed OPN. Moreover, Ets family members as well as c-Jun and \(\beta\)-catenin were highly expressed in the primary breast tumors that highly expressed OPN. We conclude that \(\beta\)-catenin, AP-1, and Ets pathways can synergize to provoke transcriptional up-regulation of OPN in a cultured rat mammary cell line. The co-incident expression of \(\beta\)-catenin, AP-1, Ets family members, and OPN in breast cancer cell lines and primary tumors suggest that they may be implicated in the dysregulation of OPN in breast cancer.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Oligonucleotides** — Expression vectors for human Ets-1 (32), Ets-2 (33), and mouse PEAs3 (34) within the expression vectors pcDNA3, pCMV5, and pSG5, respectively, were gifts of Dr. Eiji Harai (Paterson Institute for Cancer Research, Manchester, UK), with the permission of Prof. J. M. Leiden (Harvard University), Dr. C. A. Hauser (Scirps Research Institute), and Dr. Y. de Launoit (Pasteur Institute, Lille, France). Expression vectors for human c-Jun (35) and ERMI3 (30) within the expression vector pcDNA3 were purchased from ATCC (Manassas, VA). The expression vectors for human Lef-1 (36) in pCDNA3 were a gift of Prof. H. Clevers (University of Utrecht, Utrecht, Holland), and a stable mutant form of mutant \(\beta\)-catenin that lacks the N-terminal domain (37) in vector pCi-neo (Promega, Madison, WI) was a gift of Prof. B. Vogelstein (Johns Hopkins Oncology Center, Baltimore, MD). The synthetic 20-mer double-stranded oligonucleotides containing the wild type Lef-1-binding sites with sequences GGG TTA CAA AGA GTC CTC G and AGA CGA TCT AAA AGA CTT TA or a mutant Lef-1-binding site with the sequence AGA TCG ACC GCG GTA CGT TA were produced on a automated DNA synthesizer by Invitrogen. A 2.3-kilobase pair fragment of the 6-kilobase pair rat OPN promoter (38) was amplified by PCR and then cloned to a firefly luciferase reporter construct, as described previously (22, 39).**

**Mutagenesis of the osteopontin promoter** — The OPN promoter firefly luciferase reporter constructs with mutated Lef-1, AP-1, and Ets binding sites were generated in overexpression plasmids vector—directed mutagenesis kit (Stratagene, La Jolla, CA). A mutant of one Lef-1-binding site was generated in OPN at position 1954 to 1960 (Mut 1960) using TAT ACC TCC ATA ATT CGT GTC GAT TTA GAA AGA AAG and forward and reverse primers, respectively. The start of the 2285-bp promoter fragment is defined as the TATA box is in positions 28 to 23, and the start of the primary transcript from the OPN gene is at +1. A double mutant in two Lef-1-binding sites was generated at positions 1457 to 1463 in addition to the mutated site above (Mut 1960 to 1460), using GAT GCC TAG TAC CGG CGG TGG TGT ACT TAA TTC and GGA TTA TG ACAA C ACC CGG GGT CTA ATC GGC as forward and reverse primers, respectively. A triple mutant in three Lef-1-binding sites was generated at positions 1115 to 1121 in addition to the two mutated sites above (Mut 1960 to 1463) using CTT AAA GAT CAA CCG CGG TAC CTT ACA AAT C and GAT TTG TAA CGT ACC GCG GTC GAT TTA GAA AGA AAG and forward and reverse primers, respectively. The mutagenesis of the single AP-1-binding site was generated at positions 1722 to 1728 using TAT ACC TCC ATA ATT GCT GTC GAG TCG TCT CGT TCT GTC CGG GG and forward and reverse primers, respectively. The Ets binding sites was generated by mutagenesis at position 1954 to 1960 (Mut 1960 to 1460), using forward and reverse primers GTC AGT GTA AGT AGC AGT CAC CCG TGT CTA and GTC ACA GGA CTG ACT GCT TAC ACT CAG, respectively. A mutation of the second Ets-binding site was separately generated at position 1361 to 1357 (Mut 1357) using forward and reverse primers GAG CTA ATT GAA AAA AGG AAG TAA TTA CAG and GTC CAA TTA CTT CTT TTT AAT TAC CTG, respectively. A mutation of the third Ets-binding site was separately generated at position 1215 to 1211 (mut 1215) using forward and reverse primers CAC CCT ATG TAT TCT ATG AGG AAG TAC TAC and TTA GGT TTA TTA CAA TGT AGA AAG GGT, respectively.

**Northern Blotting** — Complementary DNA probes were generated by digestion of OPN, Ets-1, or PEAs3 expression vectors with EcoRI/KpnI, HindIII/NalI, or EcoRI restriction enzymes, respectively. Fifty ng of each purified cDNA probe was radioactively labeled using a random primed DNA labeling kit (Roche Applied Science) to a specific activity of 5 \(\times\) 10^8 cpm/\(\mu\)g. Total cellular RNA was isolated from the relevant cell lines using the RNeasy kit (Qiagen), and 10 \(\mu\)g of total RNA was run on a 1% (w/v) agarose denaturing formaldehyde gel. Nucleic acids were blotted onto nitrocellulose membranes by capillary transfer in 10× SSC buffer (1× SSC in 0.1 M NaCl plus 0.15 M NaCitrate) overnight. The probe was hybridized to the nitrocellulose filter at 42 °C for 1 h in low-stringency hybridization solution (Amersham Biosciences), washed high-stringency. The washed membrane was exposed to x-ray film (Fujifilm) for 24 h at 0 °C, using an intensifying screen. The result autoradiographs were analyzed by densitometry using a CS-9000 dual wavelength Flying Spot scanner (Shimadzu, Tokyo, Japan). The OPN, Ets-1, and PEAs3 densitometer readings were corrected for differences in the amounts of RNA loaded in each lane by normalizing them to the ratio of GAPDH mRNA reading for each lane (41, 42).

**Immunocytochemical Analysis** — Formalin-fixed, paraffin-embedded specimens from 29 patients with breast cancer were randomly selected from the group described previously (43), cut serially into 4-μm sections, mounted on three microscope slides, deparaffinized, and rehydrated. After staining of representative sections with hematoxylin and eosin, all of the specimens proved to be invasive breast carcinoma of no special type. For subsequent immunocytochemical staining for \(\beta\)-catenin, E-cadherin, and c-Jun, the sections were subjected to antigen retrieval by immersing them in 10 ppm of citrate buffer (pH 6.0) for 20 min. The slides were then incubated for a further 15 min in citrate buffer (pH 6.0) and stained using a 1× murine anti-c-Jun antibody (Santa Cruz, CA) and Sigma, respectively and have been characterized.
previously (22, 39). Rabbit polyclonal antibodies to Ets-1 (C-20, sc-350), Ets-2 (C-20, sc-351), c-Jun (9162), and MAb to PEA3 (sc-113) were purchased from Santa Cruz Biotechnology for three items and from New England Biolabs for c-Jun. All of these antibodies recognized the correct size antigens on Western blots of SDS-polyacrylamide gels. Blocking antigens for OPN, β-catenin, Ets-1, Ets-2, PEA3, and c-Jun, respectively, were human recombinant OPN (cc-1074), blocking peptide to β-catenin (sc-1496P), blocking peptide to Tcf-4 (sc-8631P), blocking peptide to Ets-1 (sc-350P), blocking peptide to Ets-2 (sc-351P), general blocking peptide for Ets family (sc-112P), and reticulocyte cell-free transcription translation protein synthesizing lysate primed with expression vector for PEA3 as described earlier under “Experimental Procedures” (40), and c-Jun fusion protein (6093). These items were purchased from Chemicon Europe (Chandlers Ford, UK) for OPN; from Santa Cruz Biotechnology Inc. (AutogenBioclear, Calne, Swindon, UK) for β-catenin, Tcf-4, Ets-4, Ets-2, and Ets family (PEA3); and from New England Biolabs (Hitchen, UK) for c-Jun.

Indirect immunocytochemistry was carried out using slightly different procedures of detection for different primary antibodies with a commercially available antibody biotin complex containing horseradish peroxidase, as described previously (45). The antibodies to β-catenin, Tcf-4, Ets-1, Ets-2, PEA3, c-Jun, and OPN were diluted to 1:20, 1:100, 1:500, 1:100, 1:10, 1:50, and 1:30, respectively. The slides from the 29 specimens were negatively stained; the results from two sections of each specimen with identical results in sections selected at random. Retrieval of antigens previously (8) increased the level of staining for β-catenin, Ets-1, Ets-2, and Tcf-4; for 16 h overnight at 4 °C prior to use. The slides from the 29 specimens were stained separately for all seven antibodies and analyzed independently by two observers using light microscopy. The specimens were recorded as staining positively for each antibody if >5% of the carcinoma cells/field were well stained, and the remaining specimens were classified as negatively stained; the results from two sections of each specimen with 10 fields/section at ×200 magnification were recorded, as described previously (8). Increasing the concentration of antibodies 5-fold gave identical results in sections selected at random. Retrieval of antigens (44) increased the level of staining for β-catenin, PEA3, and c-Jun but not for the other antibodies used. However, it failed to alter the observed subcellular distribution of any of the antigens under study. Photographs were recorded on a Reichert Polyvar microscope fitted with a Wratten 44 blue green filter (45). Testing for significance of the association of the staining for OPN and for each transcription factor in turn was undertaken using Fisher’s Exact test; the two-sided values of p were given.

RESULTS

Assay of Lef-1 Protein Binding to Tcf Motifs within the OPN Promoter.—The rat OPN promoter bears three consensus Tcf-binding sites (5′- (A/T)A/T/C/A/A/A/G-3′) between nucleotides −1960 and −1954 (TACAAG), between nucleotides −1463

FIG. 1 A, structure of the rat osteopontin promoter. Note the positions of the three consensus Tcf-binding sites, three Ets-binding sites, and one AP-1-binding site relative to the transcription start site (GenBank™ accession number AF017274). B, electrophoretic mobility shift assay for Lef-1 binding to oligonucleotides containing Tcf-binding sites. The synthetic double-strand 20-mer oligonucleotides containing the wild type first or second Tcf-binding sites (Wt −1960 and Wt −1463) were incubated with protein-synthesizing lysates programmed with expression vectors for Lef-1 or for Lef-1 and β-catenin (β-cat). The oligonucleotide with wild type Tcf site −1960 was incubated with unprogrammed lysate (Lysate). Also shown is a 20-mer oligonucleotide containing a mutated Tcf site (mut −1960) co-incubated with the same amount of lysate programmed by expression vector for Lef-1.
A mutant 20-mer oligonucleotide was also generated where the first two Tcf-binding sites present in the OPN promoter AACAAAG. These oligonucleotides have the same sequence as a 20-mer oligonucleotide containing the sequence TACAAAG or CTCAAAAG (see "Experimental Procedures"). The data are presented as fold induction of the OPN promoter-reporter activity relative to that for co-transfection of the promoter constructs with empty expression vectors. The values obtained were normalized by co-transfection of the same cells with constitutively active simian virus 40-driven Renilla luciferase. The data bars represent the means of experiments repeated a minimum of three times, and each transfection was performed in triplicate. The error bars represent the standard errors.

and −1457 (AACAAG), and between nucleotides −1121 and −1115 (TTCAAAAG). Nucleotide −1 fragment is at the start of the 2285-bp OPN promoter. The negative numbers represent nucleotides increasing from this point, read in a 3' direction. The primary transcript from the gene starts at nucleotide +1 (Fig. 1A). Lef-1 affinity for these binding sites with or without β-catenin was investigated in an electromobility shift assay. Lef-1 and β-catenin proteins synthesized by a reticulocyte cell-free transcription-translation-coupled protein synthesizing system ("Experimental Procedures") were incubated with a 20-mer oligonucleotide containing the sequence TACAAAG or AACAAG. These oligonucleotides have the same sequence as the first two Tcf-binding sites present in the OPN promoter (Fig. 1B). A mutant 20-mer oligonucleotide was also generated from the second Tcf-binding site bearing mutations known to diminish Tcf binding (CCGCGGT). The addition of Lef-1 protein lysate produced a slower running band than that caused by the radioactive oligonucleotide alone for either of the oligonucleotides containing the Tcf-binding sites. This result is consistent with the formation of a DNA-protein complex. Lef-1 protein lysates failed to produce a similar slower running band with the oligonucleotide containing the mutant Tcf-binding site, even at the highest protein lysate concentrations tested (Fig. 1B). The addition of β-catenin lysates produced a further slower running band for either of the oligonucleotides containing the Tcf-binding sites, the presence of which was dependent on the mixtures containing Lef-1 lysates (data not shown). These results are consistent with the formation of a ternary complex between the two proteins and either of the two 20-mer oligonucleotides containing the Tcf-binding site.

AP-1 and Ets Transactivation of the OPN Promoter—The OPN promoter has a canonical AP-1 site (5'-TGAGTCA-3') located between nucleotides −1872 and −1866 and candidate Ets-binding sites (5'-AGGA/A/T-3') located from nucleotides −2198 to −2194, −1361 to −1357, and −1215 to −1211 (Fig. 1A). To investigate the possibility of AP-1-mediated OPN transactivation, we co-transfected the Rama 37 cells with the 2.3-kilobase pair OPN promoter-luciferase reporter construct (OPN-Luc) and an expression vector for c-Jun, which when synthesized is capable of dimerizing to initiate AP-1 transcription factor activity (see "Experimental Procedures"). Luciferase reporter activity was enhanced by −6-fold by the addition of c-Jun lysates (Fig. 2). Previously determined optimal stimulating doses of mammalian expression vectors for Ets-1, Ets-2, PEA3, and its subfamily gene, ERM (200 ng/reaction) were co-transfected with OPN-Luc separately and in combination with the expression vector for c-Jun. Ets-1 and Ets-2 lysates enhanced OPN-promoter luciferase-reporter activity by approximately 5-fold and also further enhanced c-Jun-mediated activity approximately 2-fold (Fig. 2). The lysates containing PEA3 or ERM appeared more potent and enhanced OPN promoter-reporter activity by 7–8-fold, and they synergized with lysates containing c-Jun to enhance this activity by a further 5–6-fold. These latter combinations therefore caused a 38–44-fold increase above the activity of the OPN promoter-reporter alone (Fig. 2). These findings indicate a certain degree of preference of the PEA3 and ERM subclass of the Ets transcription factor family for the OPN promoter either alone or in combination with the AP-1 co-activator c-Jun.

Co-activation of β-Catenin-Lef-1-mediated OPN Promoter Activity by c-Jun or PEA3—Rama 37 cells were co-transfected with the expression vectors for c-Jun and for Lef-1 and/or β-catenin together with the OPN promoter reporter construct (OPN-Luc). Lysates containing β-catenin or Lef-1 were relatively weak individual or combined inducers of OPN promoter reporter activity. The combination of lysates increased the promoter activity by 9-fold (Fig. 3A). Co-transfection of the expression vector for c-Jun had only a relatively weak enhancing effect on β-catenin/Lef-1-mediated OPN promoter activity of approximately 1.3-fold, increasing the overall stimulation to
12-fold (Fig. 3). Co-expression of PEA3 with either Lef-1 or β-catenin increased the OPN promoter reporter activity by approximately 8-fold, whereas expression of PEA3, Lef-1, and β-catenin into 1 x 10^6 Rama 37 cells. The output of the OPN promoter was measured by the activity of the firefly luciferase reporter gene after 24 h (“Experimental Procedures”). The data are presented as fold induction of the OPN promoter reporter relative to co-transfection of the reporter with empty expression vectors. The values were normalized to those obtained with the SV40-Renilla luciferase internal. The data bars represent the means of four experiments, each performed in triplicate. The error bars represent the standard errors.

Activation of Endogenous OPN Expression by PEA3, c-Jun, β-Catenin, and Lef-1—Rama 37 cells that typically express OPN mRNA at a low level were transiently transfected with expression vectors for PEA3, c-Jun, β-catenin, and Lef-1, individually and in combination at the same optimal concentrations and for the same time period used in the OPN promoter-reporter assays. Stepwise increases in endogenous OPN mRNA were observed after transfection with combined expression vectors for PEA3/c-Jun with Lef-1 and with Lef-1/β-catenin (Fig. 3B). These increases corresponded to 2.5-, 5.2-, and 7.2-fold, respectively, over that produced endogenously after normalization for the expression of a constitutively expressed GAPDH mRNA.

c-Jun and β-Catenin-Lef-1 Act Independently to Synergize with PEA3—One possible explanation for synergy of β-catenin with PEA3 on the activity of the OPN promoter could involve up-regulation of expression of c-Jun by β-catenin (30). To address this possibility, we constructed an OPN promoter-luciferase reporter vector containing inactivating point mutations in the AP-1 site (mAP1-OPN-Luc). Mutant and wild type OPN-luciferase reporters were co-transfected with combination of PEA3, c-Jun, Lef-1, and β-catenin expression vectors into Rama 37 cells. Mutation of the AP-1 site did not alter basal OPN promoter reporter activity over that of the wild type.
promoter (Fig. 4). Expression vectors for PEA3, alone or in combination with those for Lef-1 and β-catenin, activated both the mutant (mAP1-OPN-Luc) and wild type OPN-luciferase reporter constructs to similar degrees (Fig. 4). However, no enhancement of the mutant reporter was observed by the expression vector for c-Jun, although c-Jun enhanced luciferase activity from the wild type OPN-Luc reporter, with PEA3 and Lef-1 alone or PEA3 with Lef-1 and β-catenin, by 17- and 14-fold, respectively (Fig. 4). Thus the effects of c-Jun upon mAP1-OPN-Luc activation were not additive to those of expression vectors for PEA3, alone or in combination with Lef-1 and β-catenin (Fig. 4). These results indicate that Lef-1/β-catenin synergy with PEA3 on OPN promoter reporter transactivation is independent of the expression of c-Jun.

β-Catenin-Lef-1 Synergize with PEA3/c-Jun through Tcf-binding Motifs, in the OPN Promoter—To investigate Tcf-binding motifs as potential β-catenin-Lef-1-responsive elements within the OPN promoter, we generated OPN-promoter luciferase-reporter constructs mutated at one Tcf recognition site (single mutation at position 1960/1463 Tcf, mTcf1960/1463-OPN-Luc), at two sites (double mutation at positions 1960/1463, mTcf1960/1463-OPN-Luc), and at three sites (triple mutation at positions 1960/1463/1115 Tcf, mTcf1960/1463/1115-OPN-Luc) promoter reporters, respectively. The values were normalized to those obtained with the SV40-Renilla luciferase internal control. The data bars represent the means of three experiments, each performed in triplicate. The error bars represent the standard errors.
OPN promoter reporter constructs was unaffected by expression vectors for PEA3 or c-Jun, alone or in combination (Fig. 5). However, the Lef-1 and Lef-1/β-catenin expression vector enhancement of PEA3/c-Jun-mediated OPN promoter-reporter activity was reduced by mutation of the Tcf-binding sites. Single mutation at position −1960 was associated with a decline of Lef-1 and Lef-1/β-catenin enhancement PEA3/c-Jun-mediated OPN promoter-reporter activity to 48% for Lef-1 alone (65-fold enhancement for mTcf −1960 versus 136-fold for wild type) and 46% for Lef-1/β-catenin (120-fold enhancement for mTcf −1960 versus 260-fold for wild type). The corresponding values for the Tcf double mutant versus wild type were 42% for Lef-1 (57-fold enhancement for mTcf −1960/−1463 versus 136-fold for wild type) and 29% for Lef-1/β-catenin (75-fold enhancement for mTcf −1960/−1463 versus 260-fold for wild type). Mutation of all three Tcf-binding sites in the OPN promoter effectively abolished Lef-1 and Lef-1/β-catenin enhancement of PEA3/c-Jun-mediated OPN promoter-reporter activity (Fig. 5). These results indicate that β-catenin and Lef-1 cooperate independently with c-Jun/PEA3 to transactivate the OPN promoter.

Combination PEA3, c-Jun, and β-Catenin-Lef-1 Activation of the OPN Promoter Involves Specific Ets-binding Motifs—To investigate the role of each of the three Ets-binding sites in combinatorial PEA3/c-Jun/Lef/β-catenin regulation of OPN promoter activity, we generated a series of OPN promoter luciferase-reporter constructs with inactivating point mutations in each Ets-binding domain, at positions −2198, −1361, and −1215. Single mutations at any of the three Ets sites produced no dramatic effect upon PEA3 responsiveness of the OPN promoter (Fig. 6). The expression vector for PEA3 enhanced promoter activity in each Ets mutant OPN-promoter-luciferase reporter by 5–10-fold compared with 8-fold for the wild type OPN promoter reporter (Fig. 6). However, mutation of the first Ets site (position −2198), effectively abolished c-Jun enhancement of OPN promoter activity, mediated by PEA3 alone or in combination with Lef-1 or Lef-1/β-catenin (Fig. 6). Mutations in the Ets-binding domains at positions −2194 and −1215 impaired c-Jun cooperation with PEA3, to a lesser degree (Fig. 6). For example expression vectors for PEA3 and for c-Jun together induced 26- and 22-fold increases in promoter activity from mutant Ets −2194 and Ets −1215 OPN-promoter luciferase reporters compared with a 39-fold increase for the wild type promoter reporter construct (Fig. 6). These results suggest that PEA3 binding to the Ets domain is central to PEA3/c-Jun cooperation on the OPN promoter and that transcriptional efficiency may be related to the proximity of activated domains (Fig. 1A). Mutation of any of the three Ets sites (positions −2198, −1361, or −1215) impaired Lef-1 and β-catenin synergy with PEA3 in enhancing the activity of the OPN promoter. Thus expression vectors for PEA3 in combination with those for Lef-1 and for Lef-1/β-catenin enhanced promoter activity 9–16-fold in Ets mutant promoters compared with the 16–25-fold increase induced by the wild type OPN promoter-reporter construct (Fig. 6).

Expression of OPN, PEA3, and Ets-1 in Benign and Malignant Breast Cells—We examined the expression of OPN, PEA3, and Ets-1 transcripts in total RNA isolated from human benign and malignant breast cells by Northern analysis. PEA3, Ets-1, and OPN were weakly expressed in all benign breast cells.
tested (Huma 7, Huma 109, and Huma 123). Conversely, coincident high expression of OPN, PEA3, and Ets-1 was observed in malignant breast cells (ZR-75, T47-D, and MDA-MB231) (Fig. 7).

**Immunocytochemical Staining of Human Breast Cancers for Transcription Factors and OPN**—Nearly adjacent histological sections from specimens from 29 patients with primary invasive breast carcinoma were immunocytochemically stained for OPN and for different transcription factors. All of the antibodies used against OPN and the transcription factors stained histological sections from some of these breast carcinomas (Figs. 8, A, C, and E, and 9, A, C, E, and G). The specificity of the antibodies used against OPN (Fig. 8, A and B), β-catenin (Fig. 8, C and D), Tcf-4 (Fig. 8, E and F), Ets-1, (Fig. 9, A and B), Ets-2 (Fig. 9, C and D), PEA3 (Fig. 9, E and F), and c-Jun (Fig. 9, G and H) was tested by their prior incubation with the requisite antigen described under “Experimental Procedures.” Immunocytochemical staining was suppressed completely by this treatment (Figs. 8, B, D, and F, and 9, B, D, F, and H) but not with antibodies incubated with noncognate antigens (not shown), demonstrating that the antibodies were specific. Staining of the carcinoma cells for OPN and β-catenin was predominantly cytoplasmic, although both membranous and nuclear staining was observed for the latter but not the former antigen (Fig. 10, A and B). Specific staining for OPN was also observed in the cytoplasm of reactive stromal cells/lymphocytes as previously reported (8). Staining for Ets-1, Ets-2, PEA3, and c-Jun varied from nuclear to cytoplasmic depending both on the identity of the transcription factor and the specimen (Fig. 10, C–F).

Staining for Tcf-4 was mainly nuclear (not shown). The samples were considered positively stained if more than 5% of the carcinoma cells were stained strongly by the requisite antibody (“Experimental Procedures”). The percentage of positively stained samples for β-catenin, Tcf-4, Ets-1, Ets-2, PEA3, c-Jun, and OPN were ~71, 35, 78, 78, 63, 78, and 66%, respectively. Staining for OPN was significantly correlated with that for β-catenin, Ets-1, Ets-2, PEA3, and c-Jun (p < 0.001) but not with that for Tcf-4 (p = 0.36) when cross-tabulated and assessed using Fisher’s Exact test (“Experimental Procedures”) (Table I). Similarly there was a significant correlation for samples staining positively for any one of the transcription factors in comparison with another (p = 0.01), except only a borderline correlation of staining for β-catenin and c-Jun (p = 0.06). No significant correlation between staining for Tcf-4 and any fac-

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**Fig. 8.** Specificity of immunocytochemical staining of human breast cancers for OPN, β-catenin, and Tcf-4. A and B, sections from the same area of a tumor specimen incubated with either MAb to OPN (A) or MAb preabsorbed with OPN (B). C and D, sections from a similar area of a tumor specimen incubated with either MAb to β-catenin (C) or MAb preabsorbed with β-catenin blocking peptide (D). E and F, sections from a similar area of a specimen incubated with either MAb to Tcf-4 (E) or MAb preabsorbed with Tcf-4 blocking peptide (F). Positive immunocytochemical staining in A, C, and E is abolished in B, D, and F. Magnification, ×230; bar, 50 μm.
tor (p \leq 0.19) was observed. Within the same specimens, immunocytochemical staining for β-catenin and OPN; for Ets-1, Ets-2, and OPN; and for PEA3, c-Jun, and OPN (not shown) occurred in similar areas, often in the same cells. This was not the case for staining for Tcf-4 and OPN (not shown). When staining for four or more of β-catenin, Ets-1, Ets-2, PEA3, and c-Jun was positive in the same specimen, that specimen was also positive for OPN. When staining for three or less of the same transcription factors occurred in the same specimen, that specimen remained unstained for OPN (Table II).

**DISCUSSION**

A number of mitogenic molecules including growth factors (48), oncogenes (49), and tumor promoters (10) induce the expression of OPN, which has a key role in metastasis (9) and prognosis of breast cancer (45). The understanding of regulatory mechanisms for transcription of OPN is incomplete. Studies on the mouse or human OPN promoter has revealed a highly modular structure (50) involving four regions, as follows: (i) A nuclear receptor and a Ras-responsive region encoded by the OPN promoter, nucleotide sequence \(-1000\) to \(-550\) (2); peroxisome proliferator-activated receptor γ (51), vitamin D receptor (13), and estrogen receptor-related receptor-α (52, 53) all bind to elements in this region. (ii) A regulatory region relevant to osteoblast differentiation that maps to the OPN promoter region nucleotides \(-220\) to \(-110\) and is regulated by Polyoma enhancer-binding protein, ZnA/core-binding

**Fig. 9.** Specificity of immunocytochemical staining of human breast cancers for Ets-1, Ets-2, PEA3, and c-Jun. A and B, sections from the same area of a specimen incubated with either rabbit polyclonal antibody to Ets-1 (A) or antibody preincubated with blocking peptide to Ets-1 (B). C and D, sections from a similar area of a specimen incubated with either rabbit polyclonal antibody to Ets-2 (C) or antibody preincubated with blocking peptide to Ets-2 (D). E and F, sections from the same area of a specimen incubated with either MAb to PEA3 (E) or MAb preincubated with reticulocyte protein-synthesizing lysate primed with an expression vector for PEA3 (F). Preincubation with the general blocking peptide for the Ets family gave the same results. G and H, sections from a similar area of a specimen incubated with either rabbit antibody to c-Jun (G) or antibody preincubated with c-Jun fusion protein (H). Positive immunocytochemical staining in A, C, E, and G is abolished in B, D, F, and H. Magnification, \(\times 230\); bar, 50 μm.
factor, and Ets-1 (12), and bone morphogenetic protein simulated through an interaction with a homeodomain transcription factor, Hox-8, and Smad1 (BMP2-regulated Hoxc8:Smad1) (54). (iii) A basal promoter region that maps to nucleotides −107 to +78 relative to the start site. (iv) Enhancer element +799 to +864 that supports Sox2:Oct4-regulated expression (55). Cooperation between factors interacting at multiple sites may be implicated in context-specific OPN gene expression.

We have previously shown the responsiveness of the rat OPN promoter to Tcf components of the β-catenin signaling pathway (22). AP-1 and the Ets transcription family influence OPN expression (2, 12, 14, 27) and have oncogenic potential in mammary epithelium (56–58). In a data base-assisted bioinformatics analysis of the rat OPN promoter, we have found three Tcf-binding sites at nucleotide positions −1960 to −1954, −1463 to −1457, and −1121 to −1115 and three Ets-binding domains at nucleotide positions −2198 to −2194, −1361 to −1357, and −1215 to −1211, and an AP-1 domain was confirmed at position −1872 to −1866. We have previously shown binding of Tcf-1 and Tcf-4 to an oligonucleotide representing the Tcf-binding site at nucleotide positions −1121 to −1115 (40). Here, we report assembly of Lef-1: DNA complexes and co-localization of β-catenin with Lef-1 and the two other Tcf-binding domains at nucleotide positions −1960 and −1463. Mutagenesis of the Tcf domain at nucleotide −1960 effectively blocked its binding to Lef-1.

To assess AP-1 and/or Ets regulation of OPN transcription, we performed co-transfection experiments of a wild type OPN promoter-luciferase reporter construct with expression vectors for c-Jun, Ets-1, Ets-2, PEA3, and/or the Ets subfamily gene, ERM alone, or in the combinations outlined under “Experimental Procedures.” Individually, each of the Ets factors enhanced luciferase activity of the OPN promoter reporter construct and all synergized with c-Jun. PEA3 and ERM showed the largest increase in activity within the Ets family for the OPN promoter, whether alone or in combination with c-Jun.

Functional synergy between PEA3, the AP-1 activator protein c-Jun, Lef-1, and β-catenin for promoter activity was investigated with the wild type OPN promoter-linked luciferase reporter. We found that PEA3 had a modest co-activator function with β-catenin-Lef-1. However, co-expression of c-Jun with PEA3 and either Lef-1 or β-catenin enhanced luciferase expression from the promoter by 140- and 180-fold, respectively, whereas a large 280-fold stimulation of promoter activity was
observed by co-transfection of PEA3, c-Jun, β-catenin, and Lef-1. Luciferase reporter systems provide useful information concerning transcriptional regulation of a gene of interest, although their quantitative output may be influenced by transfection efficiency, translation, and activity of the reporter protein, as well as transcription from the promoter. Confirmatory assays of endogenous gene transcription can lend weight to findings. In this study, we show that the increases in the levels of endogenous OPN mRNA triggered by the various combinations of expression vectors reflect the increases in OPN promoter-reporter activity. However, the overall combination of expression vectors for PEA3, c-Jun, β-catenin, and Lef-1 that induced a maximum increase in OPN promoter-reporter activity of 280-fold induced only a maximum increase in endogenous OPN mRNA of 7.2-fold. This difference could be a reflection of differences in and/or stability of OPN mRNA.

To investigate the specific role of AP-1 factors in this collaborative regulatory network, the activity of the OPN promoter luciferase reporter construct bearing an inactivating mutation at the AP-1 site (mAP-OPN-Luc) was tested. Mutational inactivation of the AP-1 site had no effects on transactivation on the OPN promoter by PEA3 alone or in combination with Lef-1 or Lef-1 and β-catenin. These results do not support functional overlap between AP-1, PEA3, or β-catenin/Lef-1 on the activity of the OPN promoter. However, this mutation abolished synergy of c-Jun with PEA3 alone, with PEA3/Lef-1, and with PEA3/Lef-1/β-catenin on the activity of the OPN promoter. AP-1, Ets, and Tcf transcription factors may share some common co-activator proteins (59, 60). Conceivably, protein binding to an intact AP-1 site may enable recruitment of co-activators for Ets- and Tcf-mediated OPN promoter activity.

The influence of identified Tcf-binding sites on OPN promoter-reporter transactivation was next investigated. Mutations at the defined Tcf-binding sites had no effect upon OPN promoter-reporter responsiveness to PEA3 or c-Jun, alone or in combination. However, Lef-1 synergy with these transcription factors for transactivation of the OPN promoter-reporter was affected to a variable degree. Mutation at all three Tcf-binding sites effectively abolished Lef-1 synergy on promoter activity, in co-transfection experiments. Of necessity, mutations at position −1960 and −1463 invoked major changes to the spatial organization between the single AP-1 site and the next adjacent Tcf-binding motif. The distance between these binding domains increased from −100 bp in the wild type OPN promoter to −400 and 700 bp in the single −1960 and double −1960/−1463 mutants, respectively. Conversely, the maximum distance between any single Tcf- and Ets-binding domain remained relatively constant in the single and double mutants, for either transcription factor-binding site (Fig. 1A). However, the three pairs of Tcf- and Ets-binding sites lay within 240-bp proximity in the wild type promoter. Single −1960 and double −1960/−1463 mutants of the Tcf-1-binding site had only two and one pair separated by this distance or less, respectively. PEA3/Lef-1 synergy on the activity of the OPN promoter, although diminished, remained detectable in −1960 single and double −1960/−1463 mutants. Conversely, Lef-1 synergy with c-Jun/PEA3 and c-Jun/PEA3/β-catenin on promoter activity was inhibited to a greater degree in single and double Tcf-binding site mutants of the OPN promoter. Differential effects of these mutations upon Tcf/Ets and Tcf/AP-1 synergy on the activity of the OPN promoter may be related to the positional relationships between Tcf, AP-1, and Ets-binding domains of this promoter. These studies have also shown that β-catenin synergy with PEA3/c-Jun/Lef-1 on the activity of this promoter is mediated through the identified Tcf sites and not through any cryptic Tcf domains, as has been reported for other promoters (61).

Unlike many other families of transcription factors, the Ets family does not appear to associate as homo- or heterodimers (62) and by themselves may display only weak transactivation properties. Instead, the Ets family may form complexes with unrelated factors to initiate or enhance transcription (63). Interactions between Ets factors and AP-1 proteins have variable

### Table I

**Association of immunocytochemical staining of breast carcinomas for OPN with that for individual transcription factors**

Histological sections from samples of invasive ductal carcinomas of the breast were immunocytochemically stained for β-catenin, Tcf-1, Ets-1, Ets-2, PEA3, c-Jun, and OPN as described under "Experimental Procedures." The samples where the percentage of stained carcinoma cells was >5% are recorded as positive (+), and the rest are recorded as negative (-). The statistical significance of correlated staining between paired samples was obtained from Fisher’s Exact test (two-sided value), where a probability of $p < 0.05$ was considered significant (*Experimental Procedures*).

| Transcription factor | OPN-negative | OPN-positive | Statistical significance |
|----------------------|--------------|--------------|-------------------------|
| β-Catenin -          | 7 (78)       | 1 (5)        | <0.001                  |
| β-Catenin +          | 2 (22)       | 18 (95)      | 0.36                    |
| Tcf-4 -              | 6 (86)       | 11 (58)      | <0.001                  |
| Tcf-4 +              | 1 (14)       | 8 (42)       | 0.001                   |
| Ets-1 -              | 6 (75)       | 0 (0)        | <0.001                  |
| Ets-1 +              | 2 (25)       | 19 (100)     | 0.001                   |
| Ets-2 -              | 6 (75)       | 0 (0)        | <0.001                  |
| Ets-2 +              | 2 (25)       | 19 (100)     | <0.001                  |
| PEA3 -               | 8 (100)      | 2 (11)       | 0.001                   |
| PEA3 +               | 0 (0)        | 17 (89)      | 0.001                   |
| c-Jun -              | 6 (75)       | 0 (0)        | <0.001                  |
| c-Jun +              | 2 (25)       | 19 (100)     | <0.001                  |

### Table II

**Association of immunocytochemical staining for multiple transcription factors with that for OPN in breast carcinomas**

Immunocytochemical staining of breast carcinomas for β-catenin, Tcf-4, Ets-1, Ets-2, PEA3, c-Jun, and OPN as described under "Experimental Procedures." Those sections where the percentage of stained carcinoma cells was >5% are recorded as positive (+), and the remainder are recorded as negative (-) or not determined (ND).

| Patient number | β-Catenin | Tcf-4 | Ets-1 | Ets-2 | PEA3 | c-Jun | OPN |
|----------------|-----------|-------|-------|-------|------|-------|-----|
| 1              | +         |       | +     | +     | +    | +     | +   |
| 2              | +         | +     | +     | +     | +    | +     | +   |
| 3              | +         | +     | +     | +     | +    | +     | +   |
| 4              | +         | +     | +     | +     | +    | +     | +   |
| 5              | +         | +     | +     | +     | +    | +     | +   |
| 6              | +         | +     | +     | +     | +    | +     | +   |
| 7              | +         | +     | +     | +     | +    | +     | +   |
| 8              | +         | +     | +     | +     | +    | +     | +   |
| 9              | +         | +     | +     | +     | +    | +     | +   |
| 10             | +         | +     | +     | +     | +    | +     | +   |
| 11             | +         | +     | +     | +     | +    | +     | +   |
| 12             | +         | +     | +     | +     | +    | +     | +   |
| 13             | +         | +     | +     | +     | +    | +     | +   |
| 14             | ND        | ND    | ND    | ND    | ND   | ND    | ND  |
| 15             | +         | +     | +     | +     | +    | +     | +   |
| 16             | +         | +     | +     | +     | +    | +     | +   |
| 17             | +         | +     | +     | +     | +    | +     | +   |
| 18             | +         | +     | +     | +     | +    | +     | +   |
| 19             | +         | +     | +     | +     | +    | +     | +   |
| 20             | +         | +     | +     | +     | +    | +     | +   |
| 21             | ND        |       |       |       |      |       |     |
| 22             | +         | +     | +     | +     | +    | +     | +   |
| 23             | +         | +     | +     | +     | +    | +     | +   |
| 24             | +         | +     | +     | +     | +    | +     | +   |
| 25             | +         | +     | +     | +     | +    | +     | +   |
| 26             | +         | +     | +     | +     | +    | +     | +   |
| 27             | +         | +     | +     | +     | +    | +     | +   |
| 28             | +         | +     | +     | +     | +    | +     | +   |
| 29             | ND        |       |       |       |      |       |     |
effects upon transcription (64). In the present study, muta-
tional analysis was carried out to investigate the role of each
Ets motif in PEA3-mediated transactivation of the OPN promoter,
alone or in combination with other factors. PEA3 com-
bined with c-Jun stimulated the activity of the wild type OPN
promoter reporter by an additional 5.5-fold, over that with
PEA3 alone (Fig. 2). No single mutation at any Ets site abol-
ished OPN transactivation by PEA3 alone or in combination
with other factors. Mutation at the −2198 Ets-binding site,
which was closest to the AP-1 site, had the greatest inhibitory
effect upon PEA3−, PEA3/c-Jun−, or PEA3/c-Jun/Lef-1medi-
ated OPN transactivation. For example, the combination of
PEA3 and c-Jun transactivated the OPN promoter-reporter of
each of the single Ets-binding site mutants −2198, −1361, and
−1215 by approximately 0, 2.5-, and 3-fold, respectively, over
that with PEA3 and by approximately 0, 4.5-, and 5-fold,
respectively, over that with PEA3 and Lef-1, in comparison
with stimulation of the wild type promoter (Fig. 6). Thus the
Ets-binding site closest to the AP-1-binding domain appeared
to exert the greatest effect on the activity of the OPN
promoter, by c-Jun or c-Jun/Lef-1 transactivation. One of the
most intriguing findings was that mutation at any one of the Ets-
binding sites was associated with a substantial inhibition of
cooperative PEA3/c-Jun/Lef-1 or PEA3/c-Jun/Lef-1/β-catenin
transactivation of the OPN promoter reporter construct. Thus
full occupancy of all Ets sites within the OPN promoter is prob-
able essential for maximal synergy between these cis-acting
regulatory elements, in activation of the OPN promoter.

In addition to osteopontin, other genes are co-regulated by
various combinations of PEA3, β-catenin/Tcf, and AP-1 tran-
scription factors. Cross-coupling of different signaling path-
ways through these cis-acting regulatory domains influences
expression of TWIST transcription factor (65), which inhibits
mammary cell differentiation and expression of matrilysin,
a member of the matrix metalloproteinase family (matrix metal-
loproteinase 7) (30). Co-incident expression of OPN and matrix
metalloproteinase has been reported in conditions of tissue
injury or wound repair (66) and tumorigenesis (67), whereas
matrix metalloproteinase 7 may be co-expressed with OPN in a
remodeling tissue, like the postpartum uteruus (68, 69). OPN is
proteolytically cleaved by matrilysin with a resulting increase
expression of the matrix metalloproteinase matrilysin (72), another
protein associated with tumor invasion and metastasis. The
overexpression of transcription factors AP-1 (73) and mem-
bers of the Ets (58) (73–75) family as well as β-catenin/Tcf
(76) may reflect selection in some tumors for those cells
able of mounting a coordinated response to increase the
levels of proteins important for successful development of the
cancer.

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Ets Gene PEA3 Cooperates with β-Catenin-Lef-1 and c-Jun in Regulation of Osteopontin Transcription
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