Pea3 Transcription Factor Cooperates with USF-1 in Regulation of the Murine bax Transcription without Binding to an Ets-binding Site*

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The Pea3 transcription factor (which belongs to the PEA3 group) from the Ets family has been shown to be involved in mammary embryogenesis and oncogenesis. However, except for proteinases, only few of its target genes have been reported. In the present report, we identified bax as a Pea3 up-regulated gene. We provide evidence of this regulation by using Pea3 overexpression and Pea3 silencing in a mammary cell line. Both Pea3 and Erm, another member of the PEA3 group, are able to transactivate bax promoter fragments. Although the minimal Pea3-regulated bax promoter does not contain an Ets-binding site, two functional upstream stimulatory factor-regulated E boxes are present. We further demonstrate the ability of Pea3 and USF-1 to cooperate for the transactivation of the bax promoter, mutation of the E boxes dramatically reducing the Pea3 transactivation potential. Although Pea3 did not directly bind to the minimal bax promoter, we provide evidence that USF-1 could form a ternary complex with Pea3 and DNA. Taken together, our results suggest that Pea3 may regulate bax transcription via the interaction with USF-1 but without binding to DNA.

Pea3, also called E1AF or ETV4, is the founding member of a subfamily of ets genes that includes Er81 (or ETV1) and Erm (or ETV5), which have been currently characterized in mice (1–3), humans (4–6), rats, dogs (Telgman, GenBank™ accession number AJ313194), chicken (7), zebrafish (8), and amphipath Xenopus (9). These three factors share three functional highly conserved domains: a DNA binding domain (10), an amino-terminal transactivation domain (11), and a carboxy-terminal domain involved in DNA binding and transactivation regulation (12, 13).

These three PEA3 group members are co-expressed in several tissues and organs (3, 14, 15) and are generally described as transactivators (11, 16). Their role and function are not precisely known, but deregulation of their expression is often associated with carcinogenesis (17, 18).

Numerous studies (11, 16–24) have revealed the involvement of the three factors in mammary oncogenesis, since their overexpression is observed in certain human breast cancers and in oncogene-induced mammary tumors. Pea3 ectopic overexpression in nonmetastatic human breast cancer cells increased their invasiveness and their metastatic potential in nude mice (25). Recent data confirmed the role of Pea3 in breast cancer tumorigenesis and suggest that Pea3 is a marker of tumor aggressiveness rather than a prognostic factor (26). Moreover, we have shown that Erm expression is an adverse prognostic factor for overall survival in breast cancer patients (27).

The PEA3 group members are also expressed in different stages of normal mammary gland development, from embryonic emergence to post-natal evolution (3, 14, 15), with a high level during extensive ductal outgrowth and branching, i.e. puberty and early pregnancy. Moreover, overexpression of Erm and Pea3 in a normal mouse mammary cell line confers an autonomous capacity of branching morphogenesis (15), thus supporting their role in normal mammary embryogenesis and tumor evolution.

Very few PEA3 group target genes have been currently reported. Most of them encode proteinases required for extracellular matrix degradation, such as MMP-1, MMP-9, and MMP-3 (28, 29), MT1-MMP (30), and MMP-7 (31, 32) or adhesion molecules such as Icam-1 (33). Osteopontin (opn) (24, 34), cyclooxygenase-2 (cox-2) (21, 35), p21wat1 (36), heparanase (37), neu (38), Muc4-sialomucin (Muc4/SMC) complex (39), and glutathione peroxidase (gpx) (40) are also targets of the PEA3 group members. For all these target genes, PEA3 factors act as transactivators except for neu and gpx for which a repression has been reported (38, 40).

Bcl-2 family members are key proteins that turn on the apoptotic cascade. They are programmed cell death regulators divided in two groups as follows: inhibitors of apoptosis, which comprise Bcl-2 and Bcl-XL; and activators of apoptosis, which comprise Bax and Bcl-XS. Some Bcl-2 family members are known to be target of the Ets family: bcl-x is activated by Ets-2 and PU-1 (41) and repressed by Tel (42), or bcl-2 which is regulated by Fli-1 (43).

Here we report the regulation of the murine bax gene by the PEA3 group members in normal mammary cells. In these cells, overexpression of Erm and Pea3 results in elevated bax mRNA and protein levels, which can be down-regulated by Pea3 silencing. We found that Pea3 regulates the minimal bax promoter, not by binding to a consensual Ets-binding site but via
the interaction with USF-1, which binds to the functional E boxes of the promoter.

MATERIALS AND METHODS

DNA Array Analysis—Total RNA was isolated from control or PEA3 group members overexpressing TAC-2.1 cells, described previously (1). Chouette-Leveque et al. (15). 5 μg of RNA was used to convert total RNA into 32P-labeled first strand cDNA using [α-32P]dATP and the gene-specific CDS primer mix for the Atlas array (Clontech) following the manufacturer’s recommendation. The labeled cDNA was purified using a Nucleospin Column (Clontech). A set of Mouse Atlas Array (Clontech) was used for hybridization with the labeled probes using Express Hyb solution at 68 °C for 16 h. The arrays were exposed to a Phospho-Imager and analyzed using Quantity One software (Bio-Rad).

Reverse Transcription (RT)1-PCR—Total RNA was extracted from cells using TriReagent™ (Euromesix) as described by the manufacturer. cDNA was synthesized from 2 μg of RNA using Omniscript Reverse Transcriptase (Qiagen) with oligo(dT) (Invitrogen) priming. PCR was performed using specific primers of the bax gene (sense, 5’-AGGCTGACCCCTGCCTTCCGGC-3’) and antisense, 5’-GCCA-

CAAAGATGTTGACCGTGG-3’), the pea3 gene (sense, 5’-CACA-

GATGTACGCTTCCGG-3’), and antisense, 5’-AGGGGCAACAGGG-

GAGCTG-3’), the opn gene (24) and the cyclinH A gene (sense, 5’-GCATCACGGCTCCTGGCTAATGGC-3’), and antisense, 5’-AGT-

GGATCTTCTTGCTGGTCTTG-3’), the acidic ribosomal phosphopro-

tein P0 gene (sense, 5’-CTCAAGACATTTCCCCCTTCCG-3’), and the actin gene (sense, 5’-GTTGGGCGCCGCCAGGACCA-3’), and anti-

sense, 5’-CTCTTAAATGTCAGCCAGTTCC-3’), as internal controls. 1/20 cDNA was used for the PCRs containing 2 mM MgCl2, 0.2 mM dNTP, 0.2 μM of each primer, and 1 unit of TaqDNA polymerase (MBI Fermentas). 30 cycles were carried out in a MasterCycler (Eppendorf) as follows: denaturing at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min.

Plasmids and Antibodies—The coding sequences for erm, er81, and pea3 were amplified with oligonucleotides containing NotI sites by a high fidelity DNA polymerase (Pfu DNA Polymerase, Promega). The NotI-digested PCR-derived fragments were then cloned in the NotI-linearized pTRACER plasmid vector (Invitrogen), and recombinant pTRACER constructs were sequenced. Oligonucleotide primers were referred to as pTRACER-erm, pTRACER-er81, and pTRACER-pea3, respectively. The full-length promoter construct of the murine bax gene as well as its deletion and Sp1 site mutants and the empty luciferase reporter vector were obtained from Prof. T. Sakai (44). The pCR3-USF-1, pCR3-USF-2a, and the dominant negative pCR3-TDUFS-1 expression vectors, pk-

cuc96 and pk-luc96, luciferase reporter vector, were 25 ng of the pSV-β-galactosidase vector (Promega), and if necessary, salmon sperm DNA, and 2 μl of polyethyleneimine. Cells were prepared 24 h prior to the luciferase (Promega) and β-galactosidase (Galactolight, Tropix Inc.) activities following the manufacturer’s instructions and using a Lumat 9507 Luminometer (Berthold). The measured luciferase activity was corrected by using co-transfected pSV-β-galactosidase activity as an internal control.

Site-directed Mutagenesis—Mutations of the E box binding sites were performed on PMBaxSpIId by using the QuikChange site-directed mutagenesis kit (Stratagene). 10 ng of PMBaxSpIId was combined with complementary mutant oligonucleotide primers (0.3 μM), 1 μl dNTP and 2.5 units of PfuTurbo DNA polymerase. The oligonucleotide primers used were as follows (sense strand): E box m1, 5’-TTCCGGGCGAC-

CAGTTGAGGCCGACAGCTCAGCTCAGCTACACAGG-3’; E box m2, 5’-GGCTCAATGCTCGTTTGTAGGACTG-3’; and E box m12, 5’-TTCGCGGCGAC-

CGATAGCGCCGGCCGACAGCTCAGCTCAGCTACACAGG-3’. The underlined letters represent the bases that were mutated in the E box binding sequence. The reaction mixture was temperature-cycled in a MasterCycler (Eppendorf) to amplify the mutation constructs. Cycling conditions were 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 10 min for 12 cycles. Mutation 3 on PMBaxSacII was performed on PMBaxSacII first mutated on the first and second E boxes by using the following oligonucleotide primer 5’-GAGCGATGATGATC-

AATTGACTAGTTCTCGGC-3’.

Nuclear Protein Extracts—Cells were harvested, pelleted, and resuspended in an hypotonic lysis buffer (10 mM HEPES, 10 mM KCl, 2 mM MgCl2, 0.1 mM EDTA, 10 μg/ml aprotinin, 0.5 μg/ml leupeptin, 3 mM phenylmethylsulfonyl fluoride, 3 mM dithiothreitol) and incubated on ice for 10 min. The homogenate was then centrifuged at 10,000 × g for 5 min. The precipitated pellet was suspended in 10 mM HEPES, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1% glycerol (v/v), 3 mM dithiothreitol, 3 mM phenylmethylsulfonyl fluoride) and placed on ice for 30 min. Nuclear extracts were collected by centrifugation for 10 min at 10,000 × g, quantified by the Bradford method (Bio-Rad), aliquoted, and stored at −80 °C.

Gel Mobility Shift Assay—The annealed double strand oligonucleotide derived from the bax minimal promoter (bax wt) was 5’-end labeled by [α-32P]ATP using T4 polynucleotide kinase (Invitrogen). Binding of the protein to DNA sequences was achieved in a 20-μl mixture containing 2× buffer (50 mM HEPES, 50 mM KCl, 2 mM EDTA, 4 mM MgCl2, 0.1% Nonidet P-40 (v/v), 20% glycerol (v/v), 1.5 μg of salmon sperm DNA, 1 μg of poly(dI-dC), 1 mM dithiothreitol, 12 μg of nuclear cell extract, and 60,000 cpm of the labeled probe. For competition assays, unlabeled oligonucleotide was added at 400-fold molar excess. Supershift assays were performed by adding anti-Pea3, anti-Pim-1, or anti-USF-1 antibody to the mixture. After incubation at room temperature for 20 min, samples were loaded onto a 6% denaturing polyacrylamide gel and run in 0.5× Tris borate-EDTA running buffer. The gel was dried and exposed to x-ray film (Kodak) overnight at −80 °C.

The following oligonucleotides were used as probes or competitors (sense strand): bax wt, 5’-CACCCACGTGGAGGCCGGCCGACAGCTCAGCTCAGCT-3’; nonspecific, 5’-GGGGCCACGCGGGAGAGGGCGAGCAGGGACACT-3’; and bax mt, 5’-TTCGCGGCGAC-

CGATAGCGCCGGCCGACAGCTCAGCTCAGCTACACAGG-3’.

Cell lysis buffer—Cells were lysed in a 150 mM NaCl, 50 mM Tris-

HCl, pH 7.5, 1% Nonidet P-40 (v/v), 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin buffer. After scraping, cellular debris was removed by centrifugation at 10,000 × g for 5 min. Protein concentrations were determined by the Bradford assay (Bio-Rad).

20 μg of whole cell extracts were separated in denaturing SDS-polyacrylamide gel and transferred onto nitrocellulose membrane (N+ Hybond, Amersham Biosciences). After blocking with Tris-buffered saline, 0.1% Tween, and 3% bovine serum albumin, the membrane was probed with the primary and the secondary antibodies. The enzymatic activity was detected using a chemiluminescent-enhanced kit (Pierce). Equal transfer of proteins from the gel was controlled by Ponceau (Sigma) staining of the membrane and by using an anti-Actin antibody.

RESULTS

Bax Expression Is Up-regulated in Mammary Cells That Overexpress PEA3 Subfamily Members—In order to identify new PEA3 subfamily target genes, we have compared the PEA3 subfamily overexpressing cells (15) and mock-transfected cells by different technical approaches, including a DNA macro-

1 The abbreviations used are: RT, reverse transcription; wt, wild type; RNAi, RNA interference; HLH, helix-loop-helix; EBS, Ets-binding site; EMSA, electrophoretic mobility shift assay; MMP, matrix metalloproteinase; opn, osteopontin; cox-2, cyclooxygenase-2, Muc/SCM, Muc4-ialoglucan; gpa, glutathione peroxidase.
array analysis (Atlas™ Mouse cDNA Expression Arrays, Clontech). The fold increase used as cut-off was 2-fold. Fifteen genes/588 genes on the membrane were fished out, and among them, bax was up-regulated (data not shown). This preliminary result was then confirmed by Northern blot (data not shown) and semi-quantitative RT-PCR (Fig. 1a). For this purpose, we used stably Erm- and Pea3-overexpressing mammary cells (described previously in Ref. 15) and the same mammary cells transiently transfected with expression vectors for Erm and Pea3 versus the corresponding empty vector.

As shown in Fig. 1a, bax mRNA expression level is increased in the cells that transiently overexpress Erm and Pea3 (left panel). By quantification and normalization using the control cyclophilin expression, we showed that bax expression is increased by at least 2-fold. The stably overexpressing cells present a similar increase (Fig. 1a, right panel).

Bax protein products were evaluated by Western blot analysis on protein extracts from the modified cells described previously. Fig. 1b depicts the elevated level of the 21-kDa Bax protein in the transiently Erm- and Pea3-overexpressing mammary cells, when compared with the control cells (left panel). The same modulation was observed in the stably overexpressing cells (Fig. 1b, right panel).

Regulation by Pea3 of the endogenous bax expression was confirmed by using RNA interference. TAC-2.1 cells were transfected with the pSUPER-pea3 expression vector, which allows the expression of pea3-RNAi oligonucleotides. More than 70% of the cells were transfected by the vectors in these experiments (data not shown). The levels of pea3 mRNA (Fig. 2a) and Pea3 protein product (Fig. 2c) were shown to be reduced in the pea3-RNAi-expressing cells when compared with the control cells. Most interestingly, the levels of the bax messenger (Fig. 2b) and protein (Fig. 2d) were reduced in TAC-2.1 cells in which Pea3 expression was down-regulated, when compared with the control cells. These results indicate that the murine bax gene is a target of the PEA3 group transcription factors.

Specificity of the pea3-RNAi was validated by testing its activity on the transcriptional level of a non-Pea3-regulated promoter (the 96 bp of the L-type pyruvate kinase promoter upstream of the transcriptional start site) and on the transcriptional level of control genes (opn, actin, cyclophilin, and RPP0). The opn transcription was described as up-regulated by Pea3 in mammary cell lines (34). In this model of Pea3 invalidation, we confirmed this regulation at the transcriptional level by showing a decrease of the opn RNA in the pea3-RNAi-expressing cells when compared with the control cells (Fig. 2e). Moreover, we have performed negative controls by comparing the RNA levels of three nonregulated genes in the pea3-RNAi versus control cells. No regulation was observed when we compared the actin RNA to RPP0 RNA levels or RPP0 to cyclophilin RNA levels (Fig. 2e), indicating the specificity of the Pea3 invalidation target. This pea3-RNAi specificity was also controlled on a Pea3 nonresponsive promoter in the TAC-2.1 cells. As shown in Fig. 9b, co-transfection of the pk-luci96 vector containing 96 bp of the pyruvate kinase promoter and pea3-RNAi expression vector had no effect on the transcriptional activity of the promoter. Similar data were obtained with the control gfp-RNAi.

**Pea3, Erm, and Er81 Transactivate the Murine bax Promoter**—To determine the mechanism by which the PEA3 group members regulate the expression of the murine bax gene, transient transfections were performed with luciferase reporter plasmids containing its promoter. TAC-2.1 mammary cells were co-transfected with Pea3, Erm, or Er81 expression vectors and with the PMBaxPF, which contains the full-length 2673-bp bax promoter (−2673 to −1; +1 = translation start site). We also tested the following series of deletion mutants: PMBaxKp1 (−1041 to −1), PMBaxBaxXI (−455 to −1), PMBaxSacI (−386 to −1), PMBaxSacII (−162 to −1), PMBaxPstI (−147 to −1), PMBaxSpeI (−124 to −1), and PMBaxSpI dl (−100 to −1) (Fig. 3a). The normalized luciferase activities of the different promoter constructs were first analyzed in TAC-2.1 cells, and we obtained data similar to that by Igata et al. (44) who used the NIH3T3 cells (Fig. 3b). Co-transfection experiments depicted in Fig. 3, b and c, showed that all promoter fragments (from PMBaxPF to PMBaxSpI dl) are transactivated by the PEA3 group factors, with the highest activity for Pea3. The induction on the full-length promoter (−2673 to −1) is 24-fold for Pea3, 10-fold for Erm, and 4-fold for Er81. All deletion constructs are activated by these factors with few variations. Pea3 and Erm are able to activate the transcription of the

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**Fig. 1. Bax expression in Pea3- or Erm-overexpressing mammary cells.** a, semi-quantitative RT-PCR. TAC-2.1 cells were transiently transfected with empty pTRACER, pTRACER-erm, or pTRACER-pea3 expression vectors and selected by flow cytometry for green fluorescent protein expression (left panel) or stably transfected to overexpress Erm or Pea3, previously described in Ref. 15 (right panel). cDNA were obtained by retro-transcription of 2 μg of total RNA. 1/20 of the RT product was used in PCR to amplify bax and the control cyclophilin. b, Western blot. Twenty μg of total protein extracts from TAC-2.1 cells transiently transfected with empty pTRACER, pTRACER-erm, or pTRACER-pea3 expression vectors (left panel) or stably transfected to overexpress Erm or Pea3, previously described in Ref. 15 (right panel), were loaded on a 12.5% polyacrylamide gel and transferred onto nitrocellulose membrane (N+Hybond, Amersham Biosciences). Immunoblot analysis was performed using 1/1000 of anti-Bax and control anti-Actin antibodies.
100-bp minimal promoter (PMBaxSp1dl, −100 to −1) by 10- and 8-fold, respectively, thus suggesting the presence of a responsive element for these Ets transcription factors within this promoter sequence. The same experiments were performed in the HC11 murine epithelial mammary cell line, which gave rise to the same modulation (data not shown).

**PEA3 Group Members Functionally Cooperate with USF-1 Transcription Factor on the bax Minimal Promoter**—The 100-bp minimal fragment of the bax promoter (−100 to −1) was scanned with Transfac or MatInspector software, and no consensual Ets-binding site (EBS) was identified. We then wanted to check the possibility that Pea3 could bind a nonconsensual binding site by electrophoretic mobility shift assay (EMSA). In vitro translated Pea3 was not able to bind to the PMBaxSp1dl promoter (data not shown). These results eliminate the possibility that Pea3 factor may bind to the minimal promoter on a nonconsensual site.

In contrast, this sequence contains potential binding sites for AP-1, Sp1, and USF-1 transcription factors, previously characterized as partners of Ets family members, mapped at position −63 to −56; −20 to −11 and −91 to −83; and −58 to −53 and −86 to −81, respectively. To identify the possibility of a functional interaction between Pea3 and one of these factors, we performed co-transfection experiments with the PMBaxSp1dl reporter vector, the Pea3 expression vector, and the putative co-partner expression vector: Sp1, AP-1 (Fos + Jun), and USF-1. No cooperative effect was obtained with Sp1 and AP-1 (data not shown). Moreover, on the PMBaxSacI promoter mutated on the Sp1 sites (44), the Pea3-induced transactivation is not affected (Fig. 4), and the basal activity of the mutants is in agreement with the results obtained by Igata et al. (44). Thus, the Sp1 factor is not involved in Pea3 transactivation, even if it has been hypothesized to contribute to the basal activity of the bax promoter (44).

Expression of USF-1 induced a 2- and 4-fold transactivation increase on the PMBaxSacII (−162 to −1) and PMBaxSp1dl (−100 to −1), respectively, and no significant effect on the PMBaxSacI (−386 to −1) (Fig. 5a, 7th columns). These relatively low levels of transactivation could be due in part to the high amount of endogenous USF-1 in the TAC-2.1 cells, as assessed by Western blotting (data not shown).

Co-expression of Pea3 and USF-1 was then tested on the PMBaxSacI, PMBaxSacII, and PMBaxSp1dl (Fig. 5a) reporter plasmids. Although no significant synergistic effect was found for PMBaxSacI promoter, Pea3 and USF-1 increased the transactivation of the PMBaxSacII and the PMBaxSp1dl reporter plasmids by 40 and 190%, respectively, when compared with Pea3 alone (Fig. 5a, compare the 8th and the 2nd columns). An antisense PMBaxSp1dl construct has been used as a specificity control, and no basal or synergistic activities have been detected (data not shown).

Pea3-RNA interference was used to confirm the specificity of the Pea3/USF-1 synergistic activity. Co-transfection of Pea3 and pea3-RNAi expression vectors reduced almost all the transactivation effects of Pea3 on the PMBaxSacI, -SacII, and -Sp1dl promoters (Fig. 5a, compare the 6th and 2nd columns).
When more pea3-RNAi expression vector is used (pea3-RNAi/Pea3 ratio = 1.5), this transactivation effect is completely abolished for the proximal PMBaxSacII and -Sp1dl promoters and almost completely abolished for the PMBaxSacI promoter (data not shown). Furthermore, co-expression of pea3-RNAi drastically reduced the synergistic effect of USF-1 and Pea3 for the proximal PMBaxSacII and -Sp1dl promoters and reduced the transactivation effect to those obtained for Pea3+pea3-RNAi for the PMBaxSacI promoter (Fig. 5a, 10th columns). Furthermore, the transfection of the pea3-RNAi vector induced a de-
crease in the basal transcriptional activity of the PMBaxSacI, PMBaxSacII, and PMBaxSp1dl promoters (Fig. 5a, 4th columns). The specificity of the pea3-RNAi activity was controlled by comparison with a nonspecific gfp-RNAi (Fig. 5a, 3rd, 5th, and 9th columns) for which no effect could be detected on the transcriptional activities.

These results show that Pea3 and USF-1 cooperate to transactivate the proximal bax promoter, and this effect is optimal with the minimal construct, leading to more than an additive transcriptional activation. The same experiments were performed with expression vectors for Erm, which gave rise to the same modulation but with a weaker effect. Er81 was tested with USF-1 but, as the initial level of transactivation was very weak, no significant cooperation effect could be detected (data not shown).

Here we would like to mention that the human bax promoter was shown to be transcriptionally activated by p53 (51), but this was not the case for the mouse promoter that was tested in CaCo2 (44), Saos, and HeLa cells (52). We therefore tested whether p53 could have an effect on the transactivation induced by the PEA3 group members, and we observed no cooperative effect of Pea3 and p53 on the murine bax promoter (data not shown). Moreover, the transcriptional cooperation of USF-1 with other Ets transcription factors on the regulation of bax was also tested with Ets-1, Erg, Fli-1, and Fev, but none of them functionally interacted with USF-1 (data not shown).

**Pea3-stimulated Transcriptional Activation Is Impaired on E Box Mutant bax Promoter**—To identify the molecular mechanism by which USF-1 cooperates with Pea3 to regulate bax transcription, we took advantage of the presence of three putative USF-binding sites at position -11002 to -10986, -10958 to -10953, and -10986 to -10981 of the proximal bax promoter. To assess the functional importance of these sites, we performed mutations of the E boxes alone or in combination within the PMBaxSp1dl (−100 to −1) and PMBaxSacII (−162 to −1) reporter plasmids (Fig. 6). As shown in Fig. 6b, the mutation of one (PMBaxSp1dl E box m1, m2), or both (PMBaxSp1dl E box m1/2), of the E boxes abolished the basal transcriptional activity and the USF-1-, Pea3-, and USF-1/Pea3-induced transcriptional potential, thus indicating that the two proximal E boxes are crucial for the functionality of the minimal PMBaxSp1dl promoter.

The effect of the mutation of these E boxes was also tested on the longer fragment PMBaxSacII (−162 to −1) (Fig. 6c). Although Pea3 was still able to transactivate the mutated PMBaxSacII 1/2 promoter (PMBaxSacII E box m1/2), this activation was weaker than in the nonmutated version, and no cooper-
tion between Pea3 and USF-1 was observed (Fig. 6c). Moreover, these mutations affected the basal and the USF-1-induced transcriptional activity.

We then supposed that the supplementary E box at position −136 was responsible for the Pea3-induced residual transactivation of the PMBaxSacII 1/2 promoter. To confirm this hypothesis, mutation of this E box was performed, leading to the triple E box mutant (PMBaxSacII E box m1/2/3). No significant transcrip-
tional activation could be detected with this mutated construct, either for the control or for Pea3 and/or USF-1, thus indicating that the three E boxes were as important for the basal activity of the proximal bax promoter as for the minimal one.

A USF-1 Dominant Negative Abolishes the Pea3 Transactivation Ability—To establish the role of USF-1 for Pea3 transactivation on the bax minimal promoter, we used a dominant negative version of USF-1 (TDUSF-1), and this version lacks the NH2-terminal putative activation domain (corresponding to the first 163 residues) and possesses a normal DNA binding activity (46). This protein keeps the helix-loop-helix and leucine zipper domains of USF-1 and can still dimerize with USF-1 (46, 47). However, although USF-2 can transactivate the PMBaxSp1dl promoter, it did not enhance the Pea3-induced transactivation effect (Fig. 7), thus indicating the high specificity of USF-1 and Pea3 transcriptional cooperation on the bax promoter.

Pea3 Is Not Able to Bind to the Bax Promoter but Interacts with USF-1 via the E Box Sites—To investigate the molecular mechanism by which Pea3 and USF-1 recognize the bax promoter, we performed EMSA using nuclear extracts from TAC-2.1 cells (Fig. 8). Nuclear extracts were incubated with a labeled double-strand oligonucleotide spanning the region between −48 to −92 of the bax promoter and containing two E boxes (bax wt), with or without competitors or antibodies as indicated. As shown in Fig. 8, two complexes are formed with the wt bax oligonucleotide which corresponds to USF-1 (lower complex) and USF-1 in association with Pea3 in a ternary complex formation with Pea3 (55). This USF-1 dominant negative is not able to interact with Pea3 in a co-immunoprecipitation experiment performed in TDUSF-1-overexpressing TAC-2.1 cells (data not shown). We showed here that this dominant negative was not only unable to transacti-

![Figure 5. Pea3 and USF-1 transcription factors cooperate to stimulate transcription of the minimal bax promoter.](http://www.jbc.org/)

**Fig. 5.** Pea3 and USF-1 transcription factors cooperate to stimulate transcription of the minimal bax promoter. pTRACER (ctrl), pSUPER-pea3 (pea3-RNAi), pSUPER-gfp (gfp-RNAi), and/or pTRACER-pea3 (Pea3) and/or pCR3-USF-1 (USF-1) expression vectors and luciferase reporter vector containing the bax promoter (PMBaxSacI (−386 to −1), PMBaxSacII (−162 to −1), or PMBaxSp1dl (−100 to −1)) (a) or the corresponding empty luciferase reporter vector (b) were transiently co-transfected in TAC-2.1 cells. Luciferase activities were obtained and presented as in Fig. 3b.
expected, this mutated oligonucleotide was not able to displace the formation of the complexes. These different results indicate the specificity of the formed complexes. The lower and upper migrating bands were identified as a USF-1-DNA complex and a Pea3-USF-1-DNA complex, respectively, by using Pea3 and USF-1 antibodies. Incubation with a Pea3 antibody displaced the upper band (ternary complex) almost totally (Fig. 8, lane 6), whereas incubation with a USF-1 antibody displaced the two bands (Fig. 8, lane 7). We also performed the same incubation with an unrelated antibody, and no supershift could be observed (Fig. 8, lane 8).

Taken together, these results demonstrate that Pea3 does not directly bind to the 100-bp minimal bax promoter fragment but acts via USF-1. These results also confirm that these factors play a role in the regulation of the bax promoter activity.

**Pea3 Can Act Cooperatively with USF-1 on an HLH-response Element**—As Pea3 needs USF-1 to be active on the bax promoter, we wanted to test if this property is conserved in an-
other promoter context. To assess whether Pea3 and USF-1 are able to cooperate independently of Pea3 DNA binding, transient transfections were made with USF-1 and/or Pea3 expression vectors and a reporter vector carrying binding sites for the HLH transcription factors. We used the pk-luci96(L4)3 reporter vector containing three copies of the L4 element coming from the L-type pyruvate kinase promoter consisting of a tandem of noncanonical E boxes (underlined) as follows: 5'-CACGGG-GCACTCCCGTG-3' and shown previously to be bound and transactivated by USF-1 (46, 47). As shown in Fig. 9a, USF-1 increased transcription about 2-fold through the L4 element, whereas Pea3 is not able to transactivate. Overexpression of the two transcription factors resulted in a 50% increase of the transactivation in comparison with that of USF-1 alone, thus demonstrating that Pea3 is still able to cooperate with USF-1 without binding to DNA in a context other than the minimal bax promoter.

**DISCUSSION**

In the last decade, significant works have contributed to our understanding of the function of the PEA3 subfamily transcription factors. Many reports argue for their involvement in the different stages of normal mammary gland development and in the events leading to mammary oncogenesis and metastasis (15, 54). However, few Pea3 target genes are known and most of them are encoding genes. Here we report that Pea3 can regulate the expression of the bax gene in the TAC-2.1 normal mammary cell line, by using either Pea3-overexpressing cells or cells in which Pea3 expression was abolished by RNA interference. This is the first evidence that a Bcl-2 family member is shown as a target of the PEA3 group members.

We showed that the PEA3 group members are able to transactivate the murine bax promoter from the full-length construct (-2673) to a minimal fragment of -100 bp downstream of the translational initiation codon. All the PEA3 group target genes have been currently characterized to be regulated through an EBS within their regulatory regions. Although several potential EBS are present along the bax promoter sequence, they are all situated downstream of the -162-bp fragment (PMBaxSacII). Thus, no consensual EBS could explain the Pea3-induced transactivation within the first -162- or -100-bp bax promoter constructs. We then focused the present study on the proximal -162-bp (PMBaxSacII) and minimal -100-bp (PMBaxSp1dl) fragments to elucidate how this Pea3-induced transactivation occurs. We have found that the PEA3

Fig. 8. Pea3 can form a complex with USF-1 that binds E box sites on the proximal bax promoter. Nuclear extracts were incubated with a 32P-labeled double-stranded oligonucleotide spanning the two E boxes within the murine bax minimal promoter (bax wt). The arrows indicate the position of the protein-DNA complexes. Each lane contains the labeled bax wt probe, nuclear extracts, and the following: lane 1, buffer; lane 2, excess of the unlabeled bax wt probe; lane 3, excess of the unlabeled nonspecific probe; lane 4, excess of the unlabeled mutated bax probe; lane 5, buffer; lane 6, anti-Pea3 antibody (200 ng); lane 7, anti-USF-1 antibody (200 ng); lane 8, nonrelevant Pim-1 antibody (200 ng).

Fig. 9. Pea3 can act synergistically with USF-1 on the HLH response elements of the L-type pyruvate kinase promoter. a, pTRACER (ctrl), pTRACER-pea3 (Pea3), and/or pCR3-USF-1 (USF-1) expression vectors and the pk-luci96(L4)3 or the pk-luci96 luciferase reporter vectors were transiently co-transfected in TAC-2.1 cells. Luciferase activities were obtained and presented as in Fig. 3b. b, pSUPER (ctrl), pSUPER-pea3 (pea3-RNAi), or pSUPER-gfp (gfp-RNAi) expression vectors and the empty or the pk-luci96 luciferase reporter vectors were transiently co-transfected in TAC-2.1 cells. Luciferase activities were obtained and presented as in Fig. 3b.
group members Erm and Pea3 need USF-1 transcription factor to exert their activity. In fact, Erm and Pea3 cooperate with USF-1 to transactivate the proximal −162- and minimal −100-bp promoters. We also demonstrated the importance of Pea3 in the basal activity of these proximal −162- and −100-bp promoters as the abolition of Pea3 by RNA interference drastically reduced this transcriptional activity. Furthermore, Pea3 and USF-1 cooperation effect is greatly affected by the co-expression of the Pea3-interfering oligonucleotides.

To demonstrate the requirement of USF-1 for the activity of Pea3 on the proximal bax promoter, we used E box mutated promoter versions. There are three consensual E boxes in the proximal bax promoter, two localized in the −100-bp construct and the third in the −162-bp construct. All mutations on these E boxes drastically affect the basal expression including the ability of Pea3 transactivation, leading to complete loss of activity when the three sites are mutated, thus indicating that the E box sites are crucial for the proximal bax promoter transactivation.

To prove the role of USF-1 as a key regulator of Pea3-induced transactivation, we used a USF-1 dominant negative that was able to bind the proximal E boxes of the bax promoter (data not shown) as described for a USF-MPL motif (46, 47). This USF-1 dominant negative lacked the first 163 residues of USF-1 and was unable to interact with Pea3 in our model. This lacking region was said to be essential for the formation of a stable DNA-bound USF-1-Pea3 ternary complex, which requires DNA contacts by both Pea3 and USF-1 (53). This USF-1 dominant negative reduces the transcriptional activity of the minimal −100-bp bax promoter and abolishes the Pea3 transactivation, which is in agreement with the necessity of USF-1 for the function of Pea3 on the minimal bax promoter. Nevertheless, these results are different from those obtained by Greenall et al. (53) because, in the case of the bax promoter, no DNA binding of Pea3 was required.

We next investigated whether these factors were able to interact on the proximal bax promoter. Further evidence for DNA binding of the USF-1-Pea3 complex was obtained by EMSA. These cumulative results gave evidence for the first time that an Ets family PEA3 member could exert its function of the transcription factor without binding to DNA but in association with another DNA-binding transcription factor (here USF-1).

We also performed chromatin immunoprecipitation experiments on the murine bax promoter (data not shown). Although a specific USF-1 antibody was successfully used on this promoter, we were not able to detect any immunoprecipitated complex while using any of the anti-Pea3 antibodies commercially available. We also tried the antibody previously characterized by our group (20) but without success. The absence of an immunoprecipitated complex with the Pea3 antibodies was probably because of the low affinity of these antibodies as assessed by the fact that, at the current time, no paper has reported chromatin immunoprecipitation experiments on Pea3.

Numerous regulations implicating USF-1 and Ets family members have been documented, but in all cases the two proteins interact directly with DNA on their respective binding sites (E box and EBS). Ets-1 and USF-1 are widely known to interact, for example, on the human immunodeficiency virus, type 1, long terminal repeat (55), on the BRCA2 promoter (56) or on the mannose receptor promoter (57). Since Greenall et al. (53) have shown by an in vitro glutathione S-transferase pull-down assay, a physical interaction between USF-1 and the ETS domain of Pea3, we then confirmed by co-immunoprecipitation assay in TAC-2.1 cells that these two factors interact (data not shown). Transcriptional regulation of a transcription factor without DNA binding has been described previously for few other transcription factors. For example, Oct-1 forms a complex with cAMP-response element-binding protein that is bound to the cAMP-response element of the cyclin D1 promoter, but without requiring DNA binding of Oct-1 (58). This model also exists for the bcl-2 promoter, which is regulated by a C/EBP-Myb and Cdx complex, this latter binding to DNA on the Cdx site (59). Finally, Howe et al. (21), who have demonstrated the up-regulation of Pea3 in response to Wnt-1 to activate the expression of cox-2 gene, argue for the possibility that Pea3 might bind to C/EBP proteins at the NF-IL6 site without itself binding to DNA.

According to our data on the proximal bax promoter, it was conceivable that Pea3/USF-1 cooperation occurs for other promoters. We then demonstrated this functionality on an HLH-responsive element derived from the L-type pyruvate kinase promoter, which comprises only E boxes and no EBS. Taken together, these findings revealed a novel regulation mechanism for PEAS transcription factors indicated for the bax gene promoter transactivation and probably valid for other promoters.

The data presented here for Pea3 have been confirmed for the Erm transcription factor but with a lesser effect for the USF-1-Erm-DNA and USF-1-Pea3-DNA complexes forming the same retarded band by EMSA (data not shown). We can thus hypothesize that when we only partially displaced the USF-1-Pea3-DNA complex by a Pea3 antibody, the residual higher complex could correspond to the USF-1-Erm-DNA complex (Fig. 8). The other Ets family members tested were not able to cooperate with USF-1 to stimulate transcription of the minimal bax promoter. So this novel mode of function depicted for the proximal bax promoter could be specific for Pea3 and, in a lesser extent, Erm.

Primer extension analysis of the bax promoter revealed that several transcription initiation sites are clustered between −26 and −69 bp from the translational initiation site (52). An 831-bp mRNA that began at −44 bp from the translation start site is described in GenBank™. The location of the E box and the EBS at the vicinity of the transcriptional initiation site is documented. A functional E box has been described at position −7 to −2 from the transcriptional initiation site of the cation-exchange group members has been detected (data not shown). At −18 and −58 bp on human BRCA2 gene promoter (56). By taking into account the close vicinity of the E box to the transcriptional initiation site in the context of the proximal bax promoter and the drastic effect of mutation of these E boxes on the activity of this promoter, we can suggest that the binding of Pea3 with USF-1 to these E boxes is involved in and can facilitate the formation of the transcriptional preinitiation complex. In this regard, Erm has been shown to bind TAFI160, TAFII40, and TBP (61) and USF-1 interacts with and stabilizes TFIID (62–64).

Bax belongs to the Bcl-2 family whose members are known to be important actors of apoptosis. However, in TAC-2.1 cells and TAC-2.1 cells overexpressing Erm or Pea3, we were not able to detect a significant induction of apoptosis. One hypothesis we have made is that Bcl-2 could be regulated in balance, so that the Bcl-2/Bax rheostat was not modified. We have thus checked the possibility that Bcl-2 was also overexpressed, but neither modification of transcriptional nor of translational Bcl-2 expression by PEAS group members has been detected (data not shown). According to these results, we hypothesize that Pea3-induced Bax overexpression could play another role in mammary cells, such as migration, proliferation, or invasion. These events are involved in the branching morphogenesis or invasion capacities of mammary cells overexpressing Erm or Pea3 that we have described previously (15). Moreover, few data reveal a effect of Bcl-2 or Bax on
migration, proliferation, and invasion. Wick et al. (65) have shown that Bcl-2 can promote migration and invasiveness of human glioma cells. Another report (66) defines a novel pathway for hepatocyte growth factor-induced glioma cell migration and invasion, which requires differences in the Bcl-2/Bax rheostat and the induction of transforming growth factor-$
abla$
expression in vitro. The bax-deficient mice provide evidence for an interrelationship between proliferation, differentiation, and cell death, as bax deficiency can be manifested as hyperplasia or hypoplasia, depending on the cellular context (67).

In conclusion, we present evidence for a new target gene of the Pea3 group members, the bax gene, and a novel molecular mechanism of regulation. Pea3 is able to transactivate the minimal bax promoter without binding to DNA but via its partner USF-1, which binds DNA on E boxes (Fig. 10). Further studies will be required to determine the role of bax transactivation by Pea3 in mammary cells by using in vitro and in vivo models.

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Pea3 and USF-1 Cooperate to Transactivate the bax Promoter

Fig. 10. Model of the USF-1-Pea3 complex on the minimal murine bax promoter. Pea3 acts via the USF-1 transcription factor and E box sites to transactivate the minimal murine bax promoter, without itself binding DNA.
Pea3 Transcription Factor Cooperates with USF-1 in Regulation of the Murine \textit{bax}
Transcription without Binding to an Ets-binding Site

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