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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\& Supported by the ‘Ligue Nationale contre le Cancer’

* This work was supported by the ‘Ligue Nationale contre le Cancer’, the ‘Association pour la Recherche sur le Cancer’, the ‘Institut Pasteur de Lille’, the ‘Centre National de la Recherche Scientifique’, the ‘Fonds National de la Recherche Scientifique, Belgium’ and the ‘Université des Sciences et Technologies de Lille’

Running Title: Pea3 and USF-1 cooperate to transactivate the \textit{bax} promoter
Summary

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 \textit{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 \textit{bax} promoter fragments. Although the minimal Pea3-regulated \textit{bax} promoter does not contain an Ets binding site, two functional USF-regulated E-boxes are present. We further demonstrate the ability of Pea3 and USF-1 to cooperate for the transactivation of the \textit{bax} promoter, mutation of the E-boxes dramatically reducing the Pea3 transactivation potential. Although Pea3 did not directly bind to the minimal \textit{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 \textit{bax} transcription \textit{via} the interaction with USF-1, but without binding to DNA.
Introduction

Pea3, also called E1AF or ETV4, is the founding member of a subfamily of ets genes that include Er81 (or ETV1) and Erm (or ETV5) which have been currently characterized in mice (1-3), humans (4-6), rats, dogs (Telgman, Genebank AJ313194), chicken (7), Zebrasfish (8) and amphibian 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 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 (11, 16-24). Pea3 ectopic overexpression in non-metastatic 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 member target genes have been currently reported. Most of them encode proteinases required for extracellular matrix degradation, such as \textit{MMP-1}, \textit{MMP-9} and \textit{MMP-3} (28, 29), \textit{MT1-MMP} (30) and \textit{MMP-7} (31, 32) or adhesion molecules such as \textit{Icam-1} (33). \textit{Osteopontin} (24, 34), \textit{cyclooxygenase-2} (21, 35), \textit{p21waf-1} (36), \textit{heparanase} (37), \textit{neu} (38), \textit{Muc4/Sialomucin complex} (39) and \textit{glutathione peroxydase} (40) are also targets of the PEA3 group members. For all these target genes, PEA3 factors act as transactivators except for \textit{neu} and \textit{glutathione peroxydase} 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: 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: \textit{bcl-xl} is activated by Ets-2 and PU-1 (41), and repressed by Tel (42), or \textit{bcl-2} which is regulated by Fli-1 (43).

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

\section*{Materials and Methods}

\textit{DNA array analysis}

Total RNA was isolated from control or PEA3 group members overexpressing TAC-2.1 cells, previously described in Chotteau-Lelievre \textit{et al.} (15). 5 µg of RNA was used to convert total RNA into $^{32}$P-labeled first strand cDNA using [$^{32}$P]-ATP and the gene-specific CDS-primer mix for the Atlas array (BD Clontech) following the manufacturer’s recommendation. The labeled cDNA was purified using a Nucleospin Column (BD Clontech). A set of Mouse Atlas Array (BD Clontech) was used for hybridization with the labeled probes using Express Hyb solution at 68°C for 16h. The arrays were exposed to a PhosphorImager and analyzed using Quantity One software (Biorad).

\textit{RT-PCR}

Total RNA was extracted from cells using Tri Reagent\textsuperscript{TM} (Euromedex) 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 \textit{bax} gene (sense: 5’-AAGCTGAGCGAGTGTCTCCGGCG-3’ and antisense: 5’-GCCACAAAGATGGTCTGACTGTCTGCG-3’), the \textit{pea3} gene (sense: 5’-CCCAGATGATGTCTGCATTG-3’ and antisense: 5’-AGTGGGACAAAGGGACTGTG-3’), the \textit{osteopontin} gene (24) and the \textit{cyclophilin A} gene (sense: 5’-GCATACAGGTCTGGCATCTTGCTG-3’ and antisense: 5’-ATGGTGATCTTCTTCTGCTGGTCT-3’), the \textit{acidic ribosomal phosphoprotein P0} gene (RPP0) (sense: 5’-CATGCTACACATCTCCCCTCTCC-3’ and antisense: 5’-GGGAAGGTGTAAATCCGTCTCCAG-3’), and the \textit{actin} gene (sense: 5’-GCCACAGGGTACAGTGCT-3’ and antisense: 5’-GCCACAGGGTGTCACAG-3’).
5’-GTGGGGCGCCCCAGGCACCA-3’ and antisense: 5’-CTCCTTAATGTCACGCACGATTTC-3’). As internal controls, 1/20 cDNA was used for the PCR reactions containing 2 mM MgCl₂, 0.2 mM dNTP, 0.2 μM of each primer and 1 unit of Taq DNA 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. These vectors 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 Pr T. Sakai (44). The pCR3-USF-1, pCR3-USF-2a and the dominant negative pCR3-TDUSF-1 expression vectors, pk-luci96 and pk-luci96(L4)₃ luciferase reporter vector were obtained from Dr Benoît Viollet (45-47).

The oligonucleotide sequence of pea3 used for the small RNA interference was as follows: 5’-GCAGGAAGGGATTGGAGCT-3’ and cloned in the pSUPER expression vector (OligoEngine) by following the procedure described by Brummelkamp et al. (48). This vector was referred to as pSUPER-pea3. A control vector, pSUPER-gfp, was constructed using a 19-nucleotide sequence of the gfp coding sequence (5’-GCTGACCCCTGAAAGTCATC-3’) and served as a non-silencing control.
The anti-Pea3 (sc113), anti-Bax (sc7480), anti-Pim-1 (sc13513) and anti-USF-1 (sc229) antibodies were purchased from Santa Cruz Biotechnology, the anti-Actin (A-2066) and secondary anti-rabbit coupled to horse-radish peroxidase (A-0545) antibodies were purchased from Sigma and the secondary anti-mouse antibody coupled to horse-radish peroxidase (AP192P) was purchased from Chemicon.

**Cell culture**

Wild-type TAC-2.1 cells (49), a clonal subpopulation of TAC-2 murine mammary epithelial cells (50), and their derivatives (Erm and Pea3 stable cell lines) (15) were cultured on collagen-coated tissue culture flasks (Becton Dickinson) in DMEM (Gibco, Invitrogen) supplemented with 10% (v/v) fetal calf serum (FCS, Gibco, Invitrogen), gentamycin (100 IU/ml) and non essential amino-acids (Gibco, Invitrogen).

**Cell transfections and reporter assays**

TAC-2.1 cells were seeded at 70% confluence in 12-well plates and transfected with polyethylenimine (PEI 10 µM, Euromedex). Each well was transfected for 6 hrs using a mixture of 500 µl serum free optiMEM (Invitrogen), 500 ng of DNA (including 150 ng of expression vector(s), 50 ng of luciferase reporter vector, 25 ng of the pSV-β-galactosidase vector (Promega) and, if necessary, salmon sperm DNA) and 2 µl of PEI. Cells lysates were prepared 24 hrs later for 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 PMBaxSp1dl using the QuikChange site-directed mutagenesis kit (Stratagene). 10 ng of PMBaxSp1dl was combined with complementary mutant oligonucleotide primers (0.3 µM), 1 µM dNTP and 2.5 units of PfuTurbo DNA polymerase. The oligonucleotide primers used were as follows (sense strand): E-box m1: 5’-TTGCGGGGCCACCCAATTGAGGGCCGCACGT-3’, E-box m2: 5’-GTCCACGATCAGTCAAT TGACCGTGGTGCGCC-3’ and E-box m1/2: 5’-TTGCGGGGCACCCAATTGAGGGCCGCACGTCCACGATCAGTCAATTGACCGTGGT GCGCC-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 mutant constructs. Cycling conditions were 95°C for 30 sec, 55°C for 1 min and 68°C for 10 min for 12 cycles. Mutation 3 on PMBaxSacII was performed on PMBaxSacII firstly mutated on the first and second E-boxes by using the following oligonucleotide primer: 5’-GAGCGATGATGATCAATTGACTAGTCCTGCGGC-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 [PMSF], 3 mM dithiothreitol [DTT]) and incubated on ice for 10 min. The homogenate was then centrifuged at 10,000 g for 5 min. Pelleted cells were resuspended in an hypertonic buffer (50 mM HEPES, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol (v/v), 3 mM DTT, 3 mM PMSF) 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 (Biorad), aliquotted and stored at -80°C.
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**Gel mobility shift assay**

The annealed double strand oligonucleotide derived from the *bax* minimal promoter (bax wt) was 5’end-labeled by $^{32}$P-ATP using T4 polynucleotide kinase (Invitrogen). Binding of the protein to DNA sequences was achieved in a 20 µl mixture containing 2X buffer (50 mM HEPES, 50 mM KCl, 2 mM EGTA, 4 mM MgCl$_2$, 0.1% NP40 (v/v), 20% glycerol (v/v)), 1.5 µg salmon sperm DNA, 1 µg d[I-C], 1 mM DTT, 12 µg 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% non-denaturating polyacrylamide gel and run in 0.5X 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’-CACCCACGTTAGGGCCGCACGTCCACGTCCAGTAGTCTCGTGACC-3’, non-specific: 5’-GGGCGCACCCGGCGAGAGGCAGCGGCAGTG-3’ and bax mt: 5’-TTGCGGGGCACCCAATTAGGGGCCCACGTCACGTCCAGTCAATTGACCGTG GTGCACC-3’.

**Immunoblotting**

Cells were lysed in a 150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% Nonidet-P40 (v/v), 1 mM sodium orthovanadate, 1 mM PMSF, 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 (Biorad).
20 μg of whole cell extracts were separated in denaturing SDS polyacrylamide gel and transferred onto nitrocellulose membrane (N+ Hybrid, Amersham). After blocking with TBS, 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 chemoluminescent-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.
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**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-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 (Figure 1a). For this purpose, we used stably Erm- and Pea3-overexpressing mammary cells (previously described in (15)) and the same mammary cells transiently transfected with expression vectors for Erm and Pea3 *versus* the corresponding empty vector.

As shown in figure 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 twofold. The stably overexpressing cells present a similar increase (right panel).

Bax protein products were evaluated by Western blot analysis on protein extracts from the previously described modified cells. Figure 1b depicts the elevated level of the 21 kDa Bax protein in the transiently Erm- and Pea3-overexpressing mammary cells, when compared to the control cells (left panel). The same modulation was observed in the stably overexpressing cells (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 are transfected by the vectors in these experiments (data not shown). The levels of pea3 mRNA (Figure 2a) and Pea3 protein product (Figure 2c) were shown to be reduced in the pea3-RNAi-expressing cells when compared to the control cells. Interestingly, the levels of the bax messenger (Figure 2b) and protein (Figure 2d) were reduced in TAC-2.1 cells in which Pea3 expression was down-regulated, when compared to 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-pyruvate kinase promoter upstream of the transcriptional start site) and on the transcriptional level of control genes (osteopontin, actin, cyclophilin and RPP0). The osteopontin 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 osteopontin RNA in the pea3-RNAi-expressing cells when compared to the control cells (Figure 2e). Moreover, we have performed negative controls by comparing the RNA levels of three non-regulated 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 (Figure 2e), indicating the specificity of the Pea3 invalidation target. This pea3-RNAi specificity was also controlled on a Pea3 non-responsive promoter in the TAC-2.1 cells. As shown in figure 9b, co-transfection of the pk-luci96 vector containing 96 bp of the pyruvate
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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: PMBaxKpnI (–1041 to –1), PMBaxBstXI (–455 to –1), PMBaxSacI (–386 to –1), PMBaxSacII (–162 to –1), PMBaxPstI (–147 to –1), PMBaxSpeI (–124 to –1) and PMBaxSp1dl (–100 to –1) (Figure 3a). The normalized luciferase activities of the different promoter constructs were first analyzed in TAC-2.1 cells and we obtained similar data as those obtained by Igata et al. (44) who used the NIH3T3 cells (Figure 3b). Co-transfection experiments depicted in figures 3b and 3c showed that all promoter fragments (from PMBaxPF to PMBaxSp1dl) 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 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).
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\textit{PEA3} group members functionally cooperate with USF-1 transcription factor on the \textit{bax} minimal promoter

The 100 bp minimal fragment of the \textit{bax} promoter (–100 to –1) was scanned with Transfac or MatInspector software, and no consensual EBS (Ets Binding Site) was identified. We then wanted to check the possibility that Pea3 could bind a non-consensual binding site by electrophoretic mobility shift assay (EMSA). \textit{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 non-consensual 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 the position (–63 to –56), (–20 to –11 and –91 to –83) and (–58 to –53 and –86 to –81), respectively. In order 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 vectors: Sp1, AP1 (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 (Figure 4) and the basal activity of the mutants is in agreement with the results obtained by Igata \textit{et al.} (44). Thus, Sp1 factor is not involved in Pea3 transactivation, even if it has been hypothesized to contribute to the basal activity of the \textit{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) (Figure 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 (Figure 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 to Pea3 alone (Figure 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 effect of Pea3 on the PMBaxSacI, -SacII and -Sp1dl promoters (Figure 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 (Figure 5a, 10th columns). Furthermore, the transfection of the pea3-RNAi vector induced a decrease in the basal transcriptional activity of the PMBaxSacI, PMBaxSacII and PMBaxSp1dl promoters (Figure 5a, 4th columns). The specificity of the pea3-RNAi activity was controlled by comparison with a non-specific gfp-RNAi (Figure 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 \textit{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 \textit{bax} promoter was shown to be transcriptionally activated by p53 (51), but this was not the case for the mouse promoter which 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 \textit{bax} promoter (data not shown). Moreover, the transcriptional cooperation of USF-1 with other Ets transcription factors on the regulation of \textit{bax} was also tested with Ets-1, Erg, Fli-1 and Fev but none of them functionally interacted with USF-1 (data not shown).

\textit{Pea3-stimulated transcriptional activation is impaired on E-box mutant \textit{bax} promoter}

In order to identify the molecular mechanism by which USF-1 cooperates with Pea3 to regulate \textit{bax} transcription, we took advantage of the presence of three putative USF binding sites at position (−136 to −132), (−58 to −53) and (−86 to −81) of the proximal \textit{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 (Figure 6). As shown in figure 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
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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) (Figure 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 non-mutated version and no cooperation between Pea3 and USF-1 was observed (Figure 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 transcriptional 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 \textit{bax} promoter as for the minimal one.

\textit{A USF-1 dominant negative abolishes the Pea3 transactivation ability}

In order to establish the role of USF-1 for Pea3 transactivation on the \textit{bax} minimal promoter, we used a dominant negative version of USF-1 (TDUSF-1), which 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 but lacks most of the domain (144-197) required for ternary complex formation with Pea3 (53). This USF-1 dominant negative is not able to interact with Pea3 in a co-immunoprecipitation experiment.
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performed in TDUSF-1-overexpressing TAC-2.1 cells (data not shown). We showed here that this dominant negative, not only was unable to transactivate the PMBaxSp1dl promoter, but also dramatically reduced the Pea3-induced transactivation (Figure 7). [Figure 7 location]

USF-1 is closely related to another bHLH-ZIP transcription factor, USF-2, which is able to dimerize with USF-1 (46, 47). However, although USF-2 can transactivate the PMBaxSp1dl promoter, it did not enhance the Pea3-induced transactivation effect (Figure 7), thus indicating the high specificity of USF-1 and Pea3 transcriptional cooperation on the \textit{bax} promoter.

\textit{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 \textit{bax} promoter, we performed EMSA using nuclear extracts from TAC-2.1 cells (Figure 8). Nuclear extracts were incubated with a labeled double-strand oligonucleotide spanning the region between –48 to –92 of the \textit{bax} promoter and containing two E-boxes (bax wt), with or without competitors or antibodies as indicated. As shown in figure 8, two complexes are formed with the wt \textit{bax} oligonucleotide which corresponds to USF-1 (lower complex) and USF-1 in association with Pea3 in a ternary complex (higher complex) (lane 1). The specificity of these bindings was demonstrated by competition with the same unlabeled oligonucleotide (lane 2) and the absence of a competition effect with an unrelated oligonucleotide (lane 3). Competition assay was also performed with an oligonucleotide mutated on the two E-boxes (lane 4). As 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
Pea3 and USF-1 cooperate to transactivate the \textit{bax} promoter

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 (lane 6), whereas incubation with a USF-1 antibody displaced the two bands (lane 7). We also performed the same incubation with an unrelated antibody and no supershift could be observed (lane 8).

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

\textit{Pea3 can act cooperatively with USF-1 on a HLH response element}

As Pea3 needs USF-1 to be active on the \textit{bax} promoter, we wanted to test if this property is conserved in another promoter context. In order 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)\textsubscript{3} reporter vector containing three copies of the L4 element coming from the L-pyruvate kinase promoter consisting of a tandem of non-canonical E-boxes (underlined) as follows: 5'-CACGGGGC\textbf{ACTCCCCGTG}-3', and previously shown to be bound and transactivated by USF-1 (46, 47). As shown in figure 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 \textit{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 MMP (matrix metalloproteinase) 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 bp 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 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.
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Furthermore, Pea3 and USF-1 cooperation effect is greatly affected by the co-expression of the Pea3 interfering oligonucleotides.

In order 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 which 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) since, 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 cumulated results gave evidence for the first time that an Ets family PEA3
Pea3 and USF-1 cooperate to transactivate the *bax* promoter

member could exert its function of 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. Absence of an immunoprecipitated complex with the Pea3 antibodies was probably due to the low affinity of these antibodies as assessed by the fact that, at the current time, no paper has reported ChIP 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 HIV-1 LTR (55), on the *BRCA2* promoter (56) or on the *mannose receptor* promoter (57). Since Greenall *et al.* (53) have shown by an *in vitro* GST 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 previously described for few other transcription factors. For example, Oct-1 forms a complex with CREB that is bound to the CRE element of the *cyclin D1* promoter, but without requiring DNA-binding of Oct-1 (58). This model also exists for the *bel-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 *cyclooxygenase-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 \textit{bax} promoter, it was conceivable that Pea3/USF-1 cooperation occurs for other promoters. We then demonstrated this functionality on a HLH responsive element derived from the L-pyruvate kinase promoter, which comprises only E-boxes and no EBS. Taken together, these findings revealed a novel regulation mechanism for PEA3 transcription factors evidenced for the \textit{bax} gene promoter transactivation and probably valid for other promoters.

The data presented here on Pea3 have been confirmed for the Erm transcription factor but with a lesser effect, 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 USF-1/Erm/DNA complex (Figure 8). The other Ets family members tested were not able to cooperate with USF-1 to stimulate transcription of the minimal \textit{bax} promoter. So this novel mode of function depicted for the proximal \textit{bax} promoter could be specific to Pea3 and, in a lesser extent, Erm.

Primer extension analysis of the \textit{bax} promoter revealed that several transcription initiation sites are clustered between \(-26\) bp and \(-69\) bp from the translational initiation site \((52)\). A 831 bp mRNA that began at \(-44\) bp from the translation start site is described in the Genebank data. 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 \textit{cathepsin B} gene \((60)\), as have an E-box and an Ets/E2F site respectively at \(-18\) and \(-58\) bp on human \textit{BRCA2} gene promoter \((56)\). Taking into account the close vicinity of the E-boxes to the transcriptional initiation site in the context of the proximal \textit{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 theses
E-boxes is involved in and can facilitate the formation of the transcriptional pre-initiation complex. In this regard, Erm has been shown to bind TAFII60, 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 PEA3 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 those involved in the branching morphogenesis or invasion capacities of mammary cells overexpressing Erm or Pea3 that we have previously described (15). Moreover, few data reveal an 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 defines a novel pathway for HGF-induced glioma cell migration and invasion, which requires differences in the Bcl-2/Bax rheostat and the induction of TGFβ2 expression in vitro (66). Then, bax deficient mice provide evidence for an interrelationship between proliferation, differentiation and cell death, as bax deficiency can be manifested as an hyperplasia or an hypoplasia, depending on the cellular context (67).

In conclusion, we give 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
Pea3 and USF-1 cooperate to transactivate the bax promoter on E-boxes (Figure 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.
Acknowledgements

We thank I. Damour and B. Quatannens for skillful technical assistance, and L. Brunet and G. Courtand for help in manuscript illustrations. We also thank Pr Sakai for providing bax promoter constructs, Dr B. Viollet for providing USF and pk-luci96 reporter constructs, Dr D. Monté for the ChIP experiments, Dr J. L. Baert for critical reading of the manuscript and Pr R. Montesano for helpful discussions.
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References


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Pea3 and USF-1 cooperate to transactivate the bax promoter


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\textbf{Legends}

\textbf{Figure 1 : Bax expression in Pea3- or Erm-overexpressing mammary cells.}

(a) \textit{Semi-quantitative RT-PCR}. TAC-2.1 cells were transiently transfected with empty pTRACER, pTRACER-\textit{erm} or pTRACER-\textit{pea3} expression vectors and selected by flow cytometry for GFP expression (left panel) or stably transfected to overexpress Erm or Pea3, previously described in (15) (right panel). cDNA were obtained by retro-transcription of 2 µg total RNA. 1/20 of the RT product was used in PCR to amplify \textit{bax} and the control \textit{cyclophilin}.

(b) \textit{Western blot}. Twenty µg of total protein extracts from TAC-2.1 cells transiently transfected with empty pTRACER, pTRACER-\textit{erm} or pTRACER-\textit{pea3} expression vectors (left panel) or stably transfected to overexpress Erm or Pea3, previously described in (15) (right panel) were loaded on a 12.5\% polyacrylamide gel and transferred onto nitrocellulose membrane (N+ Hybond, Amersham). Immunoblot analysis was performed using 1/1000 of anti-Bax and control anti-Actin antibodies.

\textbf{Figure 2 : Pea3 and Bax expression in \textit{pea3}-RNAi expressing mammary cells.}

(a, b, e) \textit{Semi-quantitative RT-PCR}. TAC-2.1 cells were transfected with empty pSUPER or pSUPER-\textit{pea3} expression vectors. RT-PCR was made as in figure 1a to amplify \textit{pea3} and the control \textit{actin} (a), \textit{bax} and the control \textit{cyclophilin} (b) or the following controls, \textit{actin} and \textit{osteopontin} or \textit{actin} and \textit{RPP0} or \textit{RPP0} and \textit{cyclophilin} (e).

(c, d) \textit{Western blot}. Twenty µg of total protein extracts from TAC-2.1 cells transfected with empty pSUPER or pSUPER-\textit{pea3} expression vectors were loaded as
Pea3 and USF-1 cooperate to transactivate the \textit{bax} promoter described in figure 1b. Immunoblot analysis was performed using 1/1000 of control anti-Actin and anti-Pea3 (c) or anti-Bax (d) antibodies.

\textbf{Figure 3 : The full-length and deletion fragments of the murine \textit{bax} promoter are transactivated by the PEA3 subfamily members.}

(a) Schematic representation of the murine \textit{bax} promoter and the different deletion fragments. Numbering is relative to the translation initiation start site (+1).

(b) pTRACER (ctrl), pTRACER-erm (Erm), pTRACER-er81 (Er81) or pTRACER-pea3 (Pea3) expression vectors and luciferase reporter vectors containing \textit{bax} promoter fragments or the corresponding empty luciferase reporter vector were transiently co-transfected in TAC-2.1 cells. Luciferase activity was measured 24 hrs after transfection. Values were normalized to those obtained with the co-transfected pSV-\(\beta\)-galactosidase expression vector. Each assay was performed at least three times in triplicate. Data are presented as the mean of three independent experiments ± SD.

(c) Table representing the fold induction of the indicated \textit{bax} promoter constructs by Erm, Er81 and Pea3 relative to the empty control vector.

\textbf{Figure 4 : Pea3-induced transactivation is not affected by mutation of the Sp1 sites in the PMBaxSacI (–386) \textit{bax} promoter.}

(a) Schematic representation of the murine \textit{bax} promoter sequence with the location of the Sp1 binding sites (Sp1 m3, -m4 and -m5).

(b) pTRACER (ctrl) or pTRACER-pea3 (Pea3) expression vectors and luciferase reporter vectors containing the wild-type or mutated fragments of the
PMBaxSacI bax promoter (–386 to –1) or the corresponding empty luciferase reporter vector were transiently co-transfected in TAC-2.1 cells. Luciferase activities were obtained and presented as in figure 3b.

Figure 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 figure 3b.

Figure 6: Pea3-stimulated transcriptional activation of the proximal bax promoter is impaired by mutation of the E-boxes.

(a) Schematic representation of the murine bax promoter sequence with the location of the Ets Binding Sites (EBS) and the three E-box binding sites (E-box 1,-2,-3).

(b) pTRACER (ctrl), pTRACER-pea3 (Pea3) and/or pCR3-USF-1 (USF-1) expression vectors and the luciferase reporter vector containing the wild-type or mutated fragments (E-box m1, E-box m2 and E-box m1/2) of the minimal bax promoter (PMBaxSp1dl (–100 to –1)) or the corresponding empty luciferase reporter vector were transiently co-transfected in TAC-2.1 cells. Luciferase activities were obtained and presented as in figure 3b.
Pea3 and USF-1 cooperate to transactivate the *bax* promoter

(c) pTRACER (ctrl), pTRACER-*pea3* (Pea3) and/or pCR3-USF-1 (USF-1) expression vectors and the luciferase reporter vector containing the wild-type or mutated fragments (E-box m1/2 and E-box m1/2/3) of the PMBaxSacII *bax* promoter (–162 to –1) or the corresponding empty luciferase reporter vector were transiently co-transfected in TAC-2.1 cells. Luciferase activities were obtained and presented as in figure 3b.

**Figure 7 :** A USF-1 dominant negative abolishes the Pea3-induced transactivation ability of the *bax* promoter.

pTRACER (ctrl), pTRACER-*pea3* (Pea3) and/or pCR3-USF-2a (USF-2a), pCR3-USF-1 (USF-1) or dominant negative pCR3-TDUSF-1 (TDUSF-1) expression vectors and the luciferase reporter vector containing the minimal *bax* promoter fragment (PMBaxSp1dl (–100 to –1)) or the corresponding empty luciferase reporter vector were transiently co-transfected in TAC-2.1 cells. Luciferase activities were obtained as in figure 3b and presented relative to value obtained with the corresponding expression vectors on the empty reporter vector.

**Figure 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 [*32*P]-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,
Pea3 and USF-1 cooperate to transactivate the *bax* promoter

excess of the unlabeled non-specific 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*, non-relevant Pim-1 antibody (200 ng).

**Figure 9**: Pea3 can act synergistically with USF-1 on the HLH response elements of the L-pyruvate kinase promoter.

(a) pTRACER (ctrl), pTRACER-*pea3* (Pea3) and/or pCR3-USF-1 (USF-1) expression vectors and the pk-luci96(L4) or the pk-luci96 luciferase reporter vectors were transiently co-transfected in TAC-2.1 cells. Luciferase activities were obtained and presented as in figure 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 figure 3b.

**Figure 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 DNA-binding.
Pea3 and USF-1 cooperate to transactivate the \( bax \) promoter

**Figure 1**

(a) Western blot analysis for \( bax \) and cyclophilin.

(b) Western blot analysis for Bax and Actin.
Pea3 and USF-1 cooperate to transactivate the bax promoter.

Figure 3

(a) Schematic representation of the bax promoter region showing the positions of various restriction enzyme sites. 

(b) Bar graph showing relative luciferase activity for different constructs. The constructs are labeled with their respective enzyme sites: PMBaxPF, PMBaxKpnI, PMBaxBstXI, PMBaxSacI, PMBaxSacII, PMBaxPstI, PMBaxSpeI, and PMBaxSp1dl. The expression levels are compared with control (ctrl), Erm, Er81, and Pea3 conditions.
Pea3 and USF-1 cooperate to transactivate the \textit{bax} promoter

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
 & PF & KpnI & BstXI & SacI & SacII & PstI & Spel & Sp1dl \\
\hline
Erm & 10 & 7 & 7 & 8.7 & 7.5 & 6 & 5 & 8 \\
Er81 & 4 & 3.6 & 3 & 4.2 & 3 & 2.6 & 2.6 & 3.2 \\
Pea3 & 24 & 24 & 18 & 14 & 16 & 10 & 9 & 10 \\
\hline
\end{tabular}
\end{table}

Figure 3C
Pea3 and USF-1 cooperate to transactivate the bax promoter

Figure 4
Figure 5

Pea3 and USF-1 cooperate to transactivate the bax promoter.
Figure 5

Relative Luciferase Activity
Pea3 and USF-1 cooperate to transactivate the box promoter

(a) Schematic representation of the box promoter region with indicated restriction sites (SacI, SacII, SpI1d1) and E-boxes (3, 2, 1).

(b) Bar graph showing relative luciferase activity for PMBaxSp1dl constructs with different conditions: ctrl, Pea3, USF-1, Pea3 + USF-1. The x-axis represents empty, Wild-type, E-box m1, E-box m2, and E-box m1/2.
Pea3 and USF-1 cooperate to transactivate the box promoter.

**Figure 6**

Bar chart showing the relative luciferase activity across different experimental conditions: empty, Wild-type, E-box m1/2, E-box m1/2/3. The conditions are denoted as ctrl, Pea3, USF-1, and Pea3 + USF-1.
Pea3 and USF-1 cooperate to transactivate the bax promoter.
Pea3 and USF-1 cooperate to transactivate the bax promoter.
Pea3 and USF-1 cooperate to transactivate the box promoter.
Pea3 and USF-1 cooperate to transactivate the bax promoter.

Figure 10
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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J. Biol. Chem. published online October 5, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M408017200

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