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Creation of Estrogen Resistance *in Vivo* by Transgenic Overexpression of the Heterogeneous Nuclear Ribonucleoprotein-Related Estrogen Response Element Binding Protein

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Estrogen unresponsiveness among primate species can result from overexpression of a heterogeneous nuclear ribonucleoprotein (hnRNP) that competes with estrogen receptor (ER) for binding to the estrogen-response element (ERE). This hnRNP has been coined the "ERE-binding protein" (ERE-BP). The ERE-BP is a member of the hnRNP C-like subfamily of hnRNPs, traditionally considered to be single-strand RNA binding proteins designed for the stabilization and handling of pre-mRNA. To verify *in vivo* the dominant-negative actions of the ERE-BP to inhibit ER-ERE-directed transactivation and to avoid the potential for lethality from global overexpression of an hnRNP, we generated transgenic mice that overexpressed ERE-BP in breast tissue under the control of a whey acidic protein gene promoter. Graded overexpression of ERE-BP in transgenic mice was established. Founders were viable and fertile. Female transgenics in all lines gave birth to pups, but their ability to nurse was dependent on the level of ERE-BP expression in breast; high-ERE-BP expressors were unable to lactate. A gradient of impaired breast pheno(histo-)type, from near normal to failed ductal development and lactational capacity, correlated with the relative level of transgene expression. ERE-BP, expressed either endogenously as a transgene or after transfection, colocalized with ERα in the nucleus of target cells. This work confirms that tissue-targeted overexpression of the ERE-BP can effectively block estrogen-ERα-ERE-directed action *in vivo*. (*Endocrinology* 146: 4266–4273, 2005)

NEW WORLD PRIMATES (NWPs) exhibit a compensated form of resistance to gonadal steroid hormones. In female monkeys, the hormone-resistant state is characterized biochemically by increased circulating levels of 17β-estradiol and progesterone (1) and anatomically by gonadal hypertrophy (2). In their native environment, NWPs have adapted well to this state of gonadal steroid resistance, because they exhibit normal reproductive capabilities (3). The reason why NWP species require such high circulating levels of gonadal steroids has been unclear and debated (2, 4, 5). In the past several years, we have identified, characterized, cloned, and expressed a protein that legislates estrogen resistance in NWPs (6, 7). Compared with cells from Old World primates (OWPs), including human cells, this protein is greatly overexpressed in NWP cells and competes *in trans* with estrogen receptor (ER) for binding to the estrogen-response element (ERE) (see Fig. 1). As such, the protein has been coined the "ERE binding protein" (ERE-BP).

The ERE-BP is a member of the heterogeneous nuclear ribonucleoprotein (hnRNP) C-like subfamily of hnRNPs (6, 7). There are now at least 20 different hnRNPs recognized to be present and active in mammalian cells (8, 9). Traditionally, the hnRNPs have been considered to be single-strand RNA binding proteins designed for the stabilization and handling of pre-mRNA. However, recent studies have shown hnRNPs to 1) possess the potential for both single- and double-strand DNA binding (6, 7, 10, 11) and 2) regulate transcription (12–14), as well as 3) modulate splicing events (15). When present in high enough concentration in the cell, the hnRNP-related hormone response element binding proteins can block steroid hormone action by competing with receptor dimers for binding to their cognate hormone response element (11). In the case of the ERE-BP *in vitro*, it is able to effectively compete with the liganded ERα for binding to the ERE and inhibit transactivation of estradiol (E2)-ERα-ERE-driven genes (7).

There are two approaches to document ERE-BP-mediated estrogen resistance *in vivo*: 1) relief from overexpression of ERE-BP *in vivo* restores the estrogen-responsive (wild-type) phenotype in hormone-resistant NWPs; and 2) overexpression of ERE-BP *in vivo* on a wild-type, estrogen-responsive

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Abbreviations: E2, Estradiol; ER, estrogen receptor; ERE, estrogen response element; ERE-BP, estrogen response element-binding protein; FITC, fluorescein thiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H&E, hematoxylin-eosin; hnRNP, heterogeneous nuclear ribonucleoprotein; NWP, New World primates; OWP, Old World primates; WAP, whey acidic protein.

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background transduces the same estrogen-resistant phenotype characteristic of NWPs. Because knock-out transgenesis in primates is beyond feasibility at this point and because we want to mimic the gain-of-function mutation created by nature, we used the mouse as a hormone-responsive surrogate for the OWPs to pursue the second approach. We used the whey acidic protein (WAP) gene promoter to drive ERE-BP overexpression in mouse breast to exclude the likely outcome of prenatal and/or early postnatal lethality from global overexpression of the ERE-BP transgene. In this paper, we demonstrate creation of lactation-deficient mice resulting from the targeted overexpression of ERE-BP in the breast.

Materials and Methods

Generation of transgenic mice overexpressing the ERE-BP under the control of the WAP gene promoter

A full-length ERE-BP cDNA (7) was ligated to the 3′ end of the rabbit β-globin second intron by insertion into the *Eco*RI and *Xho*I sites of plasmid pKBPA (provided by Dr. F. DeMayo, Baylor College of Medicine, Houston, TX) (see Fig. 2). The 2.6-kb WAP promoter transgene cassette provided by Dr. L. Hennighausen (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD) (16, 17) was cloned into T-Easy Vector (Promega Madison, WI), digested with *Not*I, and inserted into the *Not*I site at the 5′ end of the rabbit β-globin second-intron ERE-BP. The excised DNA fragment was sequenced and then microinjected into FVB/N fertilized eggs. The founder animals were verified by Southern analysis of tail DNA as described previously (18). Transgenic offspring were subsequently identified by PCR using a set of primers specific for the transgene, possessing the WAP-promoter forward primer 5′CAAAGTCTTCCTCCTGTGGG-3′ and rabbit β-globin second-intron reverse primer 5′-GGTGATACAAGGGACATCTTCC-3′. Five different transgenic lines were established, of which three, representing variable levels of transgene expression in the breast, are depicted in Fig. 3. All mice were cared for and used in accordance with institutional animal care policies.

RNA extraction, Northern blot, and RT-PCR analysis

Total RNA was isolated from cells and tissue using the Qiagen (Valencia, CA) RNAeasy kit, and Northern blots were performed as described previously (7). Whole ERE-BP cDNA was used as probe in Northern blots. For RT-PCR, 2 μg of total RNA were incubated with deoxyribonuclease I, and RT-PCR was performed with the one-step RT-PCR kit (Qiagen) according to the protocol of the manufacturer. The sequences for each primer set were as follows: ERα 5′ primer, AATGGAGTCTGCCA-AGGAGAC; ERα 3′ primer, CTTCAACATTCTCCCTCCTC; WAP 5′ primer, ATGCGTTG-CCTCATCAGCC; WAP 3′ primer, CTGAAGGGTTATCACTGGCA; ERE-BP 5′ primer, ACTATGTCGGAGGAGCAGTTCGGCG; ERE-BP 3′ primer, GGCTTTGGCCCTTTTAG-GATCAATCAC; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 5′ primer, AAGGCTGTGGGCAAGGTCAT; and GAPDH 3′ primer, CATACCAGGAAATGAGCTTGAC. The correct size of PCR products was verified by 2% agarose gel electrophoresis.

Western blot analysis

Western blotting was performed as described previously (6, 7). The membranes were blocked with 5% nonfat dry milk for 1 h and then incubated with a rabbit polyclonal antiprimate ERE-BP (Chen H., unpublished observation), anti-pS2 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-ERα (Santa Cruz Biotechnology) antibody for 2 h, followed by horseradish peroxidase-conjugated secondary antibody for another 1 h before detection of antibody-reactive proteins with a chemiluminescence reagent (ECL; Amersham Biosciences, Arlington Heights, IL).

Generation of cell lines stably overexpressing ERE-BP

E2-responsive SKOV-3 human ovarian carcinoma cells and MCF-7 human breast cancer cells (American Type Culture Collection, Rockville, MD) were incubated with 5.0 μg of pcDNA3.1/v5-His-TOPO ERE-BP plasmid in lipoTAXI solution for 5 h, followed by the addition of equal volume of 20% fetal calf serum-supplemented medium. After incubation overnight, cells were split (1:10 ratio) and incubated with fresh medium containing 500 μg/ml of the geneticin-selective antibiotic G418 sulfate (Invitrogen, Carlsbad, CA). This medium was replaced every 3–4 d until stable colonies formed. Single colonies were picked, transferred into a new dish, and incubated with medium containing the selection antibiotic G418 until confluence was attained.

Breast whole-mount preparation, histology, and immunohistochemistry

Mammary tissue was harvested from euthanized mice. For whole-mount examination, the mammary tissue was fixed in Carnoy's solution for 4 h and stained with carmine aluminum overnight (19). For histological analysis, the mammary tissue was fixed overnight in 10% formaldehyde and processed by standard protocols. Paraffin sections (5 mm) were stained with hematoxylin-eosin (H&E) for histologic evaluation. For immunostaining, deparaffinized slides were incubated overnight with anti-ERE-BP antibody and/or antihuman ERα antibody and anti-WAP antibody (Santa Cruz Biotechnology). A primary 2 h incubation was followed by a 30 min incubation with the appropriate biotin-conjugated secondary antibody and by a 30 min exposure to Vectastain ABC reagent (Vector Laboratories, Burlingame, CA). The slides were then incubated with 3,3′-diaminobenzidine developing solution.

For immunofluorescent staining, cells were grown overnight on coverslips in MEM and fixed by exposure to cold acetone for 5 min after washing with PBS. Coverslips were incubated with normal bovine serum for 30 min at room temperature, followed by the addition of primary antibodies (monoclonal goat antihuman ERα antibodies and polyclonal rabbit anti-ERE-BP antibody) overnight at 4 C. Specifically bound antibody was visualized using fluorescein thiocyanate (FITC) and/or Texas Red-conjugated fluorescent secondary antibodies (duck antigoat and duck antirabbit, respectively; Santa Cruz Biotechnology) under an Olympus Optical (Tokyo, Japan) BX41 microscope. Normal goat/rabbit IgG was used as negative control antiserum.

ERE-luciferase reporter activity

The 5×10^5 MCF-7 breast cancer cells were seeded into six-well plates in phenol red-free medium containing 10% charcoal-stripped fetal calf serum and allowed to proliferate to 90% confluence. Transfections were performed in triplicate with the following combinations of DNA preparations to a maximum final concentration of 20 μg DNA/ml in LipoTAXI solution (Stratagene, La Jolla, CA): 1) 5.5 μg of ERE-luciferase reporter plasmid; 2) 5.0 μg of ERE-BP plasmid (in cDNA3.1/his/v5 TOPO vector); 3) 5.0 μg of β-galactosidase expression construct as internal control; and 4) pGEM-3z vector DNA as carrier (Promega). An equal volume of 20% fetal calf serum-supplemented, antibiotic-free medium was added to each well 5 h after transfection, followed by the addition of 0.1–10 nM E2. After an additional 48 h at 37 C, the cells were lysed, and luciferase and β-galactosidase activities were measured.

Statistics

An unpaired Student's *t* test was used to compare the statistical difference of the means between experimental and control groups.

Results

Creation and characterization of WAP-ERE-BP transgenic mice

ERE-BP has been shown to inhibit ER-ERE-directed transactivation when overexpressed, either transiently or stably in wild-type, estrogen-responsive OWP cells (7) (Chen H., unpublished observation), mimicking *in vitro* the biochemical phenotype of the naturally estrogen-resistant NWPs (Fig. 1). To confirm that the transactivational inhibiting effects of ERE-BP *in vitro* could be recapitulated *in vivo*, we created transgenic mice that constitutively overexpressed the

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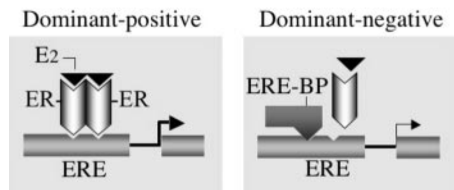

FIG. 1. Interaction of the ERE with competing *trans* factors. Shown is a simplified schematic of the proposed means by which 1) estrogen (E2) interacts with the ERα to promote ER-ERE-directed transcription of E2-responsive genes (*left*) and 2) the hnRNP-related ERE-BP inhibits, in a dominant-negative mode, ER-ERE-directed transcription of E2-responsive genes (*right*).

ERE-BP in breast tissue. Mechanistically speaking, this was an attempt to convert an ER-expressing, estrogen-responsive tissue, the breast, to one that was estrogen resistant. Use of the WAP-ERE-BP DNA (Fig. 2) for microinjection proved successful in generating five founder lines. When assessed by RT-PCR and compared with wild-type mice, lines of mice bearing relatively low, intermediate, and high levels of the transgene expression in breast were generated; data from representative mice showing relatively low, medium, and high ERE-BP transgene expression are shown in Fig. 3A. The rank order of expression of the ERE-BP transgene was recapitulated at the protein level (Fig. 3B). Whereas alterations in the level of ERE-BP transgene expression in breast were not associated with a change in endogenous, constitutively expressed genes, expression of the estrogen-responsive pS2 gene product was inversely associated with the level of ERE-BP expression.

WAP-ERE-BP transgene product expression

To confirm tissue-specific expression of ERE-BP, ovary, lung, and kidney harvested from +/− transgenic females in the high-ERE-BP-expressing line were subjected to Western blot analysis with anti-ERE-BP antibody (Fig. 4A). As expected, transgene product was encountered only in breast. Another set of controls consisted of protein extracted from male and female wild-type and high-expressing line breast. It was anticipated that the ERE-BP transgene product would be present in the male as well as the female breast of animals from the high ERE-BP-expressing line. Unexpectedly, no ERE-BP was detected in the breasts of male ERE-BP +/− mice, indicating that there existed sex difference in the expression of the ERE-BP transgene product. When assessed by RT-PCR (Fig. 4B) and Northern blot (data not shown) analysis, male breast from the transgenic line contained levels of ERE-BP mRNA only 18 ± 10% (Fig. 4C), less than that found in the breast of transgenic line females, indicating that the sex

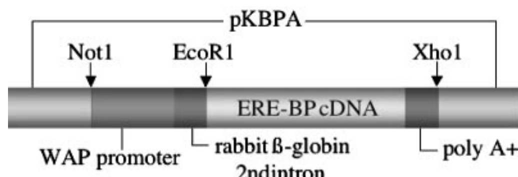

FIG. 2. ERE-BP transgenic construct. Shown in linearized format are the important elements of and restriction sites for construction of the WAP-driven ERE-BP cDNA used for generation of transgenic mice.

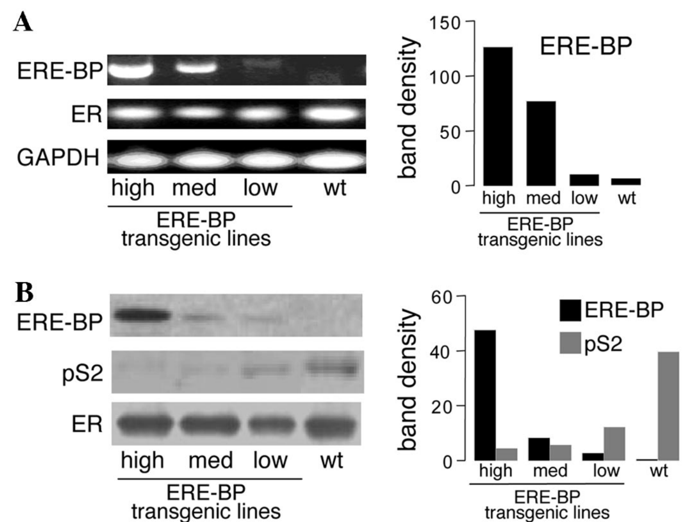

FIG. 3. ERE-BP detection in the breast of transgenic and wild-type mice. A, ERE-BP transgene product at the RNA level by RT-PCR as well as the constitutively expressed endogenous ERα and housekeeping gene GAPDH (*left*) in breast tissue extracts of representative lines of transgenic mice expressing relatively low, intermediate (med), and high levels of ERE-BP RNA and of wild-type (wt) mice. Results at the *right* are the mean of duplicate densitometric determinations of ERE-BP transcript expression in the ERE-BP-expressing transgenic and wild-type mice. B, Relative protein levels of the ERE-BP, the estrogen-responsive pS2 gene product, and the constitutively expressed ERα by Western blot of extracts of mammary tissue of representative lines of transgenic mice expressing relatively low, intermediate (med), and high levels of ERE-BP and of wild-type mice. The tissue extracts from three individual littermates from each genotype were pooled before loading onto the gel. On the *right* are the densitometric determinations of the Western blots of pooled extracts for ERE-BP, pS2, and ER protein expression in the ERE-BP-expressing transgenic and wild-type mice.

difference in ERE-BP expression was asserted principally at the posttranscriptional level.

Postnatal viability of WAP-ERE-BP transgenic animals

Figure 5A demonstrates pup viability on postnatal d 1 and 21 in five separate litters born of the various founder lines. Litter size and postnatal pup viability on postnatal d 1 were not different among the different transgenic lines compared with wild-type mice. All pups born of wild-type and low-ERE-BP-expressing mothers survived to weaning (d 21). A total of 32% of the pups born to mothers with intermediate levels of the ERE-BP transgene in breast did not survive to weaning; this was a significantly reduced survival rate (P = 0.004) compared with wild-type and low-ERE-BP-expressing mothers. Mothers from the high-ERE-BP-expressing line were unable to nurse; no milk was detected in the stomachs of pups born to mother expressing high levels of the ERE-BP transgene in the breast. No pups from mothers of the high-ERE-BP line survived to d 21. The time course of postnatal mortality of five separate litters of pups born to mothers in the high-ERE-BP expression line is shown in Fig. 5B. Most pups of high-ERE-BP expression line mothers died within the first 3 postnatal days. Mortality in these pups was 100% by postnatal d 7. Postnatal mortality of pups born to high-ERE-BP-expressing +/− mothers was due to starvation; no milk

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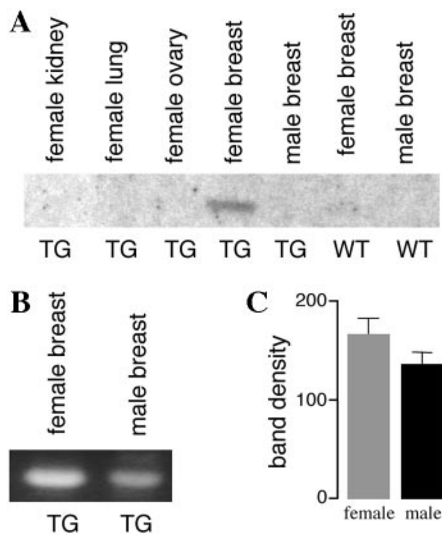

FIG. 4. Sexual differences in WAP-ERE-BP transgene product expression. A, Western blot of the anti-ERE-BP antibody-reactive protein in tissue extracts of high-ERE-BP-expressing male and female +/− transgenic (TG; lanes 1–5) and wild-type (WT; lanes 6–7) mice, demonstrating the restriction of protein expression to the breast in female transgenic mice. B, RT-PCR detection of ERE-BP-specific RNA in both female and male breast tissue from the high-ERE-BP-expressing transgenic line. C, Densinometric analyses of ERE-BP transgene expression in the breast of female and male mice from the high-ERE-BP-expressing transgenic line; values are mean ± SD of triplicate measures of ERE-BP RNA.

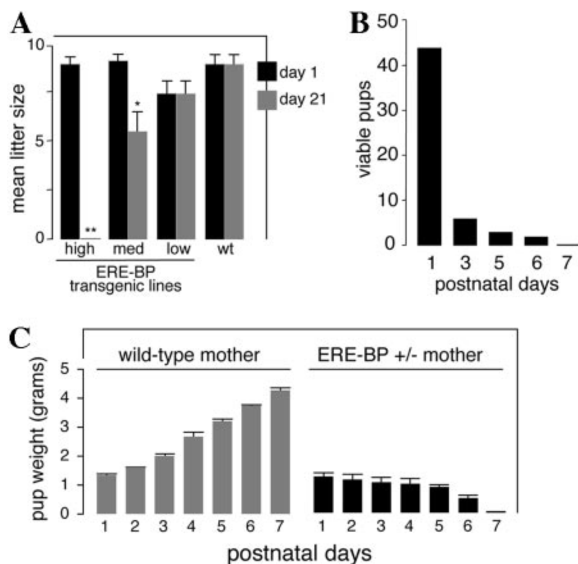

FIG. 5. Postnatal viability of WAP-ERE-BP transgenic animals. A, Mean ± SD litter size (n = 5 individual litters) on postnatal d 1 and 21 (i.e. weaning day) of pups born into the relatively low-, intermediate- (med), and high-ERE-BP-expressing founder lines compared with pups born of wild-type mothers (wt). Survival was significantly reduced in the litters born to mothers expressing intermediate (*, P = 0.04) and high (**, P = 0.005) levels of ERE-BP in the breast. B, Time course of cumulative postnatal mortality in five different litters born to a +/− mother in the high-ERE-BP-expressing line. C, Increase in body weight in pups born to wild-type mothers (*left*) and a decrease in weight in pups nursed by high-ERE-BP-expressing +/− mothers (*right*). Data are the cumulative means ± SD of pups in five different litters.

could be detected in the stomachs of suckling pups. Figure 5C shows the incremental decrease in body weight of neonates born to high-ERE-BP-expressing mothers compared with an incremental increase in weight of pups nursed by wild-type mothers. A rebound increase in body weight was observed in pups born of high ERE-BP-expressing mothers if fostered to a wild-type mother through postnatal d 1–4 (data not shown).

Mammary gland growth and development is impaired in WAP-ERE-BP transgenic mice

Figure 6A shows breast whole-mount and histology of pubertal wild-type, low-, intermediate-, and high-ERE-BP-overexpressing mice. Whole mounts of wild-type and low-ERE-BP mice pubertal mammary tissue at 8 weeks demonstrated budding and branching ductal elements extending to the periphery of the mammary fat pads. By comparison, mammary tissue from intermediate- and high-ERE-BP-expressing pubertal females showed increasingly rudimentary ductal structures with moderately to markedly reduced branching and budding, respectively; a significant reduction in pubertal ductal structures in high- and intermediate-ERE-BP +/− mice was confirmed by histomorphometric analyses (Fig. 6B). As expected, there was no difference in wild-type and transgenic breast tissue harvested from prepubertal, 3-week-old mice (data not shown), indicating no endogenous estrogen effect on the breast at the stage of development.

Figure 7A shows breast whole mounts and H&E morphology (200×) of wild-type and transgenic lines expressing relatively low, intermediate, and high levels of the ERE-BP

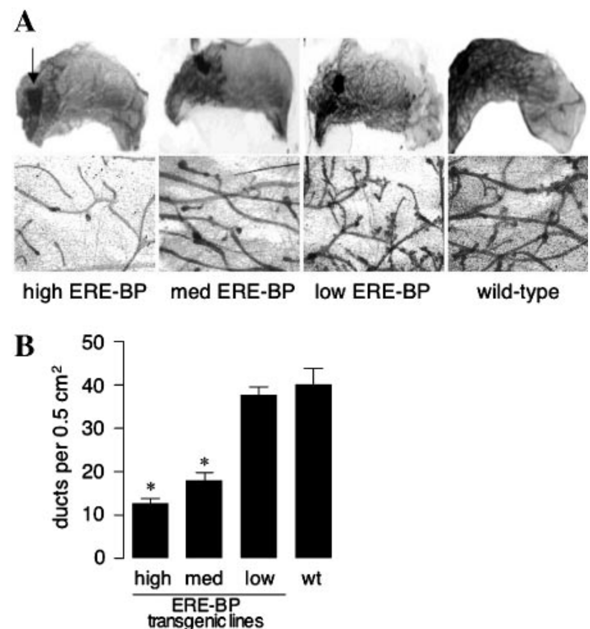

FIG. 6. Impaired mammary gland growth and development in WAP-ERE-BP transgenic mice. Shown (*left* to *right*) in panel A are breast tissue whole mounts (×2, *top row*; ×40, *bottom row*) from pubertal (8 weeks) high-, intermediate- (med), and low-ERE-BP-expressing and wild-type (wt) mice. The *arrow* indicates the lymph node from which the developing ductal structures emanate. B, Mean ± SD ductal number per 0.5 cm^2 of breast according to genotype of the host.

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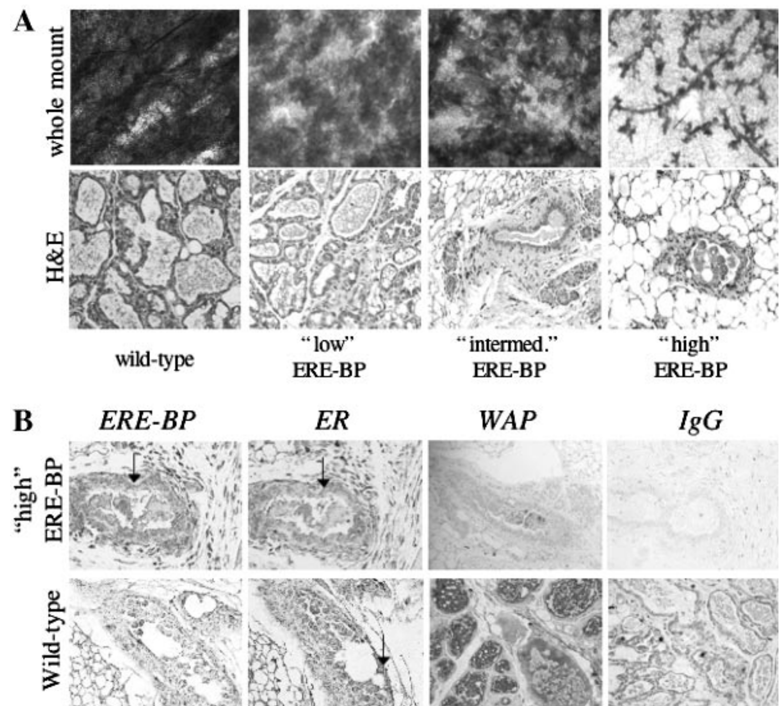

FIG. 7. Dose-dependent ERE-BP transgene effect on lactation. A, Whole-mount (×40, *top row*) and H&E (×200, *bottom row*) histology of the d 3 lactating breast of wild-type, low-, intermediate- (intermed.), and high-ERE-BP-expressing mice. The fluffy staining depicts milk in the tissue section. B, Immunostaining (×200) of the ERE-BP, ERα, WAP, and nonspecific IgG in the breast of high-ERE-BP-overexpressing mice (*top*) and wild-type mice (*bottom*) on what would be lactation d 3. ERE-BP was detected in the breast epithelium of ERE-BP +/− mice (*arrow*) but not wild-type mice, and WAP was only minimally expressed in ERE-BP +/− compared with wild-type mouse breast. ERα (*arrows*) was expressed in both lines. Specificity of ERE-BP expression was confirmed with a nonspecific IgG in methyl green-counterstained breast tissue.

transgene on what would be lactation d 3 for female wild-type mice, demonstrating a transgene effect on the lactation phenotype of breast tissue. The wild-type and, to a slightly lesser extent, low-ERE-BP-expressing breast demonstrated an abundance of dilated milk-filled ductal elements. Coincident H&E histology showed "normal" lactating mammary tissue in the wild-type and low-ERE-BP-expressing breast; these sections disclosed the typical effacement of intralobular and interlobular stroma caused by exuberant enlargement of ductal lobules containing dilated acini. Although less sparse in number, breast tissue from mice expressing an intermediate level of the ERE-BP transgene demonstrated the capacity to produce milk. By comparison, the high-ERE-BP-expressing, d 3 lactating breast demonstrated only scattered ductal elements with far less acinar formation and progressively more prominent interlobular fat reminiscent of the immature pubertal, nonlactating breast (Fig. 6). As just noted (Fig. 5), all pups born to the high-ERE-BP-expressing mothers either died or required fostering; no milk was detectable in the stomachs of pups suckling on high-ERE-BP-expressing mothers, regardless of the genotype of the suckling pups. Lactation failure in the high-ERE-BP-expressing breast was confirmed by markedly reduced immunohistochemical detection of the estrogen-responsive milk protein WAP in the breast epithelium of high-ERE-BP-expressing +/− female mice (Fig. 7), despite abundant expression of ERα in that tissue.

Histochemical colocalization of the ERE-BP and ERα in breast

If, as postulated (Fig. 1), the ERE-BP blocks ERα-directed action by competing with the ERα for binding to the ERE in the promoter of estrogen-regulated genes in the mammalian breast, then the ERE-BP and ERα should be colocalized to the same cell. Figure 8A shows by immunohistochemistry that the endogenously expressed ERα and ERE-BP transgene were both expressed in mammary epithelial cells of the adult breast from ERE-BP-high-expressing +/− female mice. Double immunohistochemistry demonstrated colocalization of the ERα and ERE-BP in the perinuclear and nuclear region of the breast epithelial cell. This finding of ERα and ERE-BP colocalization was confirmed in SKOV-3 ovarian carcinoma cells stably transfected with and expressing the ERE-BP cDNA compared with mock-transfected, non-ERE-BP-expressing SKOV-3 cells (Fig. 8, B and C). Figure 8B confirms that transfected cells constitutively expressed ERE-BP, whereas the mock-transfected cells did not. Figure 8C confirms the pattern colocalization for the transfected ERE-BP and ERα as that obtained in transgenic breast with endogenously expressed ERE-BP and ERα (see panel A); untransfected human SKOV-3 cells demonstrate a small amount of endogenous ERE-BP expression that appears to colocalize with endogenously expressed ERα.

Effect of 17β-estradiol on ERα/ERE-BP-ERE-directed transactivation

In vivo, NWPs "rescue" themselves from the overproduction of ERE-BP by boosting endogenous E2 production at the level of the ovary (1, 2). We sought to reproduce *in vitro* rescue from the dominant-negative effects of ERE-BP by exposure of ERα-positive, ERE-BP-overexpressing MCF-7 cells to increasing amounts of exogenously administered ligand E2. Figure 9 shows the response in ERE-reporter activity of mock-transfected (control; *left*) and ERE-BP-overexpressing MCF-7 (*right*) cells after exposure to increasing concentrations of E2 in the extracellular medium. Maximum reporter activity in control MCF-7 cells was achieved at 0.1 nM E2. In contrast, E2-ERE-directed reporter activity was

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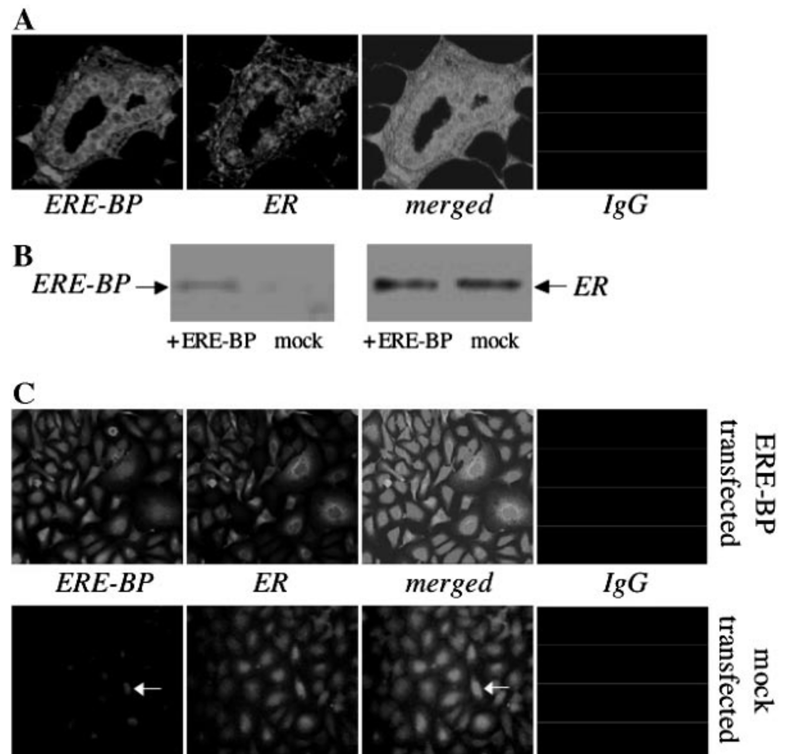

FIG. 8. Cellular localization of ERE-BP. A, Double immunofluorescence (×200) of adult mammary ductal epithelium from transgenic mice expressing high levels of ERE-BP in breast, using rhodamine-tagged anti-ERE-BP and FITC-tagged anti-ERα antibodies. The merged image demonstrates colocalization of the two proteins. Normal rabbit IgG with FITC-conjugated antirabbit antibody served as a "negative" control. B, Expression of transfected ERE-BP (*left*) and endogenous ERα (*right*) by Western blot in human ovarian carcinoma SKOV-3 cells stably transfected with ERE-BP (+*ERE-BP*) and mock-transfected (*mock*) control cells. C, Double immunofluorescence and colocalization of the ERE-BP and ERα in ERE-BP-transfected (*top row*) but not in mock-transfected (*bottom row*) cells.

significantly reduced in ERE-BP-transfected cells at all concentrations of E2 examined; half-maximal reporter activity was achieved with the same concentration of ligand E2 required to generate a maximal response in mock-transfected cells.

Discussion

The hnRNPs were first recognized for their ability to bind single-strand ribopolynucleotides and stabilize those pre-mRNAs for nuclear export and translation (8, 9). The super-

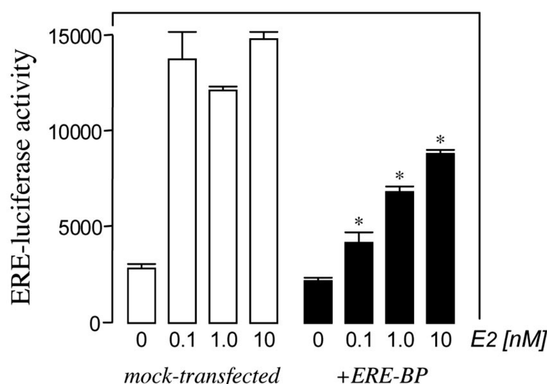

FIG. 9. Rescue from ERE-BP-mediated inhibiting of ER-ERE-directed transactivation with estrogen. Shown is ERE-directed luciferase reporter activity in ERα-positive MCF-7 human breast cancer cells mock-transfected (control; *left*) or transiently transfected with ERE-BP (*right*) after exposure to hormone-free medium or medium containing increasing concentrations of ligand E2. Data are the mean ± SD of triplicate assessments of ERE-luciferase activity; *, significant change ($P \leq 0.01$) from baseline (*i.e.* in the absence of added E2) as well as a significant reduction from comparable conditions in vector-alone-transfected cells.

family of hnRNPs have been primarily classified on the basis of their sequence-specific RNA binding potential. Although initially thought to be confined to the nuclear compartment of the cell, it is now recognized that some of these proteins can travel with their pre-mRNA cargo to the cell cytoplasm (20). That certainly appears to be the case with the hnRNP C-like-related ERE-BP under examination here; although found in the nucleus, it can be detected in the cell cytoplasm (Fig. 8). The hnRNPs have also been implicated in regulation of splicing (21–23), being able to interact with specific ribonucleotide sequences in single-strand format as well as with other protein elements of the splicesome machinery. However, only recently have the double-strand DNA binding and functional hnRNP-promoter interaction capacities of this class of molecules been recognized (6, 7, 10, 11). In all instances so far described, the hnRNPs appear to act to inhibit initiation of transcription.

In contrast to the hnRNPs, which act to inhibit transactivation, steroid hormone receptors constitute a superfamily of generally protransactivational proteins that are regulated allosterically by their high-affinity interaction with small hydrophobic sterol/steroid ligands. In the case of the ERα, the specific ligand for the receptor in most eukaryotic cells, including human cells, is E2. Interaction of the ER with E2 leads to ER dimerization and interaction of the dimer pair with its specific DNA target, the ERE (Fig. 1, *left*). Any factor(s) that competes with ER for binding to its cognate ERE would be expected to inhibit E2-directed transcriptional regulation (Figs. 3B and 7B). Although such competition for ERE occupancy can be demonstrated *in vitro* (7), there has been no previous documentation that such events, involving hnRNPs and steroid receptors as competitive *trans* elements and a hormone responsive element as a *cis* target, can occur *in vivo*.

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The results of the current transgenic study provide evidence that such a balance (Fig. 1) between the dominant-positive effects of the ERα and dominant-negative effects of the ERE-BP on E2-driven breast development can be observed *in vivo* (Figs. 5 and 6).

Because the hnRNPs are ubiquitous in their tissue distribution, crucial for normal pre-mRNA handling, and relatively highly conserved in functional domain structure (8), a major concern harbored at the outset of the transgenic experiments presented here was that the creation of a transgenic animal overexpressing ERE-BP globally would result in prenatal or immediate postnatal mortality from interference with estrogen-driven events *in utero*. As a consequence, we settled on a strategy to overexpress the ERE-BP in breast tissue using the WAP promoter as a targeting vehicle. Knowing that the WAP promoter was expressed principally during pregnancy and lactation (24–26) and its expression would not therefore be required for successful postnatal survival, we predicted that breast-targeted overexpression of ERE-BP would have its greatest adverse impact during lactation. This prediction was confirmed in that mothers expressing intermediate and high levels of the transgene in breast were deficient or devoid of lactational capacity, respectively (Figs. 5–7).

Somewhat unexpected was the finding that, compared with prepubertal 3-week-old mice carrying the transgene, breast development was inhibited phenotypically by transgene expression during puberty with the onset of gonadal steroid production. These data are compatible with previous experimental results indicating that the WAP promoter is estrogen responsive (24). These data also suggest that pubertal enhancement of the WAP promoter is either directly or indirectly modulated through an ERE, because pubertal breast development impairment is positively correlated with the level of transgene expression in that tissue (Fig. 6). Preliminary chromatin immunoprecipitation results (Chen H., unpublished observation) indicate that the ERE-BP interacts specifically with traditional ERE motifs and not with "nontraditional" estrogen-responsive activator protein-1 sites (25); both an ERE and activator protein-1 site exists in the WAP promoter (26).

Moreover, as we predicted from the dominant-negative mode of action of the ERE-BP for the ERE *in vitro* (7) (Fig. 1), there was a quantitative gradient in this effect of the breast-targeted transgene. The presence of relatively high concentrations of ERE-BP in an E2-ERα target tissue or cell, as is observed in the breast of the high-ERE-BP-expressing transgenic line *in vivo* and in the ERα-positive human ovarian carcinoma SKOV-3 cells overexpressing ERE-BP *in vitro* (Fig. 8), respectively, can "out compete" the ER-ER homodimer for a position on the ERE, giving rise to a more pronounced ERα-resistant phenotype (Fig. 1). However, when the ERE-BP is present in relatively lower concentrations and/or the ERα-selective ligand E2 is present in relatively higher concentrations in the target tissue, as was the case with our relatively low-ERE-BP-expressing transgenic mouse lines and E2-treated MCF-7 cells (Fig. 9), then mammary gland E2-ERα-ERE-directed transactivation and subsequent ductal development could proceed (Figs. 6 and 7). These data support the concept that the balance in E2 action at its target tissue can be reostatically modified by the quantity of dominant-negative-acting ERE-BP expressed in a manner similar to that described previously for the quantitative presence of the ER, ligand E2, corepressor/coactivators (27–31), as well as the heat shock protein-27-related intracellular E2 binding protein (32, 33) in the target cell. However, in contrast to the ER and intracellular E2 binding protein, the ERE-BP does not specifically bind or respond directly to ligand E2.

In addition to quantitative differences in the ligand-modified receptor, transcription coregulatory molecules and the response element interactive, hnRNP-related proteins described here, the dynamic movement and compartmental colocalization of these factors in the cell will also contribute to the integrated control of steroid hormone-modifiable transcription (34). For the ERE-BP to directly antagonize the ER as we have postulated, the two molecules must coexist in the same cell. As is now recognized for other members of the hnRNP superfamily (9), the position of ERE-BP in the cell is not static (Fig. 8). ERE-BP can be detected in the nuclear and cytoplasmic compartments of the cell in which it colocalizes with the E2-liganded ERα. These results confirm the fact that the ERα and ERE-BP *trans* factors can be traced to the same locales in estrogen-responsive cells from mammalian hosts.

Of interest was the unexpected finding of sex difference in expression of the ERE-BP transgene product. ERE-BP was detected in the breast of female, but not male, transgenic animals; although males express the transgene RNA in the breast, they do not express the protein (Fig. 4). We hypothesize that there 1) exists a conditioning factor present in the female breast that permits translation of the protein and/or 2) exists an inhibitor of ERE-BP mRNA processing/translation in the male breast. Support for the role of E2 as such a conditioning factor is provided by analysis of expression of the ERE-BP in the breast tissue of estrogen-resistant NWPs after ovariectomy (Chen H., unpublished observation); ovariectomy greatly reduces expression of ERE-BP in NWP breast to levels observed in the breast tissue of estrogen-responsive OWP females. Collectively, these results suggest that there may exist a regulatory feedback system to control estrogen action in the female breast with expression, the dominant-negative-acting ERE-BP being upregulated by the dominant-positive-acting E2-liganded ER. In other words, the ERE-BP has the potential to "brake" the stimulatory actions of estrogen in any target tissue in which it is produced.

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