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Expression of Estrogen Receptors in Estrogen Receptor–Negative Human Breast Carcinoma Cells: Modulation of Epidermal Growth Factor-Receptor (EGF-R) and Transforming Growth Factor α (TGFα) Gene Expression

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Abstract A number of studies suggest that an inverse correlation exists between the epidermal growth factor–receptor and the estrogen receptor expression in primary human breast carcinoma as well as in established human breast carcinoma cell lines. Recent studies suggest that the epidermal growth factor–receptor does not regulate the estrogen receptor gene expression. Whether the estrogen receptor regulates the epidermal growth factor–receptor gene expression is not known. We addressed this question by stably transfecting the estrogen receptor cDNA into the estrogen receptor–negative human breast carcinoma cell line MDA-MB-231. Constitutive expression of functional estrogen receptors in the transfactants resulted in increased mRNA levels of both epidermal growth factor–receptor and transforming growth factor α. Estradiol treatment of transfected cells, although enhancing transforming growth factor α mRNA levels, did not modulate epidermal growth factor–receptor mRNA levels. The estrogen receptor–transfected cells grown in estrogenic regular medium, however, exhibited lower constitutive levels of epidermal growth factor–receptor mRNA than in steroid-stripped medium, suggesting that estrogens coupled with some factors normally present in the regular medium may indeed downmodulate epidermal growth factor–receptor mRNA. Sodium butyrate treatment enhanced epidermal growth factor–receptor mRNA levels in nontransfected cells grown in regular estrogenic as well as in steroid stripped medium. Sodium butyrate enhancement of epidermal growth factor–receptor mRNA levels was completely abolished in estrogen receptor–transfected cells grown in regular estrogenic medium and blunted in steroid stripped medium. Using various epidermal growth factor–receptor gene promoter-CAT constructs in transient transfection assays, we further demonstrate that sodium butyrate enhanced transcription of the epidermal growth factor–receptor gene. The putative sodium butyrate responsive element(s) appears to localize within the proximal 384 bp of the epidermal growth factor–receptor gene promoter region. Although the interactions between estrogen receptor and epidermal growth factor–receptor are rather complex, taken together, our data suggest that estrogen receptor can indeed modulate the epidermal growth factor–receptor mRNA expression.

Key words: gene regulation, stable transfection, CAT assay, sodium butyrate, mRNA

Polypeptide growth factors EGF (epidermal growth factor) and TGFα (transforming growth factor α) are known to act as autocrine growth factors in normal and malignant mammary epithelial cells [reviewed in Dickson et al., 1992]. Both of these growth factors work through the EGF-receptor (EGF-R), which is a 170 kDa transmembrane protein with tyrosine kinase activity [Downward et al., 1984]. Although the prognostic value of EGF-R as a tumor marker remains to be established, a sizeable amount of evidence shows an inverse correlation between the ER (estrogen receptor) status of human breast carcinoma (HBC) and the EGF-R expression [reviewed in Klijn et al., 1992]. For example, it has been shown that in primary human breast tumors as well as in established cell

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lines, EGF-R is expressed at a higher level in ER-negative tumors [reviewed in Klijn et al., 1992]. The ER-negative tumors which express higher levels of EGF-R tend to be highly metastatic and exhibit a higher incidence of tumor recurrence [reviewed in Klijn et al., 1992]. Whether a causal relationship exists between EGF-R and the ER status is, however, still not clear. The issue whether the expression of ER and EGF-R is truly under reciprocal control has not been fully investigated. A recent study [Valverius et al., 1990] has demonstrated that the overexpression of an exogenously transfected *EGF-R gene* and consequent higher EGF-R expression in ER-positive HBC cells was unable to modulate ER expression. Although that study [Valverius et al., 1990] suggested that the ER expression was not under the control of EGF-R, the question remained whether indeed the ERs negatively regulate the EGF-R gene expression.

deFazio et al. [1992] have recently shown that in HBC cells sodium butyrate (NaB), which is a potent differentiation and antiproliferative agent, controls ER and EGF-R gene expression in a reciprocal fashion. NaB treatment enhanced EGF-R mRNA levels in ER-positive cells that have extremely low constitutive EGF-R mRNA levels and concomitantly downregulated the ER gene expression. Whether NaB modulation of EGF-R gene expression in turn affects ER expression was also investigated by utilizing cell lines that express only one or the other receptor—that is, MDA-MB-134 VI (ER-positive, EGF-R–negative) and MDA-MB-231 (ER-negative, EGF-R–positive). NaB treatment downregulated the ER gene expression in MDA-MB-134 VI and EGF-R mRNA levels in MDA-MB-231. These data taken together with NaB modulation of EGF-R in ER-positive cells imply that the regulation of ER gene expression was independent of EGF-R expression but the regulation of EGF-R might be dependent on ER expression. If ERs indeed negatively regulate the EGF-R gene expression and not vice versa, then the expression of functional ERs in previously ER-negative cells should result in downregulation of EGF-R gene expression.

The present study was undertaken to address this issue and to determine more specifically whether constitutive expression of exogenously transfected ERs into ER-negative cells can modulate the EGF-R gene expression.

MATERIALS AND METHODS

Materials

Dulbecco's Modified Eagle's Medium (DMEM), Ham's F-12 medium, and fetal bovine serum (FBS) were purchased from Gibco-BRL (Grand Island, NY). Sulfatase, dextran, charcoal, HEPES, acetyl CoA, and sodium butyrate (NaB) were obtained from Sigma Chemicals (St. Louis, MO). 17β-estradiol (E2) was obtained from Steraloids Inc. (Wilton, NH). [^{32}P]dCTP (3,000 Ci/mmol) and ^{14}C-chloramphenicol (53 mCi/mmol) were purchased from Amersham (Arlington Heights, IL).

Cell Lines and Cell Culture

The MDA-MB-231 cell line was provided by Dr. Marc Lippman (Lombardi Cancer Center, Washington, DC). Cells were routinely cultured in Dulbecco's Modified Eagle:Ham's F-12 (1:1) media supplemented with 5% FBS as described previously [Fontana et al., 1990]. CSS (charcoal-dextran and sulfatase treated serum) was prepared as detailed previously [Fontana et al., 1991]. Cells were treated with E2 and/or NaB in Iscove's Modified Eagle Media supplemented with 5% CSS.

Growth Experiments

For growth experiments cells were plated in regular media supplemented with 5% fetal bovine serum (FBS) for a period of 24 h. The cells were then treated with 3 mM NaB for 5 days either in the same media or in phenol red–free media supplemented with 5% CSS; control cells were not treated with vehicle alone, and the media was changed every 3 days. Subsequently, the cells were trypsinized and counted using a hemocytometer.

Stable Transfections

The stable transfection of ER-negative MDA-MB-231 cells with the estrogen receptor cDNA has been previously described [Sheikh et al., 1993a,b]. Briefly, the MDA-MB-231 cells were initially plated at a density of 1×10^5 cells/100 mm dish 24 h prior to transfection in regular media. For stable transfection, 10 μg of the expression plasmid pSG5-HEO [Green et al., 1986] and 2 μg of the dominant selection vector pSV2neo [Southern and Berg, 1982] were co-transfected using the calcium phosphate–DNA coprecipitation method as described in Sambrook et al. [1989]. The expression plasmid

pSG5-HEO (10) carries the human ER coding region under the control of a SV40 promoter/enhancer, while in the pSV2neo the neomycin-resistance gene $(neo)^R$ is under the control of the same SV40 promoter/enhancer [Southern and Berg, 1982]. Control cells were transfected with pSV2neo alone (mock transfectants). G418-resistant colonies were selected in media containing 800 μg G418/ml and were expanded into mass culture. The ER-positive colonies were identified by Southern, Northern, and Western blot analyses.

Southern, Northern, and Western Immunoblot Analysis

Genomic DNA extraction and Southern blot analysis were performed according to standard procedures [Sambrook et al., 1989]. Genomic DNA samples obtained from ER-transfected MDA-MB-231, pSV2neo-transfected MDA-MB-231, and parental MDA-MB-231 cells were digested with EcoRI; the blots were then probed with the human ER cDNA probe, and the presence of a 1.8 kb ER fragment only in ER-transfected cells identified the positive clones.

RNA extraction and Northern blot analysis were essentially as described previously [Sheikh et al., 1993c]. Briefly, total cellular RNA samples were analyzed on 1.2% agarose/2.2 M formaldehyde gels and transferred to supported nitrocellulose membranes (Gibco-BRL, Grand Island, NY). RNAs were cross-linked to membranes after exposure to ultraviolet (UV) light. The membranes were prehybridized overnight at 42°C in 50% formamide, 5X SSPE, 5X Denhardts solution, 0.5% SDS, and 500 μg/ml salmon sperm DNA. Hybridization conditions were the same except [^{32}P]-labeled probes (approximately 10^8–10^9 dpm/μg DNA) were added to the hybridization solution. The membranes were then washed twice for 10–20 min each at room temperature in 2× SSPE and 0.2% SDS and then for 15–30 min at 55–65°C in 0.1× SSPE and 0.2% SDS and exposed to Kodak X-omat films at −70°C. Probes were stripped by washing the membranes in 0.1X SSPE and 0.2% SDS at 80°C.

Western immunoblot analysis was as described by Kushner et al. [1990], and the bands were detected by employing a monoclonal antibody specific to human ER (kindly provided by Dr. G.L. Greene, University of Chicago, IL) using the nonradioactive ECL system (Amersham) according to manufacturer's instructions.

cDNA Probes

The full-length human ER cDNA was excised from the expression plasmid pSG5-HEO with EcoRI and gel purified. The EGF-R and TGFα cDNA probes were kindly provided by Dr. Anne Hamburger (University of Maryland, Baltimore, MD). The pS2 cDNA probe was purchased from ATCC (Rockville, MD). Probes were labeled by the random primer method described by Feinberg and Vogelstein [1983].

CAT Assays

Wild type MDA-MB-231 cells were transiently transfected with the EGF-R promoter CAT constructs pER2CAT, pER6CAT, and pER9CAT [Johnson et al., 1988], kindly provided by Dr. Glen Merlino (NIH, Bethesda, MD). The plasmid pER2CAT carries the proximal 2 kb of EGF-R gene promoter region fused to the promoterless chloramphenicol acetyl transferase (CAT) gene. The plasmids pER6CAT and pER9CAT carry 771 bp and 384 bp of EGF-R gene promoter regions, respectively, and are also fused to a promoterless CAT gene. Transient transfections were performed as reported by Reese and Katzennellenbogen [1992]. In brief, cells were plated in regular medium at a density of 5×10^5 to 1×10^6 cells/100 mm dish for 24 h prior to transfection. Two hours before transfection, the media was changed and the cells were cotransfected with 15 μg of pER2CAT, pER6CAT, or pER9CAT using a calcium phosphate–DNA coprecipitation method [Sambrook et al., 1989]. Six hours later, the cells were shocked with 20% glycerol for 8 min, washed several times with PBS, and plated in regular media. Twenty-four hours later the cells were treated with 3 mM NaB and harvested 24 h later for CAT assays which were performed essentially as described in Sambrook et al. [1989].

RESULTS

To determine whether the constitutive expression of ER in previously ER-negative cells results in downregulation of EGF-R, ER-negative MDA-MB-231 cells that have been previously transfected with the ER expression vector pSG5-HEO were utilized [Sheikh et al., 1993a,b]. The MDA-MB-231 cell line was chosen because it is a well-established ER-negative HBC cell line and constitutively expresses moderate to high EGF-R at both the mRNA and protein levels without any associated amplification of the EGF-R gene.

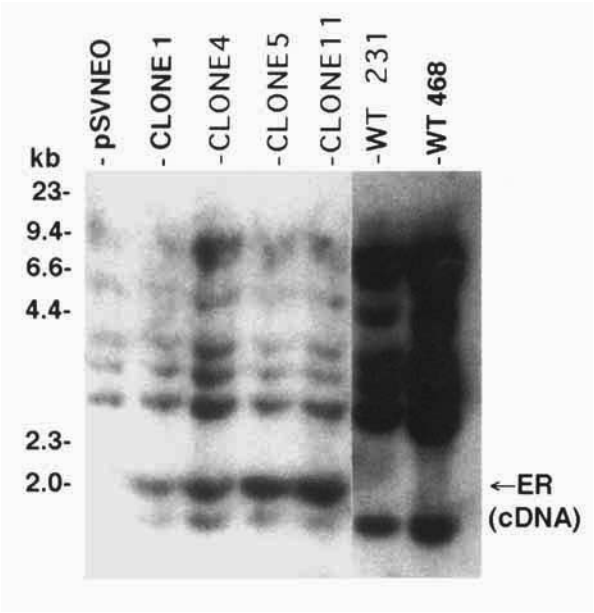

Fig. 1. Southern blot analysis of genomic DNA from ER-transfected, pSV2neo-transfected, and nontransfected MDA-MB-231 HBC cells. Approximately 10 μg DNA per sample were digested with EcoRI, subjected to agarose gel electrophoresis, and transferred to supported nitrocellulose. Prehybridization, hybridization, and washing conditions were as described in the text. As can be seen, the 1.8/1.9 kb band representing exogenously transfected ER cDNA sequences is present only in the ER-transfected cells; pSV2neo-transfected, nontransfected MDA-MB-231 and MDA-MB-468 cells do not show this band.

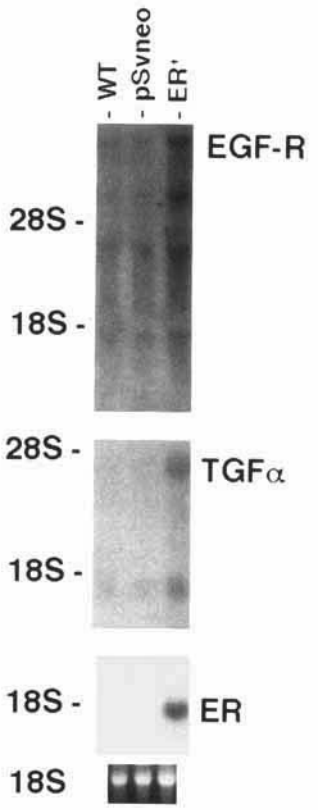

Fig. 2. Constitutive expression of EGF-R TGFα, and ER mRNA in ER-transfected, pSV2neo-transfected, and nontransfected MDA-MB-231 HBC cells. Total cellular RNA (25 μg per sample) was analyzed, and the same blot was probed with EGF-R, TGFα, and ER cDNA probes. Ethidium bromide staining of 18S RNA shows comparable loading in each lane. The experiment was repeated several times with identical results.

Two independently selected clonal populations of ER-transfected MDA-MB-231 cells expressing functional ERs and exhibiting similar growth behavior and cell morphology were analyzed in this study. Wild type nontransfected and pSV2neo-transfected MDA-MB-231 cells were used as controls. Southern (Fig. 1) and Northern blot analysis (Fig. 2) revealed that the ER cDNA had been integrated and was being expressed, while Western immunoblots demonstrated that both clones expressed ERs to levels that were comparable to that seen in ER-positive MCF-7 cells [Sheikh et al., 1993d]. The functionality of the transfected ERs was confirmed by the upregulation of the endogenous pS2 gene [Sheikh et al., 1993a,b; see Fig. 3.] and other estrogen responsive genes [Sheikh et al., 1993a,b] upon 17β-estradiol (E2) treatment in the ER-transfected MDA-MB-231 cells but not in the pSV2neo-transfected or nontransfected wild type cells.

Figure 2 depicts a representative Northern blot of ER-transfected (clone 4), pSV2neo-transfected, and parental nontransfected MDA-MB-231 cells grown in steroid-stripped phenol red–free medium. As can be seen, the constitutive expression of functional ERs in the transfectants is associated with an approximately four-fold increase the basal EGF-R mRNA levels over mock or nontransfected cells. Of note is that although the EGF-R mRNA levels in ER-transfected MDA-MB-231 cells grown in regular medium were approximately two- to threefold lower than when the cells were grown in the steroid-stripped medium, these levels were still ~ 1.5–2-fold higher than that seen in parental nontransfected cells grown in comparable culture conditions (see below in Fig. 5 compare lanes 1 to 3 and 3 to 7). Figure 2 also demonstrates that the ER-transfected cells express higher TGFα mRNA. Thus, there appears to be a basal coordinate regulation of the receptor/ligand mRNAs in the transfectants. E2 treatment of the transfectants in the steroid-stripped phenol red–free medium further enhanced TGFα mRNA levels but did not modulate EGF-R mRNA (Fig. 3). Another independent clonal population of ER-

transfected MDA-MB-231 cells (clone 5) also exhibited similar results (data not shown). We have shown previously that E2 enhances TGFα mRNA and protein levels in ER-positive MCF-7 cells [Fontana et al., 1992]. El-Ashry et al. [1991] have demonstrated that E2 regulates TGFα gene expression via two imperfect estrogen response elements (EREs) present in the promoter region of the TGFα gene. Thus, the E2-mediated increase in TGFα mRNA in the ER-transfected cells appears to reflect regulation at the gene transcription level. Figure 3 also demonstrates that E2 enhanced endogenous pS2 gene expression in ER-transfected cells, thus demonstrating that expression of functional ERs in ER-negative HBC cells results in activation of the endogenous estrogen responsive genes.

Previous studies have shown that EGF, although stimulatory to ER-positive MCF-7 and T47D cells, had no effect on the growth of ER-negative MDA-MB-231 cells [Osborne et al., 1980; Fitzpatrick et al., 1984]. To determine whether expression of functional ERs in MDA-MB-231 cells altered their growth response to EGF, the effect of EGF on growth of nontransfected and ER-transfected MDA-MB-231 cells was also investigated. Figure 4 shows that, consistent with previous reports, EGF did not modulate the growth of parental MDA-MB-231 cells at a concentration of 10 ng/ml; higher concentrations proved inhibitory. Moreover, as seen in Figure 4 the ER-transfected cells displayed a similar growth response to EGF as the parental counterparts, suggesting that presence of functional ERs has not altered their response to EGF treatment.

A recent study [deFazio et al., 1992] showed that NaB increased EGF-R mRNA levels while concomitantly decreasing the ER gene expression in the ER-positive cells. NaB, however, decreased the EGF-R mRNA levels in the ER-negative MDA-MB-231 cells [deFazio et al., 1992]. It was, therefore, concluded that NaB, by downregulating the expression of ER, relieves the EGF-R gene from negative control by the ERs [deFazio et al., 1992]. Since in our ER-transfected MDA-MB-231 cells the ER gene is under control of a heterologous promoter, we therefore sought to investigate how NaB might modulate the EGF-R mRNA in these cells. Both the parental and ER-transfected MDA-MB-231 cells were treated with NaB under different culture conditions, and Northern analysis was performed. Figure 5 depicts a representative Northern blot of parental nontransfected and ER-transfected MDA-MB-231 cells treated with 3 mM NaB for 24 h. Contrary to the findings of deFazio et al. [1992], NaB enhanced EGF-R mRNA levels approximately three- to fourfold above basal levels in the parental cells in regular as well as in steroid-stripped medium (Fig. 5, Table I). NaB enhancement of EGF-R mRNA levels was confirmed in four independent experiments. NaB, however, differentially regulated the EGF-R mRNA levels in the ER-transfected cells. NaB did not modulate the levels of the 10 and 5.6 kb EGF-R mRNA species in regular media and enhanced by only 1.5–1.8-fold the levels of these EGF-R mRNA species in steroid-stripped phenol red–free medium (Fig. 5, Table

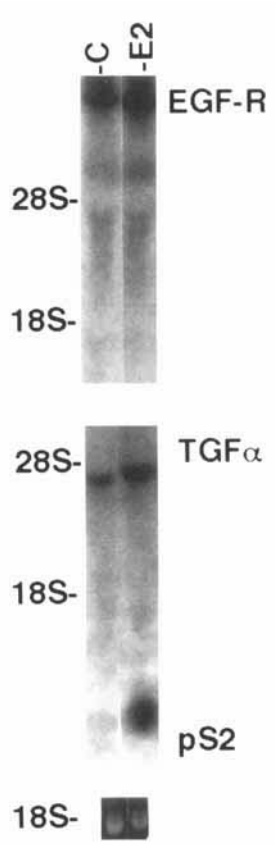

Fig. 3. Effect of 17β-estradiol (E2) on EGF-R, TGFα, and pS2 mRNA levels in ER-transfected MDA-MB-231 cells. Cells were plated in DMEM/F-12 medium for 24 h, washed several times with 1× PBS, and phenol red–free medium supplemented with 5% CSS was added for 48 h. Cells were then serum starved for an additional 24 h in phenol red–free medium and later either not treated or treated with 1 nM E2 for 48 h. Total RNA (25 μg per lane) was analyzed by Northern hybridization. The same blot was probed with EGF-R, TGFα, and pS2 cDNA probes. The experiment was performed twice with similar results. Similar results were also obtained when the cells were treated with E2 for 24 h (data not shown).

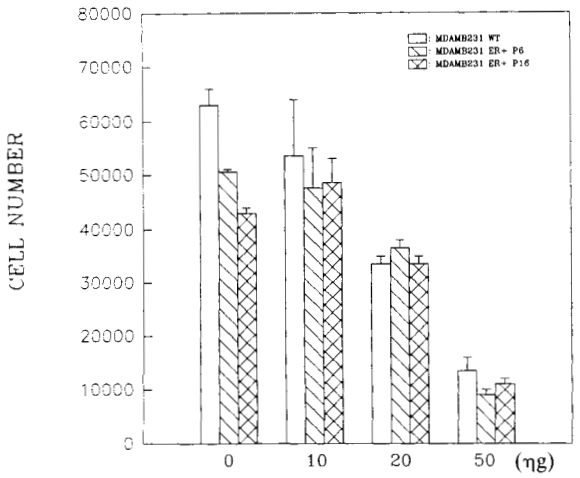

Fig. 4. Dose-dependent effect of EGF on the growth of parental and ER-transfected MDA-MB-231 cells. Cells were seeded in DMEM/F-12 supplemented with 5% serum for 24 h. After several washes with 1× PBS, the cells were treated with indicated concentrations of EGF in serum-free phenol red–free medium in the presence of 0.1% BSA for 5 days. Duplicate wells of cells were harvested and the cell numbers determined. The results represent the means ± SEM of three independent experiments. WT, nontransfected parental cells.

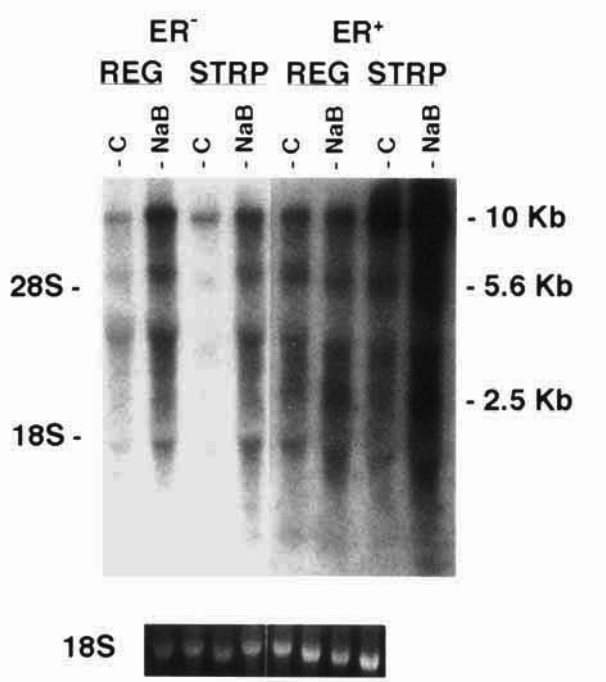

Fig. 5. The effect of NaB on EGF-R mRNA in nontransfected and ER-transfected MDA-MB-231 cells. Cells were seeded in DMEM/F-12 medium supplemented with 5% serum for 24 h. The cells were either directly treated with 3 mM NaB for 24 h, or washed several times with 1× PBS, grown for 48 h in phenol red–free medium plus 5% CSS, serum starved for an additional 24 h in the same medium, and subsequently treated with 3 mM NaB for 24 h in phenol red–free medium supplemented with 5% CSS. Total cellular RNA (approximately 25 μg per lane) was analyzed by Northern hybridization. Ethidium bromide staining of 18S RNA shows comparable loading.

I). Interestingly, NaB treatment of the ER-transfected cells resulted in an approximately four- to fivefold increase in the levels of a 2.5 kb EGF-R mRNA species in regular and stripped medium (Fig. 5, Table I). The extent of NaB-mediated increase of 10, 5.6, and 2.5 kb mRNA species in the nontransfected cells was uniform (Fig. 5, Table I).

Since phenol red and other estrogenic compounds are present in the regular medium [Berthois et al., 1986], the lower basal levels of EGF-R mRNA in the regular estrogenic medium and the inability of NaB to enhance the 10 and 5.6 kb EGF-R mRNA species in the ER-transfected cells grown in regular medium could possibly be due to ligand-mediated activation of ERs in these cells. To further investigate this issue, the transfected cells were treated either alone or in combination with NaB and E2 in steroid-stripped medium, and Northern analysis was performed. The results (data not shown) demonstrated that in contrast to expectations, E2 was unable to block the NaB-mediated increase in EGF-R mRNA levels. Figure 6 shows that NaB inhibited the growth of the MDA-MB-231 cells in regular and steroid-stripped phenol red–free medium regardless of the ER status.

The mechanism by which NaB enhanced EGF-R mRNA may involve gene transcription and/or mRNA stability. To determine whether the NaB-mediated increase in the EGF-R mRNA was at the transcriptional level, transient transfection assays using EGF-R–promoter-CAT constructs were carried out. Thus, vectors carrying various EGF-R gene promoter regions fused to a promoterless CAT gene [Johnson et al., 1988] were used to transiently transfect the MDA-MB-231 cells. Figure 7 shows that NaB was able to enhance CAT gene expression from all of the EGF-R gene promoter region tested (i.e., the 2 kb, the 771 bp, and the 384 bp 5′ promoter sequences). Our results, therefore, suggest that at least part of the NaB-mediated increase in EGF-R mRNA levels is due to increase in the EGF-R gene transcription.

DISCUSSION

A wealth of information documents an inverse correlation between the ER and EGF-R expression in HBC, both in tissue and cell culture systems. The stable transfection of the

TABLE I. Quantitation of the NaB Effect on the Steady-State Levels of EGF-R mRNA in Parental and ER-Transfected MDA-MB-231 Cells*

Experiment #	ER status	Culture conditions	Control	NaB		
				10 kb	5.6 kb	2.5 kb
1	(ER−)	Regular	1	4.3	4.2	4.0
2	(ER−)	Regular	1	3.5	3.0	3.2
1	(ER−)	Stripped	1	3.4	3.4	3.2
2	(ER−)	Stripped	1	2.8	3.1	2.9
1	(ER+)[a]	Regular	1	1.2	1.0	3.8
2	(ER+)[b]	Regular	1	1.0	1.0	3.5
1	(ER+)[a]	Stripped	1	1.5	1.8	5.2
2	(ER+)[b]	Stripped	1	1.4	1.5	4.4

*The quantitation of the mRNA signals was performed by scanning the shorter exposures of the autoradiographs using an LKB GelScan XL laser densitometer. The 10 kb, 5.6 kb, and 2.5 kb EGF-R mRNA bands were used for quantitation. The values are expressed relative to control, and the control representing each band was given an arbitrary value of 1.
[a]Passage 6 after transfection.
[b]Passage 16 after transfection

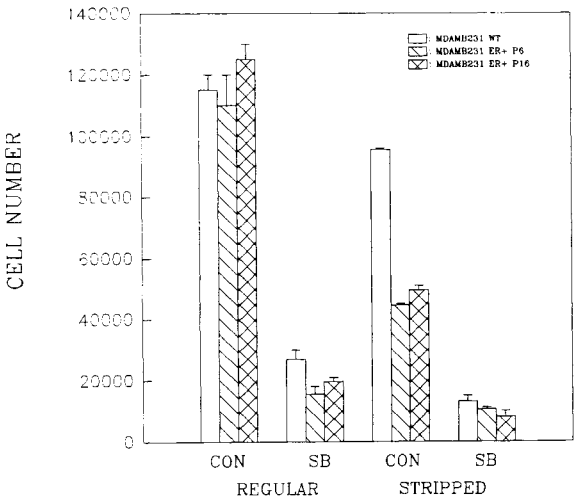

Fig. 6. Growth response of parental and ER-transfected MDA-MB-231 cells to 3 mM NaB. Cells were treated with NaB for 5 days in regular DMEM/F-12 medium supplemented with 5% serum or in phenol red–free CSS (5%) supplemented medium as mentioned in Material and Methods. The results are the means ± SEM of three independent experiments. WT, nontransfected parental cells.

EGF-R cDNA and its consequent expression in ER-positive HBC cells, however, failed to down-regulate ER levels [Valverius et al., 1990]. Although these results suggested that EGF-R does not appear to control the expression of ER, however, the question whether ERs control and regulate the EGF-R expression was still not clear. To address this question ER-negative cells stably transfected with the ER cDNA and expressing functional ERs were analyzed in this study. Our results demonstrate that the ER-mediated EGF-R mRNA regulation appears rather complex. Analysis of two independently selected clonal populations of the ER-transfected MDA-MB-231 cells demonstrated that introduction of functional ERs results in higher constitutive expression of EGF-R mRNA. Although no appreciable difference was noted in the basal EGF-R mRNA levels in parental non-transfected MDA-MB-231 cells grown in either regular or stripped medium, the ER-transfected cells had lower EGF-R mRNA levels in estrogenic regular medium.

NaB enhanced EGF-R mRNA levels in parental cells in both culture conditions. This ability of NaB to enhance the EGF-R mRNA levels was blunted when the same cells were transfected with an ER cDNA and analyzed in the steroid-stripped medium, and was completely abolished in cells treated in estrogenic regular medium. These findings suggest that the estrogenic activity of phenol red and the presence of other estrogenic compounds in regular tissue culture medium could in part be responsible for the differences noted in the levels of EGF-R mRNA in the ER-transfected cells grown in two culture conditions. Although E2 alone and in combination with NaB failed to modulate EGF-R mRNA levels, it is tempting to speculate that the ligand-activated ERs coupled with certain other factors present in the regular medium may indeed negatively regulate EGF-R mRNA levels.

EGF-R is structurally homologous to the product of the c-erbB-2/neu oncogene [Bargmann et al., 1986]. c-erbB-2/neu oncogene expression is also negatively correlated with the ER status of HBC [Zeillinger et al., 1989]. Recent evidence has shown that E2 can negatively regulate ex-

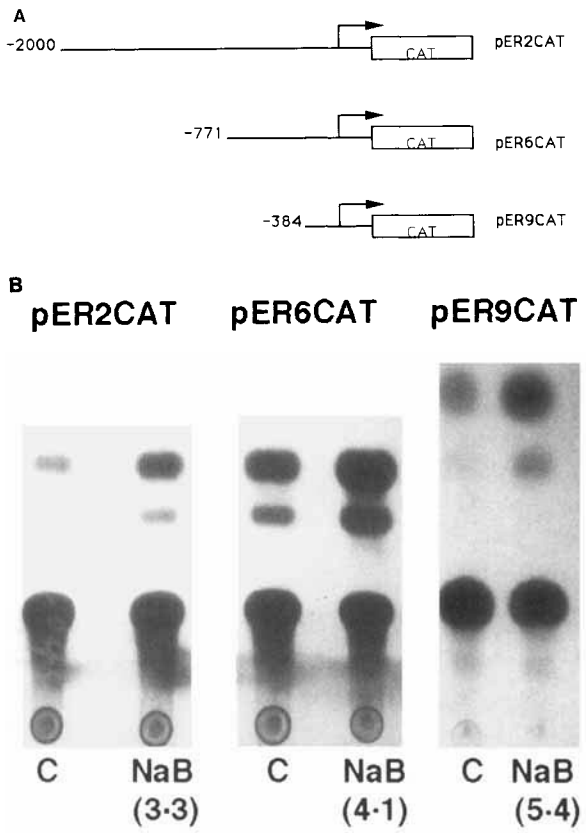

Fig. 7. NaB enhancement of EGF-R gene promoter expression. **A:** Structure of EGF-R promoter-based CAT constructs. The reporter vector pER2CAT carries the proximal 2 kb EGF-R promoter region. Reporter vectors pER6CAT and pER9CAT are deletion mutants carrying proximal 771 and 384 bp subfragments, respectively, of the 2 kb promoter fragment. All of these promoter fragments are cloned upstream of a promoterless CAT gene into the pGEM-4 plasmid. **B:** A representative CAT assay showing NaB-mediated increase in CAT activity from the EGF-R gene promoter in wild type MDA-MB-231 cells. Exponentially growing MDA-MB-231 cells were plated at a density of 5×10^5 to 1×10^6 cells/dish 24 h prior to transfection in DMEM/F-12 medium +5% serum. Cells were transfected with 15 μg of each plasmid. NaB (3 mM) treatment was for 24 h. The cells were harvested 48 h after transfection, lysed, and CAT activity, corrected for protein concentrations, was determined as mentioned in the text. The experiment using the pER2CAT reporter vector was performed once, while the experiments with pER6CAT and pER9CAT were performed twice with identical results. The numbers in parenthesis indicate fold increase in CAT activity after NaB treatment.

pression of the c-erbB-2/neu oncogene in ER-positive HBC cell lines [Dati et al., 1990]. In addition, c-erbB-2 oncogene expression was also shown to be negatively regulated by E2 in the ER-negative cells that had been transfected with ERs [Russell and Hung, 1992]. A previous study [Berthois et al., 1989] has shown that E2 treatment for 24 and 48 h decreased the EGF-R numbers by approximately 40% in ER-positive MCF-7 cells; longer treatment with E2, however, enhanced EGF-receptor numbers. Consistent with these observations Dickstein et al. [1993] have recently observed, utilizing RNase protection assay that E2 indeed decreased by approximately twofold the EGF-R mRNA levels in ER-positive MCF-7 cells. In our studies, although the ER-transfected cells exhibited differential expression of EGF-R mRNA in estrogenic and nonestrogenic tissue culture conditions, regulation of EGF-R mRNA by E2 was not observed. It is of note that the ER expression vector used in our studies contained a point mutation in the ligand-binding domain of the ER. This mutation results in a Gly-Val substitution at amino acid 400 [Green et al., 1986], and the resulting receptor [Val^{400}] has reduced binding affinity for E2 [Tora et al., 1989]. Although less likely, the possibility exists that this point mutation is partly responsible for E2's inability to down-modulate EGF-R mRNA levels in the ER-transfected cells. It should be noted, however, that physiological concentrations of E2 were able to induce expression of the ER-responsive genes in the ER-transfected cells [Sheikh et al., 1993a,b, this study]. A recent study [Smith et al., 1993] showed that both the [Val^{400}] ER as well as the wild type [Gly^{400}] ER can be activated at physiological concentrations of E2. The [Val^{400}] ER, however, was less potent than the wild type ER in activating gene expression in the absence of ligand [Smith et al., 1993]. It is possible that the MDA-MB-231 cells used in our studies may lack certain important accessory regulators of ERs which are otherwise present in the ER-positive cells as well as in regular tissue culture medium; the presence of these regulators may be necessary for the full modulatory effects of ER on EGF-R mRNA levels.

Studies by deFazio et al. [1992] also attempted to establish an inverse correlation between the ER and EGF-R gene expression in HBC cells. Using various cell lines, they were able to show that NaB concomitantly enhanced EGF-R mRNA levels and downregulated ER mRNA levels in ER-positive cells. They suggested that in the ER-positive cells, NaB by decreasing ER gene expression could relieve EGF-R gene expression from inhibitory effects of the ERs. In ER-negative MDA-MB-231 cells NaB was found to downregulate EGF-R mRNA. Contrary to their observation, we found that the EGF-R mRNA levels were increased in MDA-MB-231 cells treated with NaB (this difference might be due to different MDA-MB-231 subclones); ER-transfected MDA-MB-231 cells ex-

hibited a weaker NaB effect which was only observed in steroid-stripped phenol red–free medium. Since the ER transfected MDA-MB-231 cells treated with NaB in regular medium failed to show any EGF-R mRNA modulation, conceivably the presence of estrogenic compounds in the regular tissue culture medium (including phenol red) could activate the ERs in the transfectants and therefore interfere with NaB's ability to upregulate EGF-R mRNA. Although E2 failed to block NaB's ability to upregulate the EGF-R mRNA levels in ER-transfected MDA-MB-231 cells in steroid-stripped phenol red–free medium, the possibility exists that the activated ERs could interact with additional factors present in regular but not in stripped culture medium with which they could act in concert to block the effects of NaB on EGF-R mRNA accumulation.

NaB is a potent antiproliferative and differentiation-inducing agent, and the mechanism by which it mediates its effects appears to involve modulation of important regulatory genes [Kruh, 1982; Graham and Buick, 1988; Walker-Jones et al., 1989; Guilbaud et al., 1990]. NaB has been shown to regulate gene expression both at the transcriptional and posttranscriptional levels [Saini et al., 1990; Tichonicky et al., 1990; Bresnick et al., 1990; Pan et al., 1991]. Utilizing the EGF-R gene promoter based CAT constructs, we were able to show that the mechanism by which NaB enhances EGF-R mRNA levels is, in part, at the transcriptional level. NaB-induced promoter activity was seen when a 2 kb EGF-R gene promoter region fused to CAT, as well as when 771 bp and 384 bp subfragments of the 2 kb fragments were used in similar constructs. Our results therefore demonstrate that the regulatory element(s) required for the NaB-mediated enhancement of EGF-R gene expression is (are) present within the proximal 384 bp of the EGF-R gene promoter region.

NaB also induced differentially the expression of a ~2.5 kb EGF-R mRNA species. This effect of NaB was noted only in ER-transfected cells. The EGF-R gene has been shown to encode ~10 and 5.6 kb invariable mRNA species [Xu et al., 1984]. In addition, a 2.7/2.9 kb mRNA species is also reported in several systems [Xu et al., 1984; Petch et al., 1990]. All of these EGF-R mRNAs appear to contain a common 5′ end [Ishii et al., 1985]. The basis for the generation of multiple EGF-R mRNA species is not clear but is believed to be due to differential RNA processing [Petch et al., 1990]. The differential NaB-mediated enhancement of the 2.5 kb EGF-R mRNA species in our study warrants further investigation. Since the differential enhancement of this 2.5 kb EGF-R mRNA species by NaB was noted only in the ER-transfected MDA-MB-231 cells, it is possible that in these transfectants NaB differentially regulates processing of the EGF-R mRNA. Thus NaB can not only regulate the EGF-R gene expression at the transcriptional level, but might also exert posttranscriptional control.

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