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Estrogen Receptor-Negative Breast Cancer Cells Transfected With the Estrogen Receptor Exhibit Increased RARα Gene Expression and Sensitivity to Growth Inhibition by Retinoic Acid

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Abstract We and others have shown previously that retinoic acid (RA) selectively inhibits the growth of estrogen receptor (ER)–positive human breast carcinoma (HBC) cells and ER-negative cells are refractory to RA inhibition of growth. The ER-negative cells inherently express lower levels of RARα and retinoic acid response element (RARE)–mediated RA-induced CAT activity. In this study we report that when ER-negative MDA-MB-231 cells were transfected with the ER gene they not only expressed higher levels of RARα and RARE-mediated RA-induced CAT gene expression, but their growth was now inhibited by RA. Estrogen enhanced RARα gene expression not only in established ER-positive cell lines but also in ER-transfected MDA-MB-231 cells. The estrogen effect appears to be direct and at the gene transcription level since it did not alter the stability of RARα mRNA and cycloheximide failed to block estrogen-mediated enhancement of RARα gene expression. Our data strongly suggest that ER-mediated enhancement of RARα levels plays an important role in RA inhibition of HBC growth. In addition, we also report here that HBC cells appear to express a unique isoform(s) of RARα which was detected only when the full-length RARα cDNA was used as a probe; the RARα1 and RARα2 specific probes failed to hybridize with the HBC specific RARα message.

Key words: transfection, CAT assays, gene expression

A number of studies have now established that retinoids, the natural and synthetic derivatives of vitamin A, are highly effective in preventing the development of mammary carcinoma in a number of systems [Moon et al., 1985]. Inhibitory effects of retinyl acetate, retinyl methyl ether, and 4-hydroxy-N-phenyl retinamide (HPR) on mammary carcinoma development have been demonstrated in experimental animal models [Moon et al., 1983]. Retinoic acid (RA) has also been shown to play an important role in the differentiation of a number of cell types of endodermal, ectodermal, and mesodermal origin [Sporn and Roberts, 1984]. RA is believed to mediate its effects by binding to its specific receptors which in turn act as ligand activated nuclear transcription factors that bind to retinoic acid response elements (RAREs) present in the promoter enhancer regions of genes [Gudas, 1992, and references therein]. These nuclear receptors are known as RARs (retinoic acid receptors), and three distinct forms of RAR termed α, β, and γ, each with a number of different isoforms, are known [Gudas, 1992, and references therein]. Recently three more receptors, namely RXR (retinoid X receptor) α, β, and γ, were identified. The RXRs, although activated by all-trans RA (tRA), do not bind tRA. Recently 9-cis RA, a stereoisomer of RA and derived from tRA, was identified; 9-cis RA directly binds to RXR. The RXRs are able to form homodimers as well as heterodimers with RARs, thyroid, and vitamin D3 nuclear receptors [Gudas, 1992, and references therein]. All these receptors belong to the steroid/thyroid hormone receptor superfamily [Gudas, 1992, and references therein].

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We have shown previously that a correlation exists between RA inhibition of human breast carcinoma (HBC) cell growth and the estrogen receptor (ER) status [Fontana, 1987]. RA selectively inhibited the growth of ER-positive HBC cells; ER-negative HBC cells were refractory to the inhibitory effects of RA. Other groups [Marth et al., 1984; Koga and Sutherland, 1991] have also independently confirmed these observations. Roman et al. [1992] have recently reported that all the ER-positive cell lines tested in their study exhibited higher levels of RARα mRNA. RARγ mRNA expression was comparable in all the cell lines regardless of the ER status. Low levels of RARβ mRNA were detected only when poly A+ mRNA was utilized, and no statistically significant correlation with ER status was found [Roman et al., 1992]. More recently van der Burg et al. [1993] have reported that the ER-positive HBC cells express higher RA-induced CAT gene expression from a RARE than the ER-negative cell lines. In addition they also found that the ER-positive cell lines indeed express higher RARα mRNA than the ER-negative HBC cell lines. We have also confirmed the recent observation of Roman et al. [1992] and van der Burg et al. [1993] that the ER-positive cells express higher levels of RARα mRNA than their ER-negative counterparts and that ER-positive HBC cells express higher RA-induced CAT gene expression from a RARE than the ER-negative cell lines. Based on these findings we hypothesized that the low levels of CAT activity noted in ER-negative cells are a result of the low levels of RARα in these cells and that the differential expression of RARs and the ability of RA to modulate HBC proliferation is directly related to the ER status of these cell lines. The current study was undertaken to further investigate these hypotheses and to investigate the potential mechanism(s) by which RA selectively inhibits the proliferation of only ER-positive and not ER-negative cells.

MATERIALS AND METHODS

Cell Culture and Growth Experiments

Cell lines and culture conditions have been previously described [Fontana, 1987]. Charcoal dextran and sulfatase treated serum (CSS) was also prepared as previously mentioned [Fontana et al., 1992]. 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 1 μM RA for 5 days; control cells were treated with vehicle alone and the media was changed every 3 days. The cells were trypsinized and counted using a hemocytometer.

Southern, Northern, and Western Immunoblot Analysis

Southern blot analysis and genomic DNA extraction were performed according to standard procedures [Sambrook et al., 1989]. Genomic DNA samples from ER-transfected MDA-MB-231, neo-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 positive clones were identified by the presence of a 1.8 kb internal ER fragment only in ER-transfected cells (data not shown). RNA extraction and Northern blot analysis was essentially as described previously [Sheikh et al., 1992]. Western immunoblot analysis was performed as described by Kushner et al. [1990], and the bands were developed with a monoclonal antibody to human ER (kindly provided by Dr. G.L. Greene, University of Chicago, IL) using the nonradioactive ECL system (Amersham).

Stable and Transient Transfections

For stable transfections, ER-negative MDA-MB-231 cells were plated at a density of 1×10^5 cells/100 mm dish 24 h before transfection in regular media. Using a 5:1 molar ratio, 10 μg of the expression plasmid pSG5-HEO [Green et al., 1986] and 2 μg of dominant selection vector pSV2neo [Southern and Berg, 1982] were co-transfected utilizing the calcium phosphate DNA coprecipitation method as described in Sambrook et al. [1989]. The expression plasmid pSG5-HEO carries the human ER coding region under the control of a SV40 promoter and has been shown previously to express functional ERs in HeLa cells [Green et al., 1986]. pSV2neo is a dominant selection vector that carries the neomycin-resistance gene (neo) under the control of the SV40 early promoter-enhancer [Southern and Berg, 1982]. Control cells were transfected with the pSV2neo vector alone. G418-resistant colonies were selected in media containing 800 μg/ml G418 and were clonally expanded into mass culture. The ER-positive colonies were initially identified by Western immunoblotting and later confirmed by Southern and Northern blot analysis.

For transient transfections plasmid pBLCAT2 (RARE/tk-CAT) (kindly provided by Dr. M. Pfahl of the La Jolla Cancer Research Foundation, LaJolla, CA) and pRSVZ [MacGregor et al., 1987] were used. The plasmid pBLCAT2 [Husmann et al., 1991] is an RARE/tk-promoter-CAT reporter construct and carries a double stranded sequence

5′-gatctGTAGGGTTCACCGAAAGTTCACTCa-3′

3′-aCATCCCAAGTGGGTTTCAAGTGAGtctag-5′

containing the direct repeat RARE from the RARβ_2 promoter inserted upstream of the thymidine kinase promoter at position −105. pRSVZ [MacGregor et al., 1987] carries an *E. coli lacZ* gene under the control of a Rous sarcoma virus long terminal repeat (LTR) and codes for β-galactosidase which can be used to normalize the differences in transfection efficiencies. Transient transfections were performed as described by Reese and Katzennellenbogen [1992]. Briefly, cells were plated at a density of 3×10^6 cells/100 mm dish for 24 h in regular media, washed several times in PBS, and then plated in steroid stripped phenol red–free media for 72 h. Two hours prior to transfection, the media was changed and the cells were co-transfected with 10 μg of pBLCAT2 and 6 μg of pRSVZ using a calcium phosphate method [Sambrook et al., 1989]. Five to six hours later, the cells were shocked with 20% glycerol for 4–7 min, washed several times with PBS, and plated in steroid stripped phenol red–free media with the appropriate hormones. Twenty-four hours later the cells were harvested for CAT assay. For the analysis of CAT activity in phenol red containing media, the cells were plated in regular media and directly transfected 24 h later.

CAT Assay

Cells were trypsinized, washed several times in 1× PBS, and resuspended in 100 μl 0.25 M Tris-HCl, pH 8.0. The cells were lysed by three cycles of freezing and thawing, and the cell lysate was collected in separate tubes. CAT assays were performed essentially as described in Sambrook et al. [1989].

RESULTS

We transfected ER-negative MDA-MB-231 HBC cells with an expression vector carrying the human ER cDNA under the control of a SV40 promoter/enhancer [Green et al., 1986].

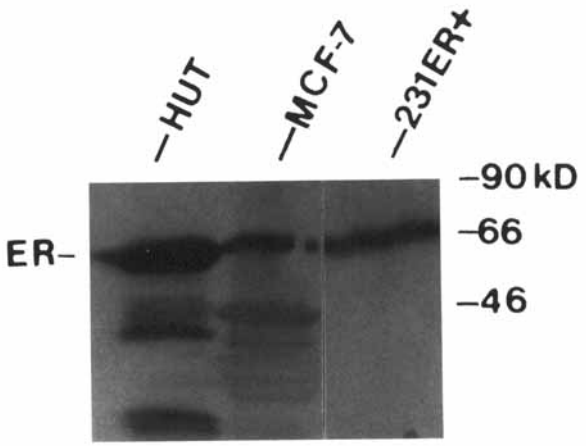

Fig. 1. Western immunoblot analysis of ER expression. Clone 4 of ER-transfected MDA-MB-231 cells was used in this study and is shown to express authentic ER of 65 kDa. Cell lysates containing equal amounts of proteins from human uterine tissue (HUT), ER-positive MCF-7, and ER-transfected MDA-MB-231 cells were analyzed on 6% SDS-polyacrylamide gels under reducing conditions.

The positive clones expressing ER were identified by Southern, Northern, and Western immunoblot analysis (see Fig. 1; some data not shown), and the functionality of the receptor was assessed by upregulation of endogenous pS2 gene expression upon estrogen treatment (see below). Among the clones expressing the functional ER, the ones that possessed ER levels comparable to the ER levels detected in MCF-7 HBC cells (Fig. 1) were selected for further analysis.

The data illustrated in Figure 2A,B demonstrate that not only are the ER-transfected MDA-MB-231 cells now growth inhibited by RA, but they also constitutively express approximately threefold (n = 2) higher levels of RARα than their parental nontransfected (Fig. 2B; compare lane 1 of 231 ER+ with lane 1 of 231 ER−) or pSV2neo transfected counterparts (data not shown); as expected, RA did not modulate the growth of the wild type and pSV2neo transfected cells. Consistent with the notion that there is a relationship between higher levels of RARα mRNA and ER positivity, the treatment of ER-transfected MDA-MB-231 cells with estrogen resulted in an approximately twelvefold increase in the levels of RARα mRNA (Fig. 2B). Similar treatment of wild type MDA-MB-231 and pSV2neo MDA-MB-231 cells with estrogen did not result in modulation of RARα mRNA (Fig. 2B). Estrogen enhancement of RARα mRNA levels was consistently observed in eight independent experiments. It is of note that the

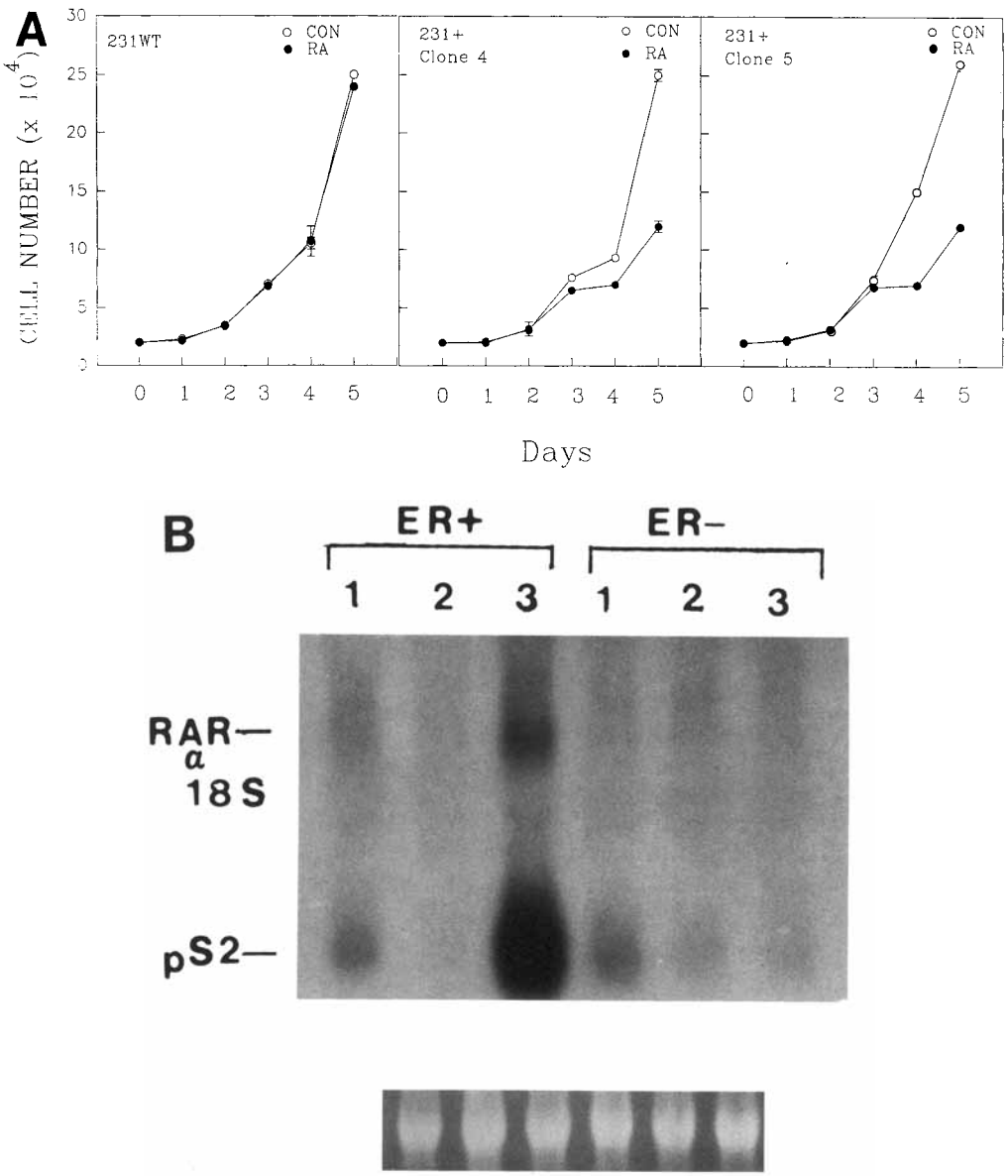

Fig. 2. **A:** RA effect on the growth of ER-negative MDA-MB-231 and ER-transfected MDA-MB-231 cells. Cells were plated in DMEM/F12 supplemented with 5% FBS in the presence or absence of 1 μM RA. The results shown are the means ± SEM of three independent experiments. **B:** RARα mRNA expression in ER-transfected MDA-MB-231 (Clone 4) and wild type MDA-MB-231. Total RNA was extracted from cells grown in regular media supplemented with 5% FBS **(lane 1)**, steroid stripped phenol red–free media **(lane 2)**, and steroid stripped phenol red–free media supplemented with 1 nM 17 β-estradiol **(lane 3)**. Approximately 35 μg total RNA per lane was analyzed by Northern hybridization, and the bands were quantitated by scanning the films with a laser densitometer. Phenol red and estrogen modulation of pS2 gene expression is also shown. Ethidium bromide staining of 18S RNA shows comparable loading.

ER-transfected MDA-MB-231 cells exhibited higher basal levels of RARα mRNA over wild type or pSV2neo-transfected cells only when grown in regular media that contains phenol red. ER-transfected cells grown in steroid stripped phenol red–free media exhibited lower levels of RARα mRNA which were comparable to the RARα mRNA levels detected in wild type or pSV2neo controls (Fig. 2B; compare lane 2 of 231ER+ with lanes 1–3 of 231 ER−). Since phenol red has been shown to contain estrogenic activity [Berthois et al., 1986], and since estrogen upregulated the levels of RARα mRNA in ER-transfected MDA-MB-231 cells, we predicted that the higher basal levels of RARα noted in ER-positive cells might actually be due to the

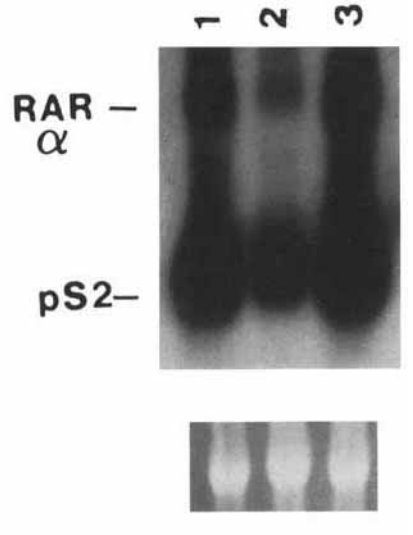

Fig. 3. E2 and phenol red modulation of RARα and pS2 gene expression in ER-positive MCF-7 cells. Total RNA was extracted from cells grown in regular media supplemented with 5% FBS (**lane 1**), steroid stripped phenol red–free media (**lane 2**), and steroid stripped phenol red–free media supplemented with 1 nM 17 β-estradiol (**lane 3**). Approximately 35 μg total RNA per lane was analyzed by Northern hybridization, and the bands were quantitated by scanning the films with a laser densitometer. Ethidium bromide staining of 18S RNA shows comparable loading.

presence of phenol red and other estrogens present in the regular media (we, Roman et al. [1992], and van der Burg et al. [1993] had analyzed the levels of RARα mRNA from cells grown in regular phenol red containing media). This possibility was further investigated utilizing the two established ER-positive cell lines MCF-7 and T47D. Results shown for MCF-7 cells in Figure 3 confirmed modulation of RARα by phenol red; that is, RARα mRNA levels were approximately 2.5-fold (n = 2) higher in regular phenol red containing media than in the phenol red–free media, and, as expected, estrogen upregulated the levels of RARα mRNA (fourfold increase over the RARα mRNA levels detected in phenol red–free media). Similar results were also obtained for the ER-positive T47D cell line (data not shown).

To demonstrate that the modulation of RARα mRNA by estrogen was also reflected in the RARα-dependent transcriptional activation of the CAT reporter gene, wild type 231, ER-transfected 231 and MCF-7 cells were transiently transfected with the RARE/tk-CAT reporter construct, and the cells were treated with RA and/or estrogen. Figure 4A,B illustrates that, consistent with the Northern blot data, the ER-

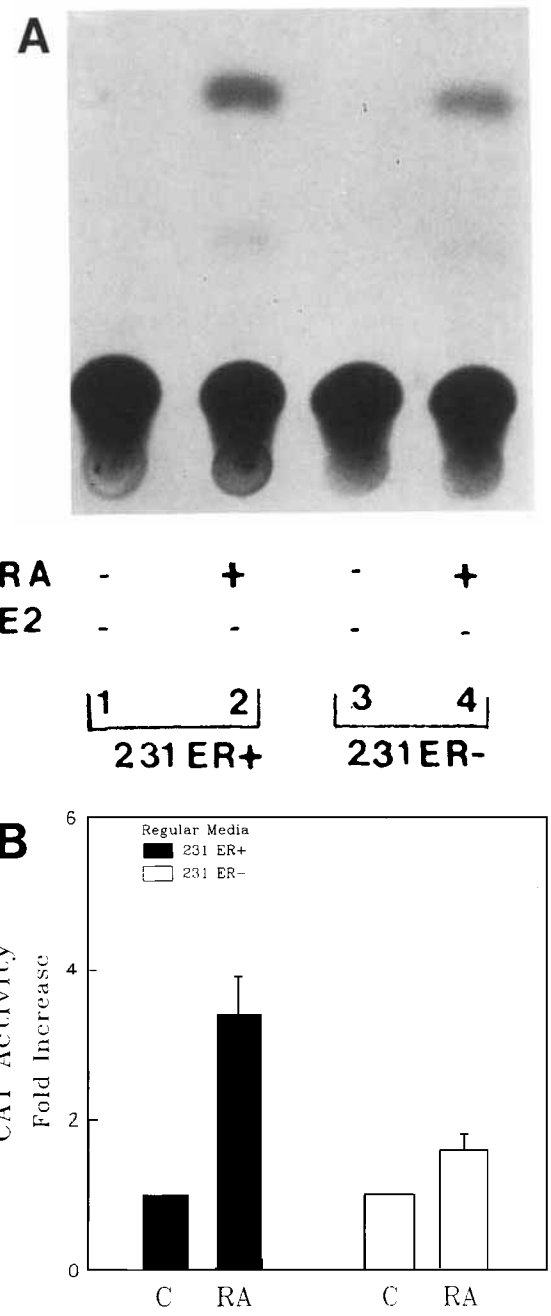

Fig. 4. RA activation of RARE-mediated transcription in ER-transfected and nontransfected MDA-MB-231 HBC cells. **A:** Representative assay of CAT activity in ER-transfected MDA-MB-231 and wild type MDA-MB-231 cells. Cells were cultured and transiently transfected in regular phenol red containing media. **B:** Graphic representation of the data shown in A. Cell extracts were normalized with respect to β-galactosidase activity and amounts of extracts containing equal β-galactosidase activity were analyzed. Activation of the CAT reporter was determined as percentage of [^{14}C]chloramphenicol converted to the acetylated form. The values are expressed relative to respective controls which were given a value of 1. The results represent means ± SEM of two independent experiments.

transfected MDA-MB-231 cells exhibited higher CAT expression when treated with RA in regular media than the wild type MDA-MB-231 cells treated under similar conditions. There was no difference in the CAT activity between the ER-transfected and wild type MDA-MB-231 cells when treated with RA in phenol red–free media (data not shown). Estrogen alone did not modulate the CAT activity in either of these cell types. Estrogen and RA in combination, however, enhanced CAT activity over that observed with RA alone only in ER-transfected MDA-MB-231 cells (Fig. 5A,B). MCF-7 cells also exhibited higher CAT activity when treated with RA in phenol red containing media than when they wer treated in phenol red–free media (data nc shown). In addition, estrogen in combinatior with RA enhanced CAT activity in MCF-7 cells above and beyond what was observed with RA treatment alone (Fig. 6). These findings clearly indicate a similarity between the pattern of RARα mRNA regulation and the CAT activity. Thus, although estrogen enhanced RARα mRNA levels, it did not modulate CAT expression alone in ER-positive cells. However, estrogen greatly potentiated the increase in CAT activity induced by RA, suggesting that RARα receptors might be involved and require ligand for their function.

The mechanism by which estrogen increases the levels of RARα mRNA could be either direct or indirect and may involve an increased rate of gene transcription, altered mRNA stability, or both. To distinguish among these alternatives, the effect of the protein synthesis inhibitor cycloheximide (CHX) on estrogen's ability to enhance RARα mRNA was investigated. Figure 7 illustrates that CHX alone also enhanced RARα mRNA levels in ER-transfected MDA-MB-231 cells and treatment with CHX plus estrogen resulted in superinduction of RARα mRNA expression, suggesting that the estrogen-mediated increase in RARα mRNA levels does not require new protein synthesis. In contrast, CHX blunted the estrogen-mediated increase in pS2 mRNA levels (Fig. 7) which suggests that part of the mechanism by which estrogen regulates expression of the pS2 gene in ER-transfected MDA-MB-231 cells may be different than in MCF-7 cells. In MCF-7 cells estrogen was shown to directly increase the expression of the pS2 gene at the transcriptional level, and CHX was unable to block estrogen-mediated increase in pS2 gene expression [Cavailles' et al., 1989]. To determine whether estrogen increases the accumulation of RARα mRNA by stabilizing its mRNA, half-life values of RARα mRNA from ER-transfected cells grown in the presence or absence of estrogen were analyzed. Our results suggest that

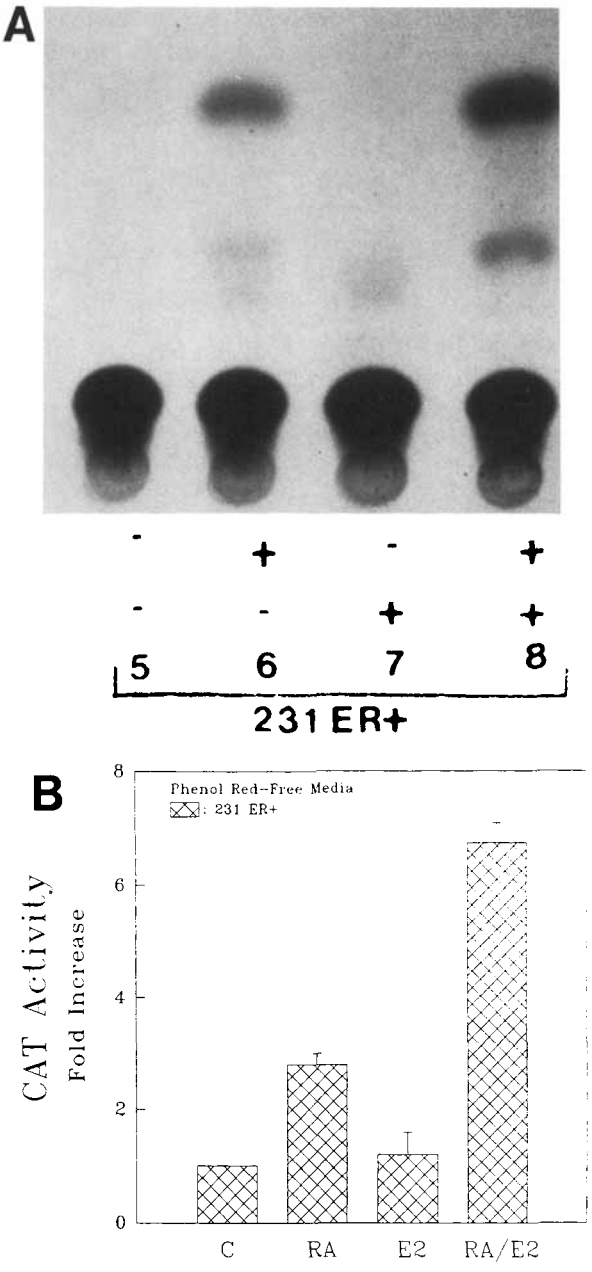

Fig. 5. RA and estrogen activation of RARE-mediated transcription in ER-transfected MDA-MB-231 cells. ER-transfected cells were cultured and transiently transfected in steroid stripped phenol red–free media. RA (1 μM) and 17 β-estradiol (1 nM) treatments were for 24 h. **A:** Representative assay of CAT activity. **B:** Graphic representation of data shown in A. CAT assay and quantitation was performed as described in Materials and Methods and in the legend to Fig. 4.

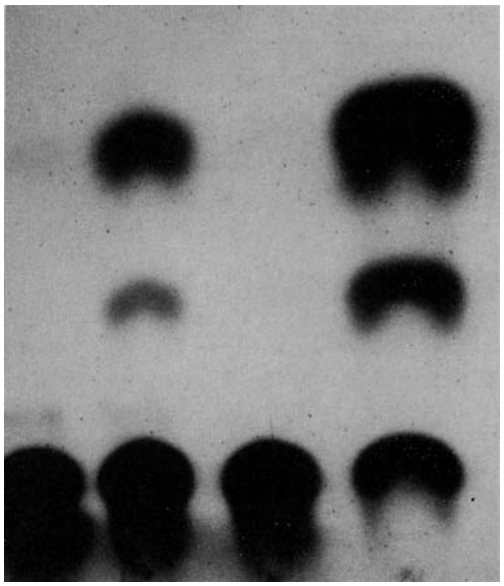

Fig. 6. RA (1 μM) and 17 β-estradiol (1 nM) modulation of RARE-mediated CAT activity in ER-positive MCF-7 cells. Cells were cultured and transiently transfected in steroid stripped phenol red–free media and treated with hormones for 24 h. Equal amounts of extracts with respect to β-galactosidase activity were analyzed in each lane. The same experiment was performed twice and similar results were obtained.

estrogen does not appear to modulate the RARα gene expression by stabilizing the message since the half-life of RARα mRNA was essentially the same (approximately 7 h) in estrogen-treated or untreated cells (data not shown).

The RARα1 and RARα2 are the only known human RARα isoforms. We utilized RARα1 and RARα2 specific probes to investigate which of the two isoforms of RARα is being expressed in HBC. Northern blot analysis revealed no detectable RARα1 or RARα2 signal in these cell lines; RARα mRNA was, however, detected on the *same blots when* the full-length RARα probe was used (data not shown). Southern blot analysis shown in Figure 8 reveals a distinct band pattern for either isoform. The RARα1 specific probe detected a major band of approximately 2.8 kb, while the RARα2 specific probe detected bands of approximately 8.5 kb and 2.8 kb, suggesting that RARα1 and RARα2 specific sequences are intact in these cell lines.

DISCUSSION

In this study we provide direct evidence that the expression of functional ERs is associated with the ability of RA to inhibit HBC cell proliferation. In addition, we also demonstrate that expression of the RARα gene appears to be under direct control of estrogen in HBC cells. Although it remains to be firmly established, we have presented several lines of evidence that the levels of RARα are critical for RA actions in HBC cells and are responsible at least in part for RA's ability to inhibit HBC proliferation. First, the ER-negative cells are not growth inhibited by RA, and they inherently express lower levels of RARα. Second, the low levels of RARα in ER-negative cells were also reflected in their function since RA was unable to induce (or induced extremely low levels) CAT gene expression from transfected RARE/tk-CAT constructs in these cells. Third, the expression of functional ERs in ER-negative MDA-MB-231 cells made these cells sensitive to RA-mediated growth inhibition. Fourth, the ER-transfected MDA-MB-231 cells not only constitutively express higher levels of RARα mRNA than their parental counterparts, but also exhibit higher RA-mediated CAT activity from RARE/tk-CAT constructs than the ER-negative parental cells. Indeed our preliminary results [work in progress] utilizing human breast carcinoma biopsy specimens suggest that the ER-positive tumors appear to express (statistically) significantly higher RARα mRNA than their ER-negative counterparts.

Estrogen appears to directly regulate the expression of RARα not only in ER-transfected cells but also in established ER-positive HBC cell lines. The difference in basal level expression of RARα mRNA might therefore be due to the estrogenic actions of phenol red and other estrogens present in the regular media. Consistent with this prediction, the ER-positive cells exhibited lower basal levels of RARα mRNA in steroid stripped phenol red–free media than in regular media. The ER-negative cells, however, did not show any change in the levels of RARα mRNA under both types of growth conditions, suggesting that the lack of functional ERs may in part be responsible for the lower basal levels of RARα mRNA.

Estrogen appears to directly enhance the levels of RARα mRNA at the transcriptional level since 1) the $t_{1/2}$ of RARα mRNA in estrogen-treated and untreated cells was essentially identical and 2) CHX did not block the estrogen-mediated increase in RARα mRNA. Treatment with CHX by itself also increased the levels of RARα mRNA, and it is not clear whether this

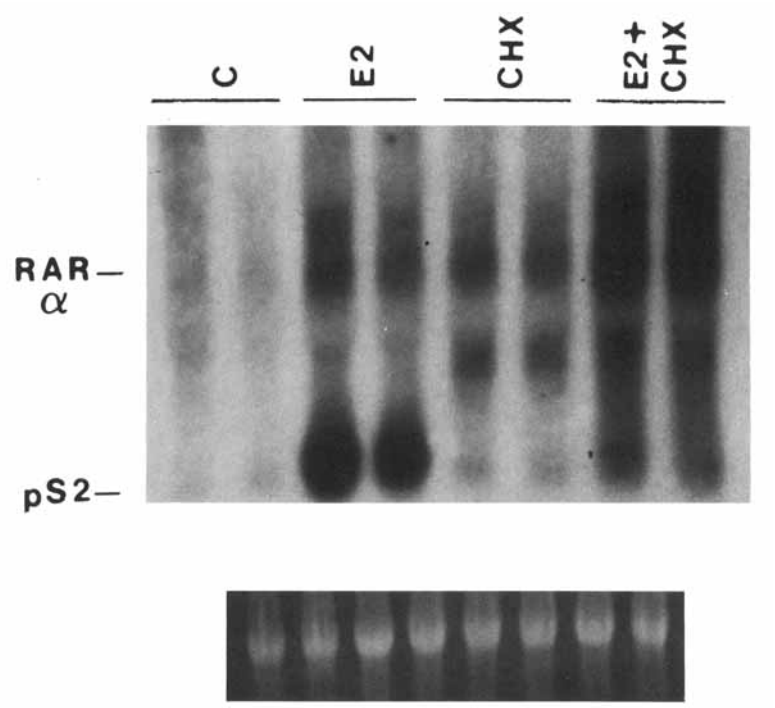

Fig. 7. CHX and/or estrogen modulation of RARα and pS2 gene expression in ER-transfected MDA-MB-231 cells. Cells were seeded in regular media overnight, washed several times with PBS, and serum starved for 24 h. Cells were then pretreated or not treated with 50 μM CHX for 1 h, and 1 nM 17 β-estradiol was then added to appropriate flasks for 12 h, while CHX was still present. The experiment was performed in duplicate, and 35 μg total RNA per lane was analyzed by Northern hybridization. The same blot was probed simultaneously with RARα and pS2 probes. Ethidium bromide staining of the 18S RNA is also shown.

effect of CHX is limited only to ER-negative cells or whether this is a generalized phenomenon. The mechanism by which CHX increases RARα mRNA levels remains to be delineated but could be due either to the release of RARα gene expression from repressive control mediated by labile factors, release from labile nucleases responsible for enhanced degradation of RARα mRNA, or both. If CHX indeed increases the levels of RARα mRNA selectively in ER-negative cells, then this would suggest one more level of control responsible for the low levels of RARα gene expression in ER-negative cells.

The cross-talk between the RA and estrogen-mediated signal transduction pathways is very intriguing. Estrogen increases RARα gene expression, but at the same time most of the estrogen-mediated actions are blocked by RA. For example, RA blocks estrogen-mediated growth stimulation of ER-positive cells [Fontana et al., 1990]. We have also shown that RA blocks estrogen-mediated increase in pS2 and TGFα gene expression in ER-positive cells [Fontana et al., 1992]. In the present study we found that RA also blocked the estrogen-mediated increase in pS2 gene expression in the ER-transfected MDA-MB-231 cells (data not shown). Conversely, RA has been shown to enhance ER mRNA and protein levels in MCF-7 HBC cells [Fontana et al., 1992; Guilbaud et al., 1990].

Estrogen, although increasing RARα gene expression in ER-positive cells, did not increase CAT activity from RARE. RA-induced CAT activity from RARE was, however, potentiated by estrogen, suggesting that ERs do not themselves bind and increase transcription from RARE and that RARs require the presence of ligand to enhance the transcription from RARE. To what extent the RXRs are playing a role in the RA-induced RARE-mediated transcription is not clear at this time; further studies are required and are in progress to elucidate the expression pattern of RXRs in HBC cells and their role, if any, in RA inhibition of HBC growth.

The steroid hormone receptors have been shown to have two transcriptional activation functions, a constitutive ligand-independent AF-1 and a ligand-dependent AF-2 [Nagpal et

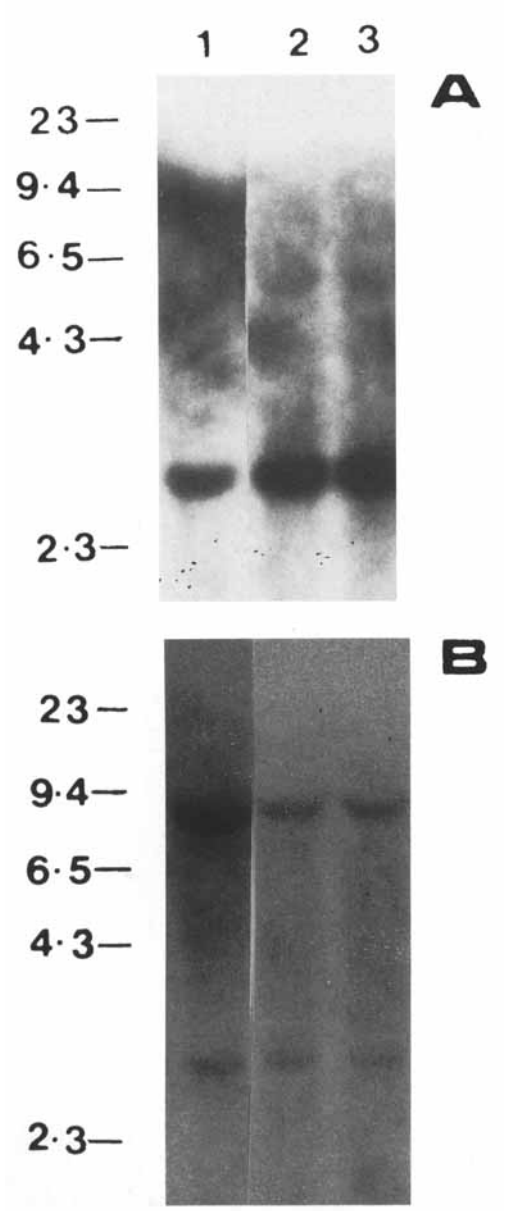

Fig. 8. Southern blot analysis of RARα1 and RARα2 genomic DNA in HBC cell lines. Approximately 10 μg DNA per sample were digested with EcoRI, subjected to agarose gel electrophoresis, and transferred to supported nitrocellulose. As shown, the same blot was probed with RARα1 and RARα2 specific probes. Prehybridization, hybridization, and washing conditions were essentially as described previously [Sheikh et al., 1992]. Migration of λ Hind III molecular weight markers is shown on the left. **A:** RARα1. **B:** RARα2. **Lane 1:** MCF-7. **Lane 2:** MDA-MB-231. **Lane 3:** Normal human mucosal DNA.

al., *1992*, and references therein]. The evidence, however, suggests that RARs and RXRs may require ligand for their optimal functioning [Nagpal et al., 1992, and references therein]. Our study further supports the notion that ligand binding is important for the function of RARs. Although our data suggest that the low levels of RA-induced CAT activity in ER-negative cells can be attributed to the low levels of RARα in these cells, the question remains as to why RARγ does not compensate for the low levels of RARα. Recent evidence suggests that while both isoforms of RARα very strongly stimulated transcription from β2RARE, RARγ failed to do so [Nagpal et al., 1992, and references therein]. Since we also used a β2RARE/tk-CAT reporter construct, it is possible that RARγ was unable to transactivate the TK promoter placed under the control of β2RARE and thus failed to compensate for low levels of RARα.

Like other family members, RARα is divided into six regions designated A–F [Leroy et al., 1991a,b]. The C and E regions contain DNA binding and ligand binding domains, respectively, and are conserved among all members of the family [Leroy et al., 1991a,b]. The murine RARα gene was shown to be expressed as several isoforms which differ in their N-terminal A region [Leroy et al., 1991a,b]. The RARα1 and α2 are the only two known human RARα isoforms and share significant homology to their murine counterparts [Leroy et al., 1991a,b]. RARα1 is believed to be expressed ubiquitously in most of the tissues, but RARα2 isoform expression is more restricted and its expression is regulated by RA [Leroy et al., 1991a,b]. Although it was shown previously that the RARα1 promoter contains an ERE (estrogen response element)-like sequence, the promoter itself failed to function in MCF-7 and T47D HBC cells [Brand et al., 1990]. To determine which isoform of RARα is being expressed and regulated by estrogen in HBC, we used RARα1 and RARα2 specific probes to probe the blots already probed with full-length RARα cDNA. Our results suggested that these cells do not appear to express either the RARα1 or RARα2 isoform. The Southern blot analysis performed on these cell lines using RARα1 and RARα2 specific probes revealed a distinct band pattern for either isoform in these cells as well as in normal human mucosal DNA, thus suggesting that the RARα1 and RARα2 specific genomic sequences were intact but were either not expressed or expressed at very low levels. This may also explain why the RARα1 promoter did not function in MCF-7 and T47D HBC cells [Brand et al., 1990] even though these

cells express higher levels of RARα. Further studies are thus required and are in progress to identify the unique isoform of RARα expressed in HBC.

In conclusion, we have demonstrated that the ER status of HBC plays an important role in RA inhibition of HBC proliferation. HBC cells apparently express a unique isoform(s) of RARα whose expression is under direct control of estrogen, and the estrogenic actions of phenol red are responsible at least in part for the higher basal levels of RARα noted in ER-positive cells.

ACKNOWLEDGMENTS

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Note Added In Proof

In the recent meeting entitled "Mechanism of Action of Retinoids, Vitamin D and Steroid Hormone," held in Banff, Canada, Roman et al. [1993] presented an abstract showing estrogen enhancement of RARα mRNA in ER-positive T47D HBC cell line. In addition, they also observed that the RARα mRNA levels were higher in regular phenol red containing media than the steroid stripped phenol red–free media.

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