Characterization of Estrogen Receptor Messenger RNA in Human Breast Cancer

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ABSTRACT

The importance of estrogen receptor (ER) determination in breast cancer is well established. Approximately 70% of ER-positive tumors are hormone responsive compared to 5–10% of ER-negative tumors. However, one-third of ER-positive tumors fail to respond, and the reasons for this are unclear. To further investigate these relationships we have determined levels of ER protein and mRNA in a number of human breast cancer biopsies. ER protein was estimated by the dextran-coated charcoal steroid binding method and by an ER immunocytochemical assay using a specific monoclonal antibody. A complimentary DNA clone (XOR3) encoding part of the human ER was used to determine mRNA levels. Dot blot analysis of twenty-seven tumors revealed a close agreement between ER mRNA and the dextran-coated charcoal assay (r = 0.75; P < 0.001). ER immunocytochemical assay staining also correlated with ER mRNA in twenty-five cases (r = 0.75; P < 0.001). Tumors from postmenopausal patients contained much higher levels of ER mRNA and ER protein than their premenopausal counterparts. ER-negative tumors produced no measurable ER mRNA. Northern blot analysis revealed a 6.4- and 3.7-kilobase species in ER-positive tumors and also in the human breast cancer cell line MCF-7. No differences in transcript sizes were found in tumors from hormone-responsive patients compared to nonresponding patients. We have also demonstrated, in tissue sections of normal and malignant breast, localization of ER mRNA by in situ hybridization to the same population of cells which exhibit immunoreactive ER.

INTRODUCTION

Normal mammary development has long been known to be under the influence of steroid hormones, particularly estrogen, presumably by interaction with specific intracellular receptors in target cells. The failure of a number of breast cell lines to respond to estrogen in vitro has been well correlated with the absence of ER in contrast to the mitogenic action of estrogen in ER-positive lines (1). Similarly, human breast neoplasms display a heterogeneity of expression of ER. About 30% of patients have undetectable levels of tumor ER as determined by a ligand binding assay (DCC) (2). Of the 70% that are ER positive, tumor regression can be achieved by endocrine therapy in about two-thirds. Only 5–10% of the ER-negative group respond to such treatment (3, 4). Overall, ER-positive patients can expect increased survival and longer disease-free intervals by a ligand binding assay (DCC) (2). Of the 70% that are ER positive, patients have undetectable levels of tumor ER as determined in ER-positive lines (1). Similarly, human breast neoplasms respond to estrogen in vitro has been well correlated with the absence of ER in contrast to the mitogenic action of estrogen in ER-positive lines (1). The recent cloning of the ER cDNA (13) has made it possible to investigate the transcription of this gene. In this study we have examined the level of ER mRNA in a number of human breast tumor biopsies and attempted to correlate this with the amount of receptor protein that can be detected either biochemically or immunocytochemically. We have also used an in situ hybridization technique to determine which cells are synthesizing message, and whether those cells which have no ER protein detectable by ERICA also lack ER mRNA. Finally, we have performed northern analysis of ER-positive tumors in order to determine whether any differences exist in the distribution of transcript sizes encoding for ER between those tumors responding and those failing to respond to endocrine therapy.

MATERIALS AND METHODS

Chemicals. [3H]Estradiol (100 Ci/mmole), [35S]dCTP (3000 Ci/mmole), and [35S]dCTP (>1000 Ci/mmole) were obtained from Amersham (United Kingdom). The ER cDNA clone XOR3 (EcoRI fragment in pBR322 encoding 1.3 kb of the open reading frame of the MCF-7 ER mRNA), was kindly provided by P. Chambon, and the rat anti-human ER monoclonal antibody H222 by Abbott Laboratories, Chicago, IL). All other reagents were of the highest grade available.

Tissue Samples. Breast biopsies were obtained from patients attending the Breast Clinics at St. George's Hospital, London and other allied hospitals. None of these had treatment prior to surgery. A total of 41 specimens (37 primary breast cancers, one metastatically involved lymph node, one fibroadenoma, and 2 normal breast) were collected following surgery, carefully dissected for material of interest, and frozen and subsequently stored in liquid nitrogen. In all cases histological confirmation of diagnosis was carried out.

RNA Extraction. Total cellular RNA was isolated by the guanidine isothiocyanate method (14) from 0.3–1.0 g frozen tissue. Yields obtained varied between tumors and were quantified spectrophotometrically. The quality of the RNA was verified by the integrity of the 28 and 18S ribosomal bands following agarose gel electrophoresis.

Biot Hybridization. As yields of RNA were generally quite low due to small tissue samples, hybridization was normally performed using total rather than poly(A)+ selected RNA. Dot blots (15) were done using serial dilutions of formaldehyde denatured RNA ranging from 20 down to 1.25 pg, spotted onto Bio-Rad nylon membranes (Pall Filtration, Portsmouth, United Kingdom) by means of a Bio-dot manifold (Bio-Rad, United Kingdom). In addition, standard dilutions of receptor complex. Experimental data to support such a hypothesis are so far unavailable. An interesting observation, however, is that immunocytochemical staining (ERICA) of nominally ER-positive breast tumors shows marked heterogeneity, in that within clusters of ER-positive cancer cells, there are often unstained cells, suggesting a lack of ER expression in these cells. However, this distribution can also be seen in patients who do respond successfully to therapy and therefore may not be of clinical significance. Moreover, it has been shown that even patients whose tumors are strongly ER positive and who initially respond to antiestrogens eventually relapse and exhibit tumor recurrence. The attractive idea that this may have reflected a clonal outgrowth from ER-negative cells within the tumor has been invalidated (10, 11) as the majority of these recurrent tumors are still ER positive. There is considerable speculation concerning this transition to a hormone-independent phenotype (12).

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The abbreviations used are: ER, estrogen receptor; DCC, dextran-coated charcoal; ERICA, estrogen receptor immunocytochemical assay; poly(A)+, polyadenylated; cDNA, complementary DNA; PBS, phosphate-buffered saline containing 10 mM sodium phosphate, pH 7.4 and 150 mM sodium chloride; kb, kilobase; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; S1, staning intensity index.
unlabeled \( \lambda OR3 \) and of a ribosomal RNA preparation were also applied to each filter to enable subsequent quantification of the signals and to determine the extent, if any, of nonspecific hybridization. Samples were washed through with 20x SSC (3 M NaCl-0.3 M trisodium citrate, pH 7) and the filter then baked for 1 h at 80°C. Filters were prehybridized at 42°C for 4–6 h in sealed bags in 50% (v/v) deionized formamide, 0.1% SDS, 5× Denhardt’s solution (1× Denhardt’s solution: 0.02% each of polyvinylpyrrolidone, bovine serum albumin, and Ficoll), 5 mM EDTA, 750 mM NaCl, and 50 mM NaH2PO4, pH 8.3, 4 mM sodium pyrophosphate, pH 6.5, and denatured sonicated salmon sperm DNA (250 μg/ml). Blots were hybridized under the same conditions for 12–16 h with addition of 105 cpm/ml denatured \( \lambda OR3 \) fragments labeled with \( \left[{^32}P\right]dCTP \) by a random primer method (16) to specific activity of 108 cpm/μg DNA.

Following hybridization, blots were washed with five changes of 2× SSC-0.1% SDS at room temperature and two changes of 0.1× SSC-0.1% SDS at 60–65°C. Filters were then exposed for autoradiography to Kodak XAR film at ~70°C with intensifying screens for 4–7 days. The autoradiographic spots were scanned using a laser densitometer and the intensities obtained were quantified by comparison with \( \lambda OR3 \) standards. Results were expressed as pg per 20 μg total RNA correcting for the fact that the ER cDNA insert constitutes only a proportion of the total plasmid.

Northern analysis of total RNA (20 μg/lane) or poly(A)^+ RNA (2.5 μg/lane) was carried out on agarose/formaldehyde gels (17). Following blotting, hybridization was performed as above. Transcripts were sized using denatured RNA and DNA markers.

**In Situ Hybridization.** Following a procedure modified from Lawrence and Singer (18) frozen sections (5–8 μm) were thaw mounted on poly-l-lysine coated slides and fixed in 4% paraformaldehyde in 1× PBS, 5 mM MgCl2, and 15 min and then stored in 70% ethanol at 4°C. Following rehydration in 1× PBS-5 mM MgCl2 for 10 min and 0.1 M glycine-0.2 M tris, pH 7.4, for 10 min, sections were placed in 50% formamide-2× SSC at 65°C for 15 min. Meanwhile, overnight-dried aliquots of random primer \( \left[{^32}P\right]dCTP \) labeled \( \lambda OR3 \) (specific activity, 0.5–3× 108 cpm/μg) containing 10 μg of each of Escherichia coli (RNA) and sonicated salmon sperm DNA, were reconstituted in 5 μl formamide and heated to 70°C for 10 min and then adjusted (in a total volume of 10 μl) to 2× SSC-2 mg/ml bovine serum albumin-20 mM Vanadyl complex-10% dextran sulfate-0.1× Denhardt’s. Dithiothreitol was also included at 20 mM. This hybridization buffer was pipetted onto the section and overlaid with a coverslip for incubation at 37°C in a humidified chamber for 4 h. The following regimen was used for washing: 30 min at 37°C in 50% formamide-2× SSC; six times in 2× SSC for 2 min each at 20°C with agitation; 30 min in 50% formamide-1× SSC at 37°C; three times in 1× SSC for 10 min each at 20°C with agitation. Sections were then sequentially dehydrated in 70, 80, and 100% ethanol, and air dried.

In all experiments, parallel incubations were carried out using labeled \( HpaII \) fragments of pBR322 as a nonspecific probe to determine background hybridization. Random sections were also checked for RNA retention by acridine orange staining.

Autoradiography was performed by dipping slides in a 50% aqueous solution of K5 nuclear emulsion (Ilford, Ltd., Mobberly Cheshire, United Kingdom) at 45°C, followed by exposure for 2–4 weeks at 4°C. Development was by standard procedure, followed by counterstaining with sections with Mayer’s hematoxylin.

**Ligand Binding Assay for ER.** This was performed by a modification of the DCC method described by McGuire and De La Garza (2). Briefly, aliquots of breast tissue cytosol were incubated with varying concentrations of \( \left[H^3\right]\)estradiol in the presence and absence of the competitor diethylstilbestrol. Bound radioligand was determined by liquid scintillation counting after removal of free label with DCC. Data were analyzed using Woolf and Scatchard plots to determine dissociation constant and maximal receptor levels; these were expressed as fmol per mg cytosol protein, which was determined by the method of Bradford (19) using Bio-Rad protein dye reagent.

Receptor concentrations >10 fmol/mg protein were regarded as a positive result.

**Immunocytochemical Assay for ER.** Details of this method have been described previously (20, 21). Frozen sections were fixed in 4% formal-PBS, cold methanol and acetone, and treated with 2% normal goat serum to reduce nonspecific staining, prior to incubation with a rat anti-human ER monoclonal antibody, H222 (22). A goat anti-rat bridging antibody was then used to amplify the signal, followed by incubation with peroxidase antiperoxidase rat antibody complex. Visualization of receptor was achieved by the diaminobenzidine hydrochloride-hydrogen peroxide procedure. Sections were subsequently counterstained with dilute Harris hematoxylin.

Results were expressed in the form of a SII as previously described (21). This was calculated as

\[
\text{(SII) = } \frac{\text{(% tumor cells stained at intensity A \times 0)}}{\text{(% tumor cells stained at intensity B \times 1) + (% tumor cells stained at intensity C \times 2) + (% tumor cells stained at intensity D \times 3)}}
\]

where intensity A is no staining, intensity B is weak staining, intensity C is moderate staining, and intensity D is strong staining. SII values greater than 0.1 were regarded as positive.

**Statistical Method.** The relationship between ER mRNA and protein levels was examined by Spearman’s rank correlation test (r) which makes no assumptions about the population from which samples are drawn. A correction was included for tied scores where applicable (23).

**RESULTS**

Total RNA was extracted from 27 primary tumors, one involved lymph node, and one fibroadenoma, and shown to be undegraded as reflected by sharp intact ribosomal bands. The majority (72%) of the primary tumors exhibited histopathological features associated with infiltrating ductal carcinoma. The variable yields of RNA were, in part, accountable by the differences in cellularity; the range was from 182–963 μg/g of frozen tumor tissue. The amount of ER mRNA in these samples detected by dot blot hybridization and estimated as in “Materials and Methods” displayed a considerable range of values from 0 (in 7 cases) to 4180 pg/20 μg total RNA, with a mean value around 900 pg (omitting the negatives). A typical autoradiogram is exhibited in Fig. 1 showing the variable expression of ER mRNA in a group of 10 tumors.

**Relationship between ER mRNA Level and ER Protein Content.** Of the 7 of 27 tumors containing no measurable ER mRNA, 6 had undetectable levels of ER protein as assessed by

![Fig. 1. Dot blot analysis of total RNA from 10 primary breast cancers showing hybridization of \( \left[{^32}P\right]labeled \lambda OR3 \) to serial dilutions of 20–1.25 μg total RNA for each sample. Note that a 20-μg aliquot was not applied for tumor G. Blots were hybridized and washed as in “Materials and Methods” and exposed at –70°C for 4–7 days on Kodak XAR film with intensifying screens. Lanes A–H and J, variable levels of ER mRNA; lane I, absence of significant expression of ER message.](image-url)
the DCC method (<10 fmol/mg). The remaining one was only borderline positive at 11 fmol/mg. From the 20 which expressed ER mRNA, 19 were ER protein positive and one was negative. However, the latter was found to give a strongly positive reaction with ERICA. Overall, there was a strong positive correlation \( r_s = 0.9; P < 0.001 \) between levels of ER mRNA and protein (Fig. 2). Even when double negative values are excluded the relationship is still highly significant \( r_s = 0.8; P < 0.001 \).

In addition to the DCC measurement, we also assessed protein in 25 samples (23 primary carcinomas, one metastatic lymph node, and one fibroadenoma) using ERICA. Seven carcinomas were ERICA negative and also had no ER mRNA.

The other 18 tumors, including the fibroadenoma, expressed significant amounts of ER mRNA and all but 2 were ERICA positive; because these were strongly DCC positive, it is likely that the lack of staining was due to the premature thawing of these particular sections which occurred prior to fixation. Unfortunately no more tissue was available. The relationship between ER mRNA levels and ERICA (SII) was a good one overall (Fig. 3) and the correlation was highly significant \( r_s = 0.75; P < 0.001 \). However, in this case if negative values are excluded the relationship among the results of the positive group is not significant \( r_s = 0.34; P < 0.2 \).

**In Situ Hybridization.** Following dot blot analysis, cases expressing varying levels of ER mRNA were selected for *in situ* hybridization. A number of tumors and normal breast specimens were examined using a 35S-labeled cDNA encoding part of the ER mRNA sequence. The slides were assessed by one of the authors (Y. L.) without prior knowledge of ER status. Comparison of *in situ* hybridization and dot blot analyses revealed a good agreement using a visual assessment scale of strongly positive, positive weak reaction, and negative, compared to a pBR322 control in each case. In all of the normal breast sections, we observed an accumulation of autoradiographic silver grains preferentially over the cells which form the ductal and lobular alveolar units, i.e., the parenchymal epithelia and the interposed myoepithelia (Fig. 4, A and B). Due to the close proximity of these cell types and the level of background obtained after the long exposures required, we were unable to resolve grain distribution between them with any degree of certainty. However, no specific reaction was seen elsewhere, either in the adipose cells or in the stromal connective tissue elements which predominate the normal resting gland. Vascular structures were likewise unlabeled.

A similar reaction was seen in the tumor sections, which were ER protein positive. In these, the grains were found to be accumulated over clusters of tumor cells interspersed in the stroma, with no other reaction significantly above background (Fig. 4, C and D). A similar pattern was observed with the immunocytochemical staining obtained with ERICA on sections of the same tumor (Fig. 4F). In ER protein-negative tumors no specific hybridization was seen (Fig. 4E). In one case of a benign biopsy, mRNA localization was clearly observed in areas of cellular hyperplasia. For comparison, we also performed hybridization with MCF-7 and MDA-MB-231 cells and observed an accumulation of grains in the cytoplasmic regions of the former and no reaction in the latter (Fig. 4, G and H).

**Analysis of ER Transcript Size.** We screened a small number of tumors of varying ER protein status by northern hybridization. Two bands were found to show hybridization with ER cDNA in most cases (Fig. 5). The higher one, which was also more abundant, was estimated to be between 6.4 and 6.9 kb in size depending upon whether RNA or DNA markers were used. This transcript was also clearly demonstrated in MCF-7 RNA (Fig. 5). The lower band of 3.7 kb (best seen in lane 2, Fig. 5) could also be detected in MCF-7 RNA but only with a much longer exposure time than was necessary to visualize the higher band. MDA-MB-231 cell RNA showed no hybridization.

We also examined RNA from ER-positive tumors obtained from patients at primary surgery who subsequently failed to respond to endocrine therapy for relapse according to Union Internationale Contre le Cancer criteria (24). Three such tumors showed no abnormal transcripts compared to tumors from responding patients.

**ER Expression and Menopausal Status.** From a group of 24 patients, we found that 5 of 9 (56%) of premenopausal women had tumors which were positive for both ER protein (DCC) and mRNA, while from 15 women of postmenopausal status, this was the case for 13 of them (87%). A comparison of the positive groups in the two categories (Table 1) showed that postmenopausal women had much higher levels of tumor ER protein as measured by DCC (mean, 219 fmol/mg) than premenopausal women (mean, 59 fmol/mg). This was paralleled by a disparity of similar magnitude in ER mRNA levels (postmenopausal mean, 1382 pg; premenopausal mean, 245 pg).

**DISCUSSION**

We have shown in a random group of 27 breast tumors that there is a direct correlation between the levels of ER protein measured by the DCC method and the presence within these tissues of mRNA transcripts coding for it. As is now well documented (25) with far larger sample numbers, we found that the majority (about 73%) of this group had positive ER status.
Fig. 4. Demonstration of ER mRNA in frozen sections by in situ hybridization (A–E and G–H) and of ER protein by immunocytochemistry (F). Normal human breast sections were hybridized with λOR3 (A) and pBR322 negative control (B) (original magnifications, × 400). ER-positive human breast carcinoma was hybridized with λOR3 (C) and pBR322 (D) (original magnifications, × 350 and 400, respectively). ER-negative human breast carcinoma hybridized was with λOR3 (E) (original magnification, × 400). Section of ER-positive breast cancer (same patient as in C and D) was stained with monoclonal antibody to ER (F) (original magnification, × 400). MCF-7 (G) and MDA-MB-231 (H) breast cancer cells were hybridized with λOR3 (original magnifications, × 250 and 420, respectively). A–E, G, and H were hybridized with 35S-labeled λOR3 at 37°C for 4 h; autoradiographic exposure of nuclear emulsion coated slides was for 2–4 weeks. Sections were counterstained with Mayer's hematoxylin eosin. F was stained using an indirect immunoperoxidase procedure with a rat anti-human monoclonal antibody to ER. Visualization was by the diaminobenzidine hydrochloride-hydrogen peroxide procedure followed by counterstaining with dilute Harris hematoxylin.
obtained from in situ hybridization with radiolabeled probes, is both in normal tissues and in tumors. Discounting the possibility of an artifact of antibody penetration the question arises of whether this protein is actually expressed in cells of the negative tumors, or whether they contain untranslated ER mRNA. Unfortunately, the scatter of the silver grains, obtained from in situ hybridization with radiolabeled probes, is such that it is not possible to say whether there is heterogeneous hybridization. Sometimes it is possible to see some cells with no grains around them in the midst of other cells that are clearly positive, but such observations are too subjective to be reliable. We intend therefore, to approach this problem by using smears of fine needle aspirates so that single cells can be resolved. Both in situ hybridization and ERICA could then be done on parallel smears. Furthermore it may be possible to do a sequential analysis on the same smear, because some preliminary investigation has shown that samples which have already been subjected to ERICA still show acridine orange induced fluorescence with intensities similar to those from untreated samples, surprising perhaps in view of the fact that no precautions against RNase were taken. It remains to be seen whether ER mRNA also survives.

The in situ hybridization technique does however clearly show, in both normal and malignant breast, that it is the same population of cells which expresses ER mRNA and exhibits staining for ERICA.

We have found no major differences in the mRNA transcript sizes within our samples. We did however observe two bands in most tumors in contrast to the single species previously reported (13). Some background track binding occurred which may be due to our use of total RNA as opposed to selected MCF-7 poly(A)+ RNA used by these authors. We also found that the smaller 3.7-kb band was more abundant in most human breast tumors compared to MCF-7 RNA; the significance of this remains a matter of speculation. The discrepancy in the size of the upper band from the 6.2 kb reported previously (13) is probably not significant and may be due to the use of different gel systems and variations in measuring diffuse bands. We did consider the possibility that there was cross-hybridization with mRNA coding for the glucocorticoid receptor, which is in the region of 7 kb (27). However, we observed no hybridization to poly(A)+ RNA from MDA-MB-231 (a cell line known to express this receptor) with the XOR3 clone.

We also examined transcript sizes from an interesting group of patients who although having ER-positive tumor status, did not respond to antiestrogen therapy. No abnormalities were observed in the 14 cases we were able to analyze, which might have resulted in an altered protein unable to bind tamoxifen. These tumors may well have growth properties related more to estrogen-independent ER-negative cells.

It is now well established from numerous studies that a greater proportion of postmenopausal patients have ER-positive tumors than their premenopausal counterparts (21, 28-30). Furthermore, the level of receptors is lower in the tumors from postmenopausal patients. One explanation was that receptor levels, as measured by ligand binding assays, were not correctly assessed due to receptor occupancy by the high levels of circulating estrogen in premenopausal women. However, studies using monoclonal antibodies to ER which are not subject to such limitations have largely invalidated this idea. Our study confirms that the ER-negative tumors in both groups also lack ER mRNA. We also show that not only is the incidence of ER positivity greater in tumors from postmenopausal patients but that actual ER content is several times higher than in the corresponding premenopausal ER-positive group. This is closely correlated with ER mRNA content. It is worthy of note that our mean DCC values are very similar to those of Martin et al. (29) whose study involved a large number of patients (n = 672) and showed similarly high SDs. In both instances this was due to inclusion of tumors with very high ER protein and, as we have shown, of mRNA levels. It is tempting to speculate that events associated with the meno-

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**Table 1 Menopausal status and ER expression**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Premenopausal</th>
<th>Postmenopausal</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>9</td>
<td>15</td>
</tr>
<tr>
<td>No. ER positive</td>
<td>5+</td>
<td>13</td>
</tr>
<tr>
<td>DCC of positive group</td>
<td>59 ± 27**</td>
<td>219 ± 184*</td>
</tr>
<tr>
<td>ER mRNA of positive group</td>
<td>245 ± 359c</td>
<td>1382 ± 1193c</td>
</tr>
</tbody>
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* Mean ± SD.
+ fmol [3H]estradiol bound per mg cytosol protein.
+ pg ER mRNA per 20 μg total RNA hybridized.

The 27% that were ER negative had no detectable ER mRNA clearly showing that the absence of receptor was not due to any inconsistency in the ligand binding method or to a translational phenomenon, but involves a transcriptional switching off at the DNA level.

Although the overall correlation with ERICA was also significant it was obtained largely from the inclusion of the negative tumors; a similar conclusion could be drawn from previous studies using monoclonal antibodies to ER which are not subject to such limitations. The discrepancy in the size of the upper band from the 6.2 kb reported previously (13) is probably not significant and may be due to the use of different gel systems and variations in measuring diffuse bands. We did consider the possibility that there was cross-hybridization with mRNA coding for the glucocorticoid receptor, which is in the region of 7 kb (27). However, we observed no hybridization to poly(A)+ RNA from MDA-MB-231 (a cell line known to express this receptor) with the XOR3 clone.

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**Fig. 5.** Northern analysis of ER transcripts. Twenty μg total RNA (lanes 1-8) or 2.5 μg poly(A)+ mRNA (lanes 9 and 10) were separated on agarose/formaldehyde gels as described in "Materials and Methods." Lanes 1-3, breast carcinomas from nonresponders to hormone therapy; lanes 4-8, breast carcinomas from responders; lane 9, MCF-7 cells; lane 10, MDA-MB-231 cells. All were hybridized to 32P-labeled XOR3 and exposed for 7 (lanes 1-8) or 3 (lanes 9 and 10) days at —70°C with intensifying screens. Sizes of the bands in kilobases as determined by DNA markers are shown on right.

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pause could trigger changes in breast cancer cells leading to constitutive expression of ER mRNA, possibly reflecting a response to the marked reduction in circulating levels of estrogen.

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