A Variant Estrogen Receptor Messenger Ribonucleic Acid is Associated with Reduced Levels of Estrogen Binding in Human Mammary Tumors

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An analysis of the human estrogen receptor (ER) mRNA was performed on 71 human breast tumors using an RNase protection assay. Complementary DNA clones to the human estrogen receptor (λR8 and λR3) were used to generate small antisense ^{32}P-labeled RNA molecules that were hybridized to the tumor RNA. We determined the relative amounts of ER mRNA in each tumor by measuring the amount of RNases A and T1 resistant hybrids. Moreover, because RNase A has the ability to cleave single-base mismatches within RNA/RNA duplexes, we were able to use the assay to screen for possible mutations or deletions in the ER mRNA.

A significant correlation was found between the ER mRNA levels and the estrogen binding concentrations determined by a dextran-coated charcoal assay ($r = 0.68$; $P < 0.0001$; $n = 58$). We also identified a subpopulation of tumors in which a mismatch in the ER mRNA was detected. This message modification, in the B region of the message, significantly correlated with low levels of estrogen binding. This result suggests that the observed B variant might lead to the production of receptors with altered properties. (Molecular Endocrinology 2: 785–791, 1988)

INTRODUCTION

The biological changes observed in the normal mammary gland during puberty, pregnancy, and lactation are under the physiological influence of circulating estrogens. Recently, estrogen receptor (ER)-containing cells have been found by immunocytochemical techniques in the normal nonlactating mammary gland (1), and the mitogenic action of estrogens has been well documented in the MCF_7 human mammary cell line (for review see Ref. 2).

Human breast cancers display a considerable heterogeneity in ER concentration, and there is evidence that ER levels constitute an important prognostic factor for patient selection and endocrine therapy. Seventy percent of breast cancer patients are ER positive, and more than half of these respond to hormone therapy (3). Furthermore, 5–10% of the ER-negative group are also expected to respond to such treatment (4). Overall, patients with ER-positive primary cancer are more likely to have a longer disease-free interval than those whose tumors lack ER (5).

Several methods are currently available to quantify the ER levels in mammary tumors. The classical biochemical determination that uses radiolabeled ligands has been confirmed by the use of specific monoclonal antibodies (6). These two complementary techniques have provided considerable insight into ER tissue distribution and intracellular localization (7, 8). However, even the combination of biochemical and immunocytochemical assays currently fails to conclusively predict which patients will actually respond to endocrine therapy.

More recently, the study of estradiol-regulated proteins, used as additional tumor markers, has provided new approaches in the definition of hormonally responsive tumors (9, 10). Numerous speculations, including the proposed existence of malfunctioning receptors, have been put forward to explain why up to 40% of ER-positive patients fail to respond to antiestrogen therapy. However, experimental data to support the association of mutated estrogen receptors and tumori-

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genesis of estrogen sensitive tissues are still not available.

The recent cloning of the human ER cDNA (11) has made it possible to investigate ER gene structure and expression. Two recent publications describe ER mRNA detection by Northern (9) and dot-blot (12) hybridization to human mammary tumor RNA. In our study, using several clones corresponding to portions of the different ER functional domains in a solution hybridization/RNase protection assay, we performed both a quantitative and qualitative analysis of the tumor ER mRNA. The levels of ER mRNA were measured and compared to the levels of estrogen binding protein. We also performed a fine structural analysis of the ER mRNA in order to screen for possible message deletions or mutations which could produce abnormal ERs.

RESULTS

Human cDNA clones (λOR8 and λOR3) provided by Chambon and co-workers (11) were used to prepare several subclones corresponding to the ER functional domains. Figure 1 shows a partial restriction map of the human ER cDNA and its subdivision into six functional domains (11, 13). Because the RNase protection method works optimally using fragments of less than approximately 600 base pairs inserted into riboprobe generating vectors, we divided the original clone into 8 fragments. Three clones were obtained from the A/B regions (ab0, ab1, ab2), one from the C (d0) and D (d1) regions, and three from the E and F regions (e1, e2, ef) (Fig. 1).

Total RNA from 71 individual tumors was isolated and hybridized in solution to antisense ^{32}P-labeled RNA (cRNA) generated from the above mentioned clones. After digestion by the combination of two ribonucleases, RNase A and T1, the nuclease resistant RNA duplexes were fractionated by nondenaturing polyacrylamide gel electrophoresis and analyzed by autoradiography. The labeled probes were also hybridized to nonspecific yeast RNA to assess the digestion efficiency. A reference positive control sample (MCF_7 or human endometrial RNA) was also included to standardize the obtained signal. Samples were loaded on the slab gel in decreasing order according to their previously determined estrogen binding levels.

Typical results are shown in Fig. 2, in which a combination of three different antisense probes was used. Three major bands representing the fully protected RNA fragments were observed after RNase digestion. A background of minor bands was also observed, probably due to enzymatic cleavage of complementary duplexes, especially in the A + U rich regions more sensitive to RNase A digestion (14). Considering this difference in sensitivity, the relative amounts of the protected fragments were found to be comparable within each tumor sample. A significant correlation was found between the estrogen binding values and ER mRNA levels in each tumor.

Figure 3 represents the results obtained with another set of samples analyzed with a different combination of probes. In this case, using milder digestion conditions, we observed that relative intensities of the three protected fragments were more similar. Moreover, using the fragments e1 and e2, we observed variable relative amounts of smaller protected fragments. These bands probably result from differences in the amount of larger precursor mRNAs containing unprocessed intron sequences that are not protected by the cRNA probes.

The partial and random analysis we performed (see *Materials and Methods*) did not reveal any major message truncation or deletion that would indicate the presence of abnormal receptors. However, we did observe the existence of a tumor subpopulation presenting a different digestion pattern for region B. Figure 4 shows three examples of additional cleavage of the RNA duplex generated with the ab1 subclone. This additional cleavage, indicating the presence of a few or a single base mismatch, was observed in eight out of 66 ER-positive tumors. No additional mismatches were

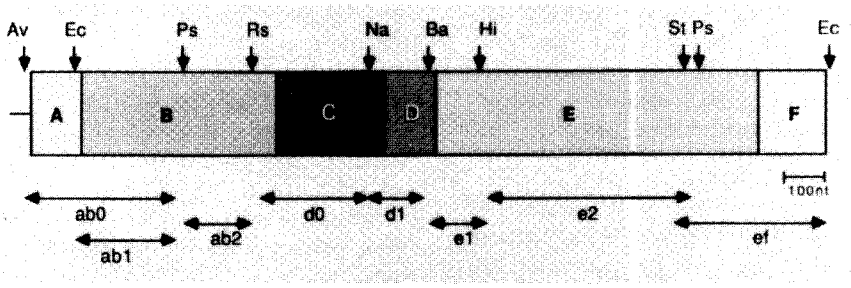

Fig. 1. The Human ER cDNA and its Subdivision into eight Clones

Schematic representation of the human ER cDNA that has been divided into six regions (A–F) based on its homology to the chicken ER (13). The ER cDNA, from the λOR8 or λOR3 clones, was divided into eight smaller fragments using the indicated into the polylinker sequence of pGEM vectors. The vectors were linearized at the end of the insert, and antisense transcripts were *Nae*I; Ba, *BAl*I; Hi, *Hin*dIII; St, *Stu*I. The *Eco*RI sites are linkers added to the λOR3 or λOR8 clones. DNA fragments were inserted into the polylinker sequence of pGEM vectors. The vectors were linearized at the end of the insert, and antisense transcripts were synthesized *in vitro*, thus generating ^{32}P-labeled complementary cRNA probes of the size of the subcloned fragments.

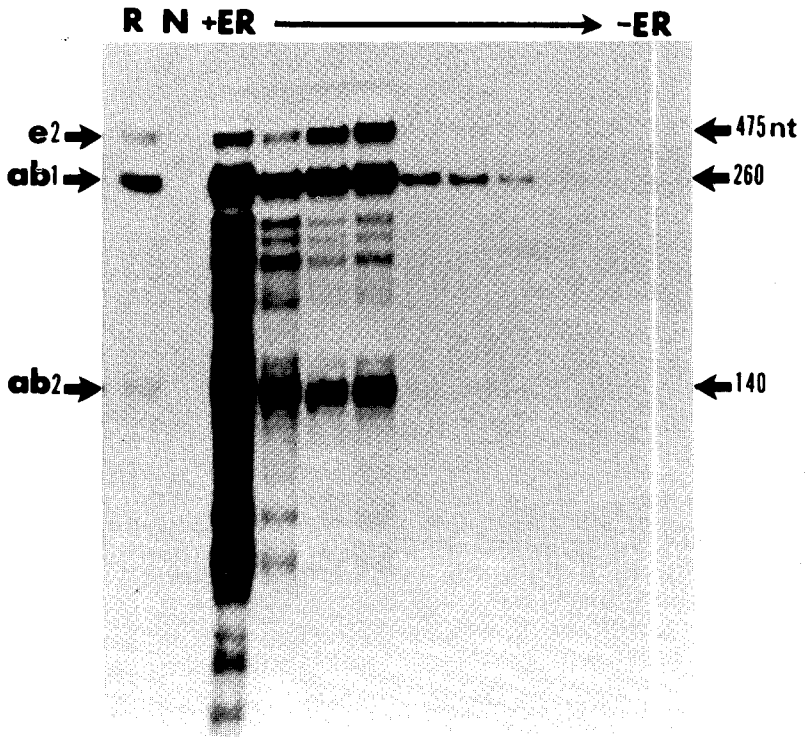

Fig. 2. Analysis of the ER mRNA with the ab1, ab2, and e2 Subclones

Total cellular RNA prepared from tumor tissue was hybridized to 2×10^5 cpm of each cRNA probe. After hybridization the samples were incubated at 37 C for 1 h with a mixture of RNase A and T1. Ribonuclease resistant hybrids were analyzed by gel electrophoresis on a 6% polyacrylamide nondenaturing gel. An autoradiogram after a 20-h exposure at −70 C with intensifying screens is shown. First lane (R), reference sample (15 μg MCF_7 total RNA); second lane (N), negative control (60 μg yeast RNA); +ER to −ER, 15 μg total RNA from different tumors containing decreasing amounts (346, 193, 283, 300, 31, 17, 17, >2, >2 fmol/mg) of estrogen binding protein.

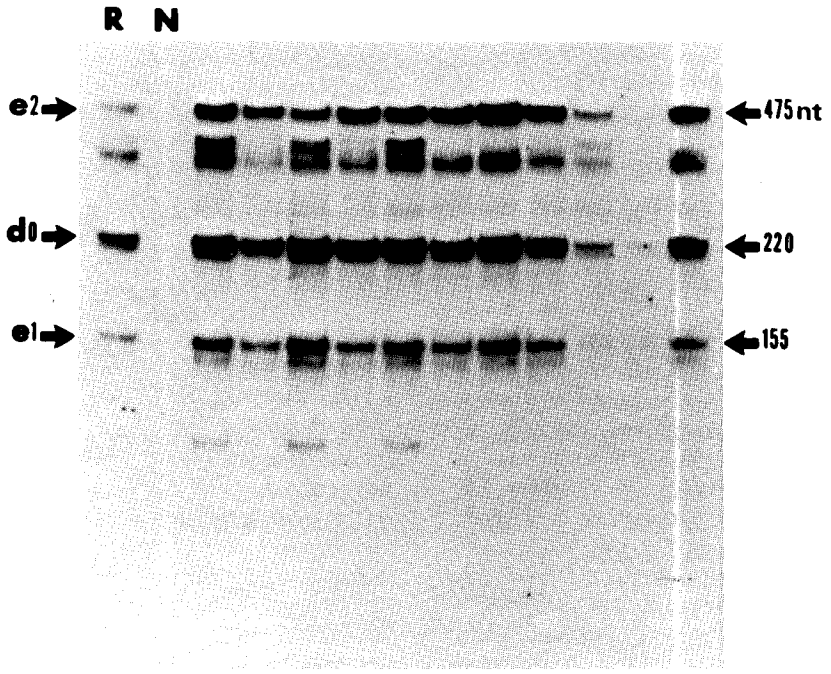

Fig. 3. Analysis of the ER mRNA with the e1, e2, and d0 Subclones

RNA extracted from individual tumors was hybridized with ^{32}P-labeled antisense RNA, digested at 32 C for 1 h, and run on a 6% polyacrylamide nondenaturing gel. An autoradiogram after a 20-h exposure at −70 c is shown. First lane (R), reference sample (15 μg MCF_7 RNA); second lane (N), negative control (60 μg yeast RNA). All the other lanes represent the hybridization to 15 μg total RNA from different tumors.

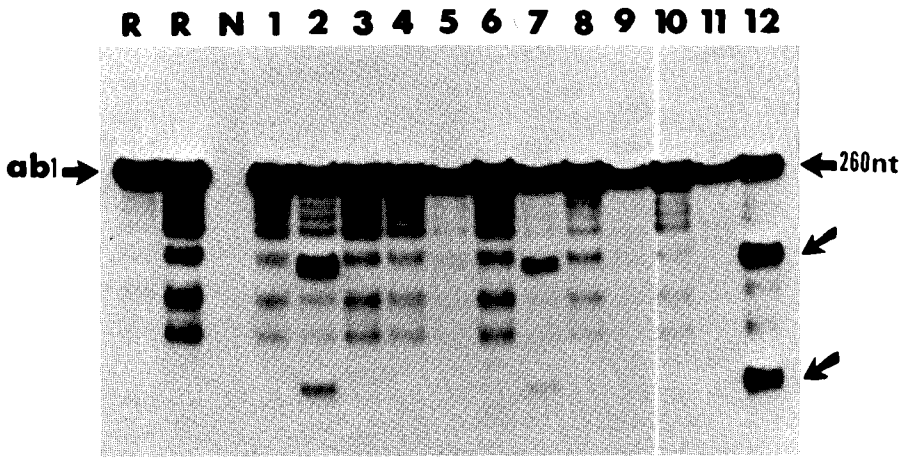

Fig. 4. RNAse Mismatch Cleavage of ER mRNA from Human Breast Tumors

Total cellular RNA from the tumors (15 μg) was hybridized to radioactively labeled antisense RNA corresponding to the first half of the B region (clone ab1: nt 100 to 363) at 55 C for 20 h. The hybridized RNA was digested at 38 C with RNase A and T1 for 1 h, and analyzed on polyacrylamide nondenaturing gels. First and second lanes (R), reference samples; third lane (N), negative control; lanes numbered 1–12, 15 μg tumor RNA. In lanes 2, 7, and 12 an additional cleavage was observed generating two small fragments indicated by the *arrows*.

detected in the DNA (d0 and d1 clones) or estrogen binding portions (e1, e2, and ef clones) of this tumor subpopulation. A more accurate localization of the structural difference in the ab region was obtained by using the overlapping ab0 subclone. As seen in Fig. 5, the approximate location of the mismatch is around nucleotide 250 of the ER cDNA.

A relative ER mRNA quantification was obtained by excising the radioactive bands corresponding to the resistant RNA duplexes from acrylamide gels, and counting them. The amount of ER mRNA estimated was compared to the previously determined values of estrogen binding. Both measures displayed a wide range of values, from 0 to 500 fmol/mg for the ER binding and from 0–50 U for the ER mRNA. All ER-positive samples contained readily detectable levels of ER mRNA while eight of 12 samples, classified as ER negative (<10 fmol/mg), had low but measurable amounts of ER mRNA.

Because the mean of ER binding values was significantly lower in the B variant group than in the rest of the ER-positive samples ($t = 2.94$; $P < 0.01$), a separate measure of the correlation was performed for these two groups. A positive correlation between the ER mRNA and estrogen binding levels was found in the common group (Fig. 6A) ($r = 0.68$; $P < 0.0001$; $n = 58$) after the exclusion of the samples containing no ER protein and no ER mRNA. In contrast, no significant correlation was found between ER mRNA and ER binding in the B variant group ($r = 0.1$; $P > 0.8$; $n = 8$) (Fig. 6B). This is also illustrated by the fact that a comparison of the ER mRNA means in the variant and common groups showed no significant difference ($t = 0.48$; $P > 0.6$).

No correlation was found between the presence of the B variant mRNA, and either the levels of progesterone receptor, a particular tumor histology, the presence of metastatic nodes, or the incidence of breast cancer in the patient's family. As previously stated, the only affected variable noted was the level of estrogen binding, significantly lower in the variant than in the common group.

The ab1 subclone was subsequently used to analyze RNA preparations from normal human myometrium. Two specimens out of 19 did present the same protection pattern previously observed in the variant group, demonstrating that the polymorphism is not restricted to malignant tissue.

DISCUSSION

We have analyzed 71 human breast tumors with a recently developed method, the RNase protection assay, used for the diagnostic detection of single point mutations in transcribed genes (14). This highly sensitive method allows both quantitative and qualitative analysis of the ER mRNA with a limited amount of total cellular RNA. We have shown, in samples containing ER mRNA structurally undistinguishable from MCF_7 ER mRNA, that there is a direct correlation between the levels of estrogen binding protein and the levels of ER mRNA. Numerous factors may interfere with the determination of tumor ER levels, such as estrogen binding lability, receptor extractibility, tumor cellularity, and heterogeneity of staining intensity by antibodies (4, 8). In the current study, the occasional disparity observed between the two measures is similar to the one reported comparing biochemical and immunological assays (8), and slightly less than the one comparing a biochemical assay with messenger quantification by dot-blot (12).

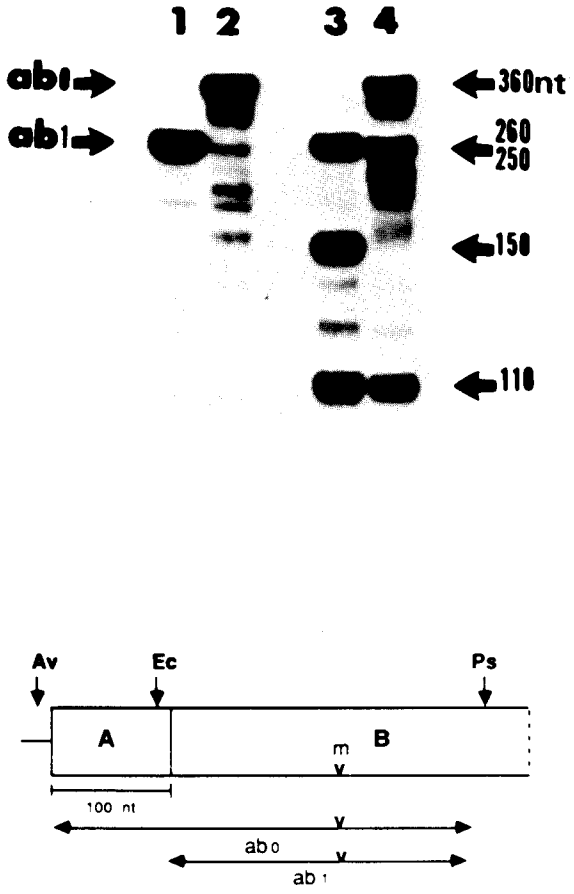

Fig. 5. RNase Mismatch Cleavage Localization on ER mRNA from Human Breast Tumors

Total cellular RNA from two different breast tumors, of the common (lanes 1 and 2) or of the B variant type (lanes 3 and 4), was hybridized to labeled antisense RNA corresponding to the ab1 and ab0 subclones, represented at the *bottom of the figure*. The ab1 clone generated a fully protected fragment of 260 bp (lane 1) and for the B variant ER mRNA two additional cleavage products migrating at approximately 150 and 110 bp, respectively (lane 3). The ab0 clone generated a fully protected fragment of 360 bp (lane 2) and for the B variant ER mRNA two additional fragments migrating at approximately 250 and 110 bp, respectively (lane 4). The estimated site of mismatch is indicated by the letter *m* at the *bottom of the figure*.

With the RNase protection assay, the detection of low levels of ER mRNA in eight out of 12 tumors classified as ER-negative suggests that this assay might be more sensitive than those previously reported. Moreover, the RNase protection assay readily distinguishes the ER mRNA from the other RNAs which, because they have partial sequence homology, might cross react in dot-blot hybridizations.

Previously, the RNase protection assay had been used for the diagnostic detections of point mutations in the first exon of the c-K-ras oncogene, that have been correlated with human colon tumorigenesis and tumor invasiveness (14, 15). RNase A recognizes and cleaves more of the possible RNA/RNA or RNA/DNA mismatches than S1 nuclease does (16). Nevertheless, RNase A may also fail to efficiently cleave certain RNA/RNA mismatches (14, 15). Despite these technical limitations, the RNase protection assay is useful as a diagnostic screening procedure to detect, localize, and characterize point mutations within RNA transcripts.

By performing a partial screening of the ER mRNA from 71 mammary tumors with the eight subclones that were constructed, we observed and confirmed that major ER mRNA alterations are not frequently encountered in human breast cancer (9). However, we found a tumor subpopulation presenting a detectable mismatch in the RNA coding for the B region of the ER. The existence of other mutations, either resistant to RNase A digestion or in regions that were not analyzed, cannot be ruled out.

In the group of samples presenting a variant form of the ER mRNA, the mismatch was localized around position 250, as deduced from the size of the additional cleavage products. Since the efficiency of the cleavage may depend on the nature of the mismatch and on the surrounding nucleotides, it is uncertain whether the fully protected fragment, still present in these samples, is the result of an incomplete cleavage or represents the expression of the normal allele in the same tissue.

Available data suggest that alterations of the A/B region do not affect estrogen binding (13, 17). This implies that the relatively low level of ER binding detected in the tumor samples containing the B variant ER mRNA does not result from a modification of the binding capacity of the receptor. Moreover the estrogen binding portion of the message (e1, e2, and ef subclones) did not present any structural alterations.The B variant might therefore correspond to a particular subclass of ER-positive tumors carrying a biologically significant missense mutation. Such a mutation could affect the half-life of the protein, its solubilization properties, or could correspond to an imperfect regulation of the ER gene by its own product. Alternatively, the variant gene may contain a frame shift or nonsense mutation that will interfere with the synthesis or markedly alter the structure of the receptor molecules.

Although the amino acid sequence of the A/B region of the ER does not show a high degree of similarity with the corresponding regions of other steroid receptors (18, 19), it may be important for activation of gene transcription (13, 18). Mutagenesis within this region affects the activation of transcription in both the human glucocorticoid receptor (20, 21) and the chicken progesterone receptor (22). Moreover, a mutated human estrogen receptor, lacking a portion of region B, is unable to induce maximal transcription of the estrogen regulated pS2 gene (17).

No correlations were found between the presence of a B variant ER mRNA, and either the degree of tumor differentiation, the progesterone binding or a family history of breast cancer. Normal human myometrium, obtained from hysterectomies, was also found to con-

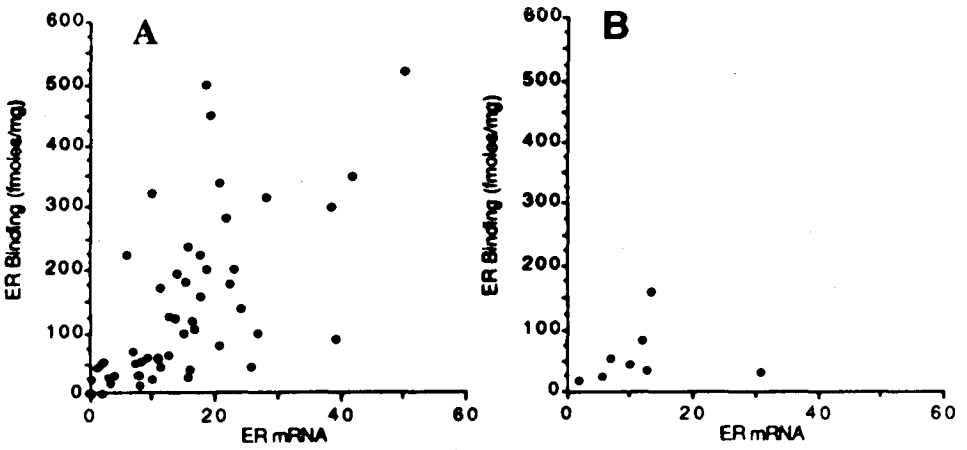

Fig. 6. Correlation between ER mRNA and ER Binding Determinations

Values of ER mRNA were determined after excising the fully protected cRNA probes from the gels and counting them by Cerenkov. Values are given in arbitrary units and standardized to the reference sample (10 U = cpm hybridized cRNA/μg MCF_7 RNA). ER binding values are given in femtomoles per mg total cytosolic proteins. A Pearson correlation was calculated after excluding the samples containing no ER binding and no ER mRNA. A, Common group: $r = 0.68$; $P < 0.0001$; n = 58. B, B variant group: $r = 0.1$; $P > 0.8$; n = 8. Most of values for ER mRNA represent average of duplicates measures.

tain ER mRNA from the variant type, thus ruling out the association of this mutation with the tumorigenic process. However, since the presence of this mutation is associated with moderate to low levels of ER, it could have an indirect influence on the potential success of hormonal therapy.

The detection of the B-variant form in genomic DNA, by restriction endonuclease digestion, or by selective oligonucleotide hybridization, could be used to screen larger populations. Since this polymorphism is present in a coding region of the ER gene, its influence on pathological processes, involving malfunctioning ERs, should then be investigated. Moreover, the determination of the exact nature of the modification would allow the construction of B variant ERs and the study of their functional properties.

MATERIALS AND METHODS

Enzymes and Chemicals

[^{32}P]dCTP (800 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Restriction enzymes were obtained from New England Biolabs (Beverly, MA) and IBI (New Haven, CT). The vectors used for subcloning (pGEM) and all the reagents for the cRNA probe synthesis were from Promega Biotec (Madison, WI). RNase A (type IIIA), RNase T1 (grade IV), and proteinase K (type XI) were from Sigma (St. Louis, MO). The human ER cDNA clones λOR3 and λOR7 (*Eco*RI Fragments encoding, respectively, 1.3 kilobases and 1.8 kilobases of the MCF_7 ER open reading frame) were provided by Dr. Pierre Chambon and co-workers (11). All of the other reagents were molecular biology grade.

Tissue Samples and Steroid-Binding Determination

Solid breast tumors were kindly provided by Dr. Demetrius Pertsemlidis and were obtained from patients in Mount Sinai Hospital (New York, NY). Samples were collected after surgery, immediately frozen, and stored at −70 C. The estrogen and progesterone binding levels of each sample were determined and provided by Dr. Pertsemlidis' group. A dextran-coated charcoal assays on the tumor cytosolic fraction was performed for each sample as described (23).

Recombinant Plasmid and *in Vitro* Transcription

Recombinant subclones of λOR3 or λOR8 were constructed using pGEM vectors that contain the transcriptional promoter for the SP6 and T7 polymerases. The original clones were digested at the restriction sites indicated in Fig. 1. After agarose gel fractionation, the appropriate DNA fragments were either electroeluted prior to ligation, or the gel band was melted and ligated to the plasmid without further purification (24).

Not all the collected tumors were screened with all the available subclones. Two were analyzed with the ab0 subclone, 69 with the ab1, 26 with the ab2, 37 with the d0, 25 with the d1, 32 with the e1, 44 with the e2, and 10 with the ef subclone. Radioactive cRNA probes were synthesized as described by Melton *et al.* (25) in the presence of 10 μM unlabeled CTP. The quality of each probe was assessed on a 4% polyacrylamide gel.

Hybridization to Total RNA and RNase Digestion

Total cellular RNA was isolated by the guanidine isothiocyanate method (26) from 0.2 to 1 g frozen tissue. The RNA recovery was determined by UV spectrophotometry; 15 μg of each RNA sample were used per data point. Hybridizations were performed as described (14) and as recommended by Promega Biotec. Briefly, 2×10^5 cpm of each ^{32}P-labeled cRNA probe were mixed with 30 μl hybridization solution containing 15 μg tumor total RNA. The mixture was heated at 85 C for 5 min, then kept at 55 C for 16–20 h. After hybridization, the samples were chilled on ice and 0.3 ml digestion buffer, containing RNase A (40 μg/ml) and RNase T1 (2 μg/ml), was added. The digestions were performed between 30 C and 38 C depending on the probes' G + C content. After 1 h the reaction was terminated by adding 20 μl 10% $NaDodSO_4$ and 50 μg Proteinase K (10 mg/ml) and the mixture was further incubated at 37 C for 15 min. Carrier tRNA (25 μg) was added before extraction with phenol-chloroform and isoamylalcohol, and 380 μl aqueous phase were precipitated with 2.5 vol ethanol in a dry ice-ethanol bath for 15 min. The precipitates were washed with 70% ethanol and dissolved in 15 μl gel-loading buffer (Tris-Borate, 50 mM, pH 8, 1 mM EDTA, 0.1%

(wt/vol) bromophenol blue, and 0.1% (wt/vol) xylene cyanol blue). The samples were heated twice at 65 C for 5 min, loaded on a 6% polyacrylamide gel, and electrophoresed at 250 V for 3–4 h. The gels were dried and exposed to x-ray film (Kodak X-Omat) at −70 C overnight with two intensifying screens.

For quantitative determinations, the radioactive bands which corresponded to the fully protected cRNA probe(s) were excised from the gels and counted for Cerenkov radiation. The assay was calibrated with a reference sample included in each determination. When two independent measurements were available for the same sample, the variability of the assay was about 10%; an average of the two values was used in further calculations.

Statistical Analysis

A Pearson correlation coefficient between the levels of ER binding and the levels of ER mRNA was calculated separately for each of the groups (B common and variant). A comparison of the measured variables in the two groups was performed using Student's t test (unpaired, two tailed): $P < 0.05$ was taken as significant.

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