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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Improving the diagnosis and clinical management of breast cancer requires access to biomarkers able to reflect the molecular phenotype of breast tissue. Indeed, the knowledge of the genetic changes that manifest themselves as alteration of gene expression occurring during breast tumorigenesis and breast tumor progression, will allow the identification of targets for new preventive and curative strategies. Three such genes are currently studied in our laboratory: Mammaglobin (MGB1), hSBEM (Human Small Breast Epithelial Mucin) and SRA (Steroid receptor RNA activator).

MGB1 expression, previously found to be restricted to breast epithelial cells, is increased in tumor cells compared to normal and correlates with ER levels even though not modified by estrogen or antiestrogen treatment in estrogen positive breast cancer cell lines. We suggest that MGB1 could be a new independent breast cancer prognostic marker.

We identified a novel breast-specific gene (hSBEM) that represents an attractive candidate for a new breast tumor marker with obvious potential for cancer diagnostics.

We identified new SRA isoforms which encode stable SRA proteins in vitro. As SRA has been shown to act as an RNA to activate steroid receptor activity, the potential translation product could indirectly interfere with estrogen signaling pathway during breast tumorigenesis.

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INTRODUCTION

Breast cancer remains one of the most frequently diagnosed cancers today. One in eight women is expected to present with breast cancer within her lifetime in developed countries. An estimated 1,000,000 cases are detected each year worldwide and in Canada alone, an estimated 20,700 women will be diagnosed in 2002 with breast cancer, and 5,400 women will be lost to this disease (1). For women with recurrent disease, the median time of survival is about two years (2). Despite such striking statistics, breast cancer related mortality is slowly decreasing as continuing research has led to earlier detection, more treatment options for breast cancer patients and an improved chance of long-term survival. Improving the diagnosis and clinical management of breast cancer requires access to and characterization of biomarkers that are able to reflect the molecular phenotype of breast tissue. Indeed, It is believed that the knowledge of the genetic changes that manifest themselves as alteration of gene expression and that occur during breast tumorigenesis and breast tumor progression, will allow the identification of targets for new preventive and curative strategies.

During the first year of this award, we focused our research on two breast specific genes (***mammaglobin*** and h***SBEM***) and on genes involved in estrogen signaling pathway (***ER-beta isoforms*** and ***co-regulators***).

BODY

Mammaglobin

Mammaglobin A was first identified in 1996, using differential display analysis, as a breast specific member of the uteroglobin gene family overexpressed in some breast tumors (3, 4). Today, a search for breast specific ESTs performed using the Differential Gene Expression Displayer Tool at the CGAP website (http://cgap.nci.nih.gov/Tissues/GXS) shows that mammaglobin A related ESTs have been found in 9 different breast cDNA libraries but only two non breast libraries, further confirming the relatively restrained breast specificity of mammaglobin A expression. Using a subtractive hybridization approach, I previously identified mammaglobin A mRNA as overexpressed in the in situ compared to the invasive element within an individual breast tumor (5, 6). Further in situ hybridization analysis (6), performed in breast tumors selected to include normal, in situ and invasive primary tumor elements and in most cases axillary lymph node metastases, revealed that mammaglobin A expression can be detected in all elements (normal, DCIS, invasive

and nodal metastasis). Mammaglobin A expression was found to be restricted to epithelial cells and to be significantly increased in tumor cells compared to normal cells (6). This suggests that mammaglobin A expression is differentially regulated in normal and breast tumor tissue, and could be involved in the mechanisms underlying breast tumorigenesis. Analysis of mammaglobin A expression within independent primary breast tumors and their corresponding axillary lymph nodes further revealed that all lymph node positive, but none of the nodes negative for metastatic breast carcinoma by histology, were mammaglobin A positive by reverse transcription-PCR (6). Moreover, it has recently been demonstrated that circulating mammary carcinoma cells can also be detected in the blood of breast cancer patients via PCR detection of mammaglobin A mRNA (7-10). Mammaglobin A is now therefore considered as being a specific marker of axillary lymph node breast metastases as well as of occult breast cancer.

As shown in **APPENDIX 1** (Submitted to Brit. J. Cancer), we evaluated MGB1 expression at the mRNA and at the protein level by reverse-transcription polymerase chain reaction and immunocytochemistry in 52 and 32 breast tumors, respectively. Both MGB1 mRNA and protein expression were significantly higher in estrogen receptor positive compared to estrogen negative tumors (Mann-Whitney rank sum test, $p = 0.04$; Chi-square test, $p = 0.01$; respectively). In contrast, MGB1 expression did not correlate with progesterone receptor levels or Notthingham grade. As estrogen and antiestrogen treatment of estrogen positive breast cancer cell lines does not modify MGB1 expression we suggest that MGB1 could be a new independent breast cancer prognostic marker.

hSBEM

There is an urgent need to identify novel genes whose expression is restricted to the mammary epithelium, as these genes have the greatest potential to enhance the detection of micrometastatic disease and the potential to report on proliferative changes in the breast.

As detailed in **APPENDIX 2** (11), we have identified a novel putative breast-specific gene (hSBEM, Human Small Breast Epithelial Mucin), that belongs to a recently regrouped cluster (UniGene identifier Hs.348419), and which represents an attractive candidate for a new breast tumor marker with obvious potential for cancer diagnostics. We showed that hSBEM gene is expressed in most breast cancer cell lines and widely expressed in epithelial cells from both normal and tumor breast tissues. In normal tissues hSBEM gene is only

expressed in breast and salivary gland. Moreover, hSBEM mRNA can be detected in axillary lymph node metastases but not in normal lymph nodes, suggesting that hSBEM mRNA could be used to detect micro-metastases.

Among the primary breast tumors examined in this study (representing mostly invasive ductal carcinoma), SBEM mRNA was observed by RT-PCR analysis in the majority (>90%) of cases. Despite a significant overall correlation between the expression of SBEM and mammaglobin A mRNA, a significantly higher SBEM:mammaglobin-1 ratio was observed in primary tumors associated with positive axillary lymph nodes as compared with node-negative tumors. This was mostly attributable to a trend toward higher SBEM expression in node-positive tumors. Although further analysis of a larger number of tumors will be required to confirm these observations, this may suggest differences in the biology of these tumors and also a possible role of SBEM and mammaglobin A in the mechanisms involved in tumor metastasis. Our findings indicate, however, that SBEM expression is a common feature of breast cancer and can furthermore serve as a useful marker for breast nodal metastasis, both for detection of micrometastatic cells within lymph nodes as well as in the differential diagnosis of the primary origin of an unknown metastasis. This potential is enhanced by the conserved SBEM expression in high grade and ER/PR-negative tumors that are most likely to metastasize.

ER-beta isoforms

Estrogens regulate the growth and development of normal human mammary tissue and are also involved in breast tumor progression (12). Indeed, estrogens are thought to promote the growth of breast tumors through their mitogenic effects on breast cancer cells. The ability of antiestrogens such as tamoxifen or raloxifene to inhibit estrogenic action provides the basic rationale for the use of endocrine therapies. Estrogen action is believed to be mediated mainly through two estrogen receptors (ERs): ER-alpha (13) and ER-ß1 (14, 15). These two receptors, which are encoded by two different mRNAs containing eight exons each (16, 17), belong to the steroid/thyroid/retinoic acid receptor superfamily (18). Several variant forms of ER-ß1 mRNAs have been identified (for reviews see Refs. 19-22). ER-ß2 variant, deleted of regions encoded by ER-ß1 exon 8 sequences, has been shown to heterodimerize with both ER-ß1 and ER-alpha and to inhibit ER-alpha DNA binding capability (23, 24). The ability of ER variants to potentially interfere with ER-alpha and ER-beta

signaling pathways raised the question of their possible involvement in mechanisms underlying breast tumorigenesis and tumor progression.

In collaboration with Dr. Murphy, we investigated the putative functional characteristics of human receptor beta isoforms (see **APPENDIX 3**, submitted to Endocrinology). We showed that only ER- ß1 was able to bind ligand whereas all ER-beta isoforms bind to DNA even though their binding abilities differ. ER-beta isoforms inhibition of ER-alpha and ER- ß1 transcriptional activity is promoter specific. Overall, our data suggest that ER-beta isoforms may have a differentially modulating estrogen action. We hypothesize that the modification of the ratio between the different ERs (25, 26), together with these different modulating actions play a role in the mechanisms underlying breast tumorigenesis and tumor progression.

Co-regulators

Recent observations using laboratory models (27-30) have demonstrated that altered levels of ER isoforms and/or alteration of expression of co-activators and co-repressors can result in altered estrogen and antiestrogen activity in target cells, suggesting the hypothesis that altered levels of ER isoforms and/or co-regulators *in vivo* could be a mechanism of tamoxifen resistance. As mentioned earlier in the text, we have previously demonstrated that the relative expression of ERα/ERβ as well as the relative expression of some ER coactivators to corepressors is significantly altered during breast tumorigenesis *in vivo* (26, 31). Furthermore, since these alterations parallel the marked changes in estrogen action that accompany breast tumorigenesis, they may have a role in this process.

* In collaboration with Dr. Murphy we explored the hypothesis that such changes could underlie *de novo* tamoxifen resistance *in vivo*. The expression of ER isoforms, two known co-activators (steroid receptor RNA activator, SRA, (28) and amplified in breast cancer-1, AIB1 (32)) and one co-repressor (repressor of estrogen receptor activity, REA (29) of ER activity have been investigated in primary breast tumors from node negative patients whose tumors were ER positive and that subsequently responded or had disease progression while on adjuvant tamoxifen therapy. As shown in **APPENDIX 4** (In press, Brit J. Cancer) we found no evidence for altered coregulators expression in breast tumors that are de novo resistant. However, our data provide preliminary evidence that the expression of ER-beta protein isoforms may differ in primary tumors of breast cancer patients who prove to have differential sensitivity to tamoxifen therapy.

* When characterizing SRA, which was found to function as an RNA transcript and to be specific for steroid receptors, Lanz et al. reported that none of the different SRA transcript isoforms isolated encoded detectable levels of SRA protein following *in vitro* translation experiments (28). We have identified three new SRA isoforms encoding *in vitro* stable SRA proteins. One of these isoforms contains a point mutation followed by a codon insertion that is found in both normal and tumor breast tissue as well as in some breast tumor cell lines. This insertion is present at the DNA level and is likely to represent a genetic polymorphism. We hypothesize that wild-type and inserted SRA mRNA and protein could play a role in the mechanisms underlying breast tumorigenesis and tumor progression (**APPENDIX 5**, submitted BBRC).

KEY RESEARCH ACCOMPLISHMENTS

* Mammaglobin expression correlates with ER levels but not with progesterone receptor levels or Notthingham grade in primary breast tumors.
* Estrogen/antiestrogen treatment does not modify Mammaglobin expression.
* We identified a novel putative breast-specific gene (hSBEM, Human Small Breast Epithelial Mucin).
* We investigated the putative functional characteristics of human receptor beta isoforms.
* We found that expression of ER-beta protein isoforms differ in primary tumors of breast cancer patients who prove to have differential sensitivity to tamoxifen therapy.
* We have identified new SRA isoforms encoding stable SRA proteins.

REPORTABLE OUTCOMES

* Two articles published or in press (APPENDICES 2 and 4)
* Three articles submitted (APPENDICES 1, 3 and 5)

CONCLUSION

As only two projects are currently funded (SBEM and SRA), the initial statement of work will be changed and set up in coordination with the Grants Officer.

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APPENDIX 1
Submitted, Brit. J. Cancer

Relationship between mammaglobin expression and estrogen receptor status in breast tumors.

Running title: MGB1 expression in breast tumors.

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Abstract

Mammaglobin (MGB1) has previously been found over-expressed in breast tumors but possible associations between its expression and established prognostic tumor characteristics such as the levels of estrogen and progesterone receptors have not yet been investigated. We evaluated MGB1 expression at the mRNA and at the protein level by reverse-transcription polymerase chain reaction and immunocytochemistry in 52 and 32 breast tumors, respectively. Both MGB1 mRNA and protein expression were significantly higher in estrogen receptor positive compared to estrogen negative tumors (Mann-Whitney rank sum test, $p = 0.04$; Chi-square test, $p = 0.01$; respectively). In contrast, MGB1 expression did not correlate with progesterone receptor levels or Notthingham grade. As estrogen and antiestrogen treatment of estrogen positive breast cancer cell lines does not modify MGB1 expression we suggest that MGB1 could be a new independent breast cancer prognostic marker.

Key words: MGB1, estrogen receptor, progesterone receptor, grade, breast cancer.

Introduction

Mammaglobin (MGB1) was first identified in 1996, using differential display analysis, as a breast specific member of the secretoglobin (SCGB) gene family overexpressed in some breast tumors (Watson and Fleming, 1994; Watson and fleming, 1996). Today, a search for breast specific ESTs performed using the Differential Gene Expression Displayer Tool at the CGAP website (http://cgap.nci.nih.gov/Tissues/GXS) shows that MGB1 related ESTs have been found in 9 different breast cDNA libraries but only two non breast libraries, further confirming the relative breast specificity of MGB1 expression. Using a subtractive hybridization approach, we previously identified MGB1 mRNA as overexpressed in the in situ compared to the invasive element within an individual breast tumor (Leygue et al., 1996a; Leygue et al., 1999). Further in situ hybridization analysis, performed in breast tumors selected to include normal, in situ and invasive primary tumor elements revealed that MGB1 expression, restricted to epithelial cells, could be detected in all elements and was significantly increased in tumor cells compared to normal cells (Leygue et al., 1999). This higher MGB1 expression in malignant versus non malignant breast epithelium has also been confirmed at the protein level by immunocytochemistry (Watson et al. 1999). In this latter study, Watson et al. concluded that MGB1 expression was independent of tumor grade and histological type.

It has recently been demonstrated that circulating mammary carcinoma cells can also be detected in the blood of breast cancer patients via PCR detection of MGB1 mRNA (Zach et al., 1999; Zach et al. 2001; Gal et al., 2001; Silva et al., 2001). Even though its biological function remains unknown, MGB1 is now considered as a relatively specific marker of axillary lymph node breast metastases as well as of occult breast cancer (Kataoka et al., 2000; Ooka et al., 2000; Silva et al., 2002; Marchetti et al., 2001). Interestingly, Zach et al. detected MGB1 mRNA expression by nested reverse-transcription PCR (RT-PCR) more frequently in the blood of patients with estrogen receptor positive (ER+) breast tumor than in the blood of estrogen receptor negative (ER-) breast cancer patients, suggesting a possible relationship between MGB1 and ER levels in primary breast tumors (Zach et al., 1999). In order to investigate further possible associations between MGB1 expression and estrogen and progesterone receptors in primary breast tumors, we assessed MGB1 expression at the mRNA and at the protein level in a cohort of breast tumors.

Materials and methods

Human breast tissues and cell lines

All breast tumor cases used for this study were selected from the NCIC-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). As it has been previously described (Hiller et al., 1996), tissues are accrued to the Bank from cases at multiple centers within Manitoba, rapidly collected and processed to create matched formalin-fixed-embedded and frozen

tissue blocks for each case with the mirror image surfaces oriented by colored inks. The histology of every sample in the Bank is uniformly interpreted by a pathologist in Hematoxylin/Eosin (H&E) stained sections from the face of the paraffin tissue block. This information is available in a computerized database along with relevant pathological and clinical information and was used as a guide for selection of specific paraffin and frozen blocks. Fifty two tumors were selected, spanning a wide range of estrogen and progesterone receptor levels, as determined by ligand binding assay. Within these tumors, 9 were ER-/PR- (ER < 3 fmol/mg total protein; PR < 10 fmol/mg), 10 were ER- /PR+ (ER < 3 fmol/mg; PR > 10 fmol/mg), 10 were ER+ / PR- (ER > 3 fmol/mg; PR < 10 fmol/mg) and 23 were ER+/ PR+ (ER > 3 fmol/mg, PR > 10 fmol/mg). These tumors also spanned a wide range of Nottingham grade for ER- (n = 19, grade ranging from 5 to 9, median 8) and ER+ (n = 33, grade ranging from 5 to 9, median 6) tumors. MGB1 mRNA expression was assessed by RT-PCR on total RNA extracted from frozen tissue sections. Paraffin blocks corresponding to 32 out of these 52 tumors were sectioned and processed for immunohistochemical analysis of MGB1 expression.

ZR-75 cells, ER positive breast cancer cells known to express MGB1, were grown. and treated with Estradiol-17β 10^{-8}M in charcoal-stripped medium or with the antiestrogen ICI 182,780 (10^{-6}M) in regular medium for 6h, 24 h or 48 h, as previously described (Coutts et al., 1999). Total RNA was extracted from frozen tissue sections or cell lines using Tri-reagent (MRCI, Cincinnati, OH).

RT-PCR analysis

One μg of total RNA was reverse transcribed in a final volume of 20 μl and one μl of the reaction mixture subsequently amplified by PCR as previously described (Leygue et al., 1996b; Leygue et al., 1996c). Primers used corresponded to: MGB1 (sense 5'-CCGACAGCAGCAGCCTCAC-3', located in MGB1 sequence between bases 41-59, and antisense 5'-TCCGTAGTTGGTTTCTCAC-3', located between bases 401-383 (Watson et al., 1996); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (sense 5'-ACCCACTCCTCCACCTTTG-3' and antisense 5'-CTCTTGTGCTCTTGCTGGG-3'); and to psoriasin gene (sense 5'-AAGAAAGATGAGCAACAC-3' and antisense 5'-CCAGCAAGGACAGAAACT-3') To amplify cDNA corresponding to MGB1, GAPDH and psoriasin, 30 cycles (30 seconds at 94°C, 30 seconds at 55°C and 30 seconds at 72°C) of PCR were used. Ten microliters of PCR products were loaded on prestained (15 μg/ml ethidium bromide) 2% agarose gels. Identity of fragments corresponding to MGB1, GAPDH and psoriasin had previously been confirmed by sequencing.

Three independent PCRs were performed using MGB1, psoriasin and GAPDH primers and signals, visualized with U.V. irradiation on a GelDoc2000/ChemiDoc System (Biorad), were quantified by densitometry using the Quantity One software (Version 4.2, Biorad). MGB1 and psoriasin expression was expressed relative to GAPDH expression as previously described (Leygue et al, 1998). Briefly, three independent PCRs were performed using each set of primers. In order to control for variations between experiments, a value of 1 was

arbitrarily assigned to the signal of one particular tumor measured in each set of PCR experiments (always the same tumor) and all signals were expressed relative to this signal. Levels of MGB1 were then expressed relative to the GAPDH signal corresponding to each individual tumor sample. Correlation between normalized MGB1 expression and tumor characteristics was tested by calculation of the Spearman coefficient, r. Comparison between tumor subgroups was performed using the Mann-Whitney rank sum test, two sided.

Immunohistochemical analysis of MGB1 expression.

Detection of MGB1 protein was performed using an antibody previously characterized and kindly provided by Dr. Timothy Fleming (see Watson et al., 1999). Paraffin-embedded breast tissue sections were processed using the automated Discovery Staining Module, Ventana System (Tucson, Arizona) and the Research IHC DAB paraffin protocol according to the manufacturer's instructions. All steps were performed automatically: briefly, following deparaffination of tissue sections, slides were incubated 60 minutes at 42°C in the presence of rabbit anti-MGB1 antibody (1/1000 final concentration), washed, incubated with Biotinylated secondary anti-rabbit antibody (14 minutes 42°C), washed, incubated 8 minutes with Avidin-HRPO complex subsequently detected with DAB-H_2O_2 solution. Counter-staining was also performed automatically by the Ventana apparatus (hematoxylin/bluing reagent).

Levels of mammaglobin expression were assessed by brightfield microscopic examination at low power magnification and using a previously described semi-quantitative approach

(Leygue et al., 1998). Scores were obtained by estimating average signal intensity (on a scale of 0 to 3) and the proportion of epithelial cells showing a positive signal (0, none; 0.1, less than one tenth; 0.5, less than one half; 1.0 greater than one half). The intensity and proportion scores were then multiplied to give an overall score. Cases with a score lower than or equal to 1 were considered negative or weakly positive, whereas tumors with scores higher than 1.0 were classified as positive for MGB1 expression. Statistical comparisons between tumor subgroups have been performed using the Chi-square test.

Results

Assessment of MGB1 mRNA expression in a cohort of 52 human breast tumor samples.

To establish whether MGB1 mRNA expression paralleled established known prognostic parameters such as ER and PR levels, a cohort of 52 cases was selected from the NCIC-Manitoba Breast Tumor Bank. For each case, clinical characteristics of the tumor (i.e ER and PR levels, Nottingham grade) were known (see Materials and methods section for a summary of tumor subgroup characteristics). Total RNA was extracted from frozen primary tumor sections, reverse-transcribed and analyzed by RT-PCR using primers recognizing specifically MGB1 cDNA and chosen to span intronic regions. As shown Figure 1, MGB1 corresponding signal can be detected in the majority of cases, even though levels of expression varied from one sample to another. Amplification of the ubiquitously expressed GAPDH cDNA in the same cDNA samples was performed in parallel and for each case, a normalized MGB1 mRNA expression value was calculated (see Materials and

Methods). MGB1 expression was found to strongly correlate with ER levels (n = 52, Spearmann coefficient r = 0.282, p = 0.042) but not with PR levels or Grade (data not shown). Similarly (Figure 2), using the established clinical cut-off of ER positivity (ER positive tumors have a binding higher than 3 fmol/mg of total protein), MGB1 mRNA expression was significantly (Mann-Whitney rank sum test, two sided, p = 0.040) higher in ER+ (n = 33, median value MGB1 = 0.62) than in ER - (n = 19, median MGB1 value = 0.33). Interestingly, even though not statistically significant, a trend towards a higher MGB1 expression in PR + (median value MGB1 = 0.56, n =33) than in PR- (median value MGB1 = 0.36, n = 19) was observed. However, as this trend disappears when comparing PR+ and PR- subgroups within ER- and ER+ cohorts, we concluded that this apparent trend resulted from sampling biases (data not shown).

Similarly, the apparent trend of a higher MGB1 expression in Low grade tumors (median values MGB1: Low grade = 0.56, n =29; High grade = 0.36, n = 23) likely results from the strong inverse correlation between grade and ER levels observed within the cohort.

Assessment of MGB1 protein expression in a cohort of 32 human breast tumor samples.

In order to determine whether MGB1 protein expression correlated with MGB1 mRNA expression and whether a similar association between ER status and MGB1 expression could be observed at the protein level, paraffin blocks corresponding to 32 out of these 52 tumors were sectioned and processed for immunohistochemical analysis of MGB1 expression (see Materials and Methods). Slides were scored blindly for MGB1 protein

expression by a pathologist as described in the Materials and Methods section. Some sections showed no (Figure 3A, MGB1 score = 0) or low (Figure 3B, MGB1 score = 1) MGB1 expression whereas others presented strong MGB1 protein signal (Figure 3C, MGB1 score = 3, Figure 3-D, MGB1 score = 2). Comparison of MGB1 protein scores and previously obtained normalized MGB1 mRNA levels revealed a strong correlation (n = 32, Spearman r coefficient r = 0.575, p = 0.0006) between protein and mRNA levels. Tumors were classified as low (scores between 0 and 1) and high (1.5 to 3) MGB1 protein expressors, and differences between tumor subgroups (ER+/ER-, PR+/PR-, Low grade/high grade) were assessed using Chi-square test. As observed for MGB1 mRNA, MGB1 protein positivity was associated (Chi-square test, p = 0.017) with ER status but not with PR status or grade (Figure 4).

Absence of estrogen regulation of MGB1 expression.

These data suggested that estrogen might regulate MGB1 expression. In order to address the question of a possible regulation of MGB1 expression in breast cancer cells, ZR-75 cells, known to express MGB1 (Min et al., 1998) were treated by estradiol-17β 10^{-8}M or the antiestrogen ICI-182,780 10^{-6}M for 6, 24 and 48 hours as described in the Materials and Methods section. Total RNA was extracted and analyzed by RT-PCR using primers recognizing either GAPDH, MGB1 or psoriasin cDNAs. Psoriasin was chosen as its expression has previously been shown to be regulated by estrogen treatment (Moog-Lutz et al., 1995). MGB1 mRNA expression was not changed under any treatment condition (data

not shown), whereas, as expected, psoriasin signal was found to be increased by estradiol and decreased by antiestrogen treatment as soon as 6 hours of treatment, with a maximum effect after 24 and 48 hours of treatment (estradiol treatment: 1.5, 2.8 and 4.5 fold control and antiestrogen treatement 0.90, 0.80, and 0.70 fold control, respectively).

Discussion

Assessment of MGB1 expression at the mRNA and at the protein levels in a cohort of breast tissue samples showed a statistically significant relationship between MGB1 levels and ER status. However, within the same cohort, no association was found between MGB1 expression and other known prognostic marker such as PR levels or Notthingham grade.

To the clinician, a factor is considered a prognostic factor when it is associated with the outcome of the disease, i.e predicts how the disease would evolve if not treated, whereas a predictive factor is associated to the degree of response to therapy, i.e predicts the likelihood of response to a particular treatment. A high level of ER in tumor tissue has a good prognostic value and also predicts a good likelihood of responding to hormonal adjuvant therapy such as tamoxifen (Aapro, 2001; Bundred, 2001). As PR expression is positively regulated by estrogens, higher PR levels in ER+ tumors support the hypothesis of an operational ER signaling pathway and is therefore also considered as a good prognostic and predictive parameter. Whereas the parallel between MGB1 and ER expression suggested that MGB1 could be a new ER target gene, the lack of association with a known regulated gene such as PR suggested that MGB1 expression was

independent of ER signaling pathway. This later hypothesis was further supported by the absence of estrogen and antiestrogen regulation of MGB1 expression in ZR-75 cells, even though ER signaling pathway appears functional, as shown by the induction of a known ER regulated gene, psoriasin. It should be noted that a similar absence of regulation was also observed in another ER positive breast cancer cell line MCF-7 cells (our unpublished results and Watson et al., 1998). However, even though MGB1 gene was not grossly rearranged in MCF-7 cells (Watson et al., 1998), these cells do not express endogenous MGB1 (our unpublished results and Watson et al., 1996). It might therefore be hypothesized that MGB1 expression in MCF-7 cells is negatively regulated by other factors, resulting in an absence of estrogen regulation in these cells.

Interestingly, the general expression of MGB1 as well its association with ER levels observed in vivo in breast tissue contrasts with in vitro observations made on mammary epithelial cancer cell lines. Indeed, looking at a panel of different breast cancer cell lines, Watson et al. reported the detection of MGB1 transcripts only in few cell lines (MB361, MB415, MB468, BT474, MB175) with no expression in MCF7, MB134, MB231 or MCF10A cells (Watson et al., 1996). Similarly, we did not detect MGB1 expression in breast cell lines such as BT20, T47D or MCF10AT1 even though a strong signal was seen in ZR-75 (our unpublished observation). As cells such as MB468 and MB361 are ER- and cells such as ZR-75 or BT474 are ER+, MGB1 expression does not appear related to ER status in cells grown in vitro. Overall, this suggests that most of cell lines, through selection, medium conditions and/or dedifferentiation lost their ability to express MGB1 in vitro.

Presently, no data are available regarding the possible biological function of MGB1. It has however recently been reported that MGB1 existed in a tetrameric complex with BU101 (lipophilin B), another member of the secretoglobin family expression of which correlated with MGB1 expression in breast tissue (Colpitts et al., 2001). The role of this complex as well as the possible regulation of its components remains to be determined.

In conclusion, we found that MGB1 expression correlated with ER levels in breast tumor tissue. As ER is considered as a good prognostic factor and as MGB1 does not appear to be directly regulated by the ER signaling pathway, we hypothesize that MGB1 expression could be a new independent prognostic marker in breast cancer.

Acknowledgments:

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Figure legend

Figure 1

RT-PCR analysis of MGB1 and GAPDH mRNA expression in primary breast tumors. Total RNA was extracted from frozen tissue sections corresponding to ER positive (ER+) and ER negative (ER-) cases, reverse-transcribed and PCR amplified as described in the Materials and Methods section using MGB1 or GAPDH specific primers. PCR products were then separated on 2% agarose gels prestained with ethidium bromide. . Black arrow: product corresponding to MGB1, grey arrow: product corresponding to GAPDH. M: Molecular weight marker (Φx174 RF DNA/Hae III fragments, Gibco BRL, Grand Island, NY).

Figure 2

Quantification of MGB1 mRNA expression in different breast tumor subgroups. Total RNA was extracted from frozen tissue sections corresponding to 52 cases and analyzed as described in Figure 1. MGB1 mRNA expression was quantified relative to GAPDH mRNA as described in the Materials and Methods section. Tumors were grouped according to their ER status (ER+, ER-), their PR status (PR+, PR-) or their Grade (Low Gr: Notthingham scores between 5 and 7, High Gr: Notthingham scores between 8 and 9). Difference between subgroups were tested using the Mann-Whitney rank sum test, two sided.

Figure 3

Detection of MGB1 protein in breast tumors by immunohistochemistry.

MGB1 protein was detected on paraffin-embedded breast tumor tissue sections using a rabbit polyclonal primary anti-MGB1 antibody (Kindly provided by Dr. T Fleming) and the Ventana-Discovery system as described in the Materials and Methods section. Panel A and B : Two independent ER- cases showing no (A) or low (B) MGB1 protein expression in tumor epithelial cells. Panel C and D: two independent ER + cases presenting a strong MGB1 signal. Blue bar: 20 μm.

Figure 4

Quantification of MGB1 protein in breast tumor subgroups.

Paraffin-embedded tissue section corresponding to 32 cases were processed as shown Figure 3. Slides were independently reviewed and scored as described in the Materials and Methods section. For each tumor subgroup (ER-, ER+, PR+, PR-, Low Grade and High Grade) the number of cases negative (White columns) or positive (black columns) is shown. Differences between subgroups were tested using the Chi-square test.

Figure 1

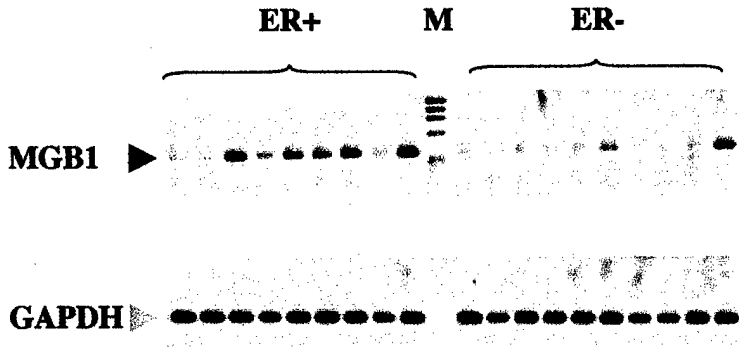


Figure 2

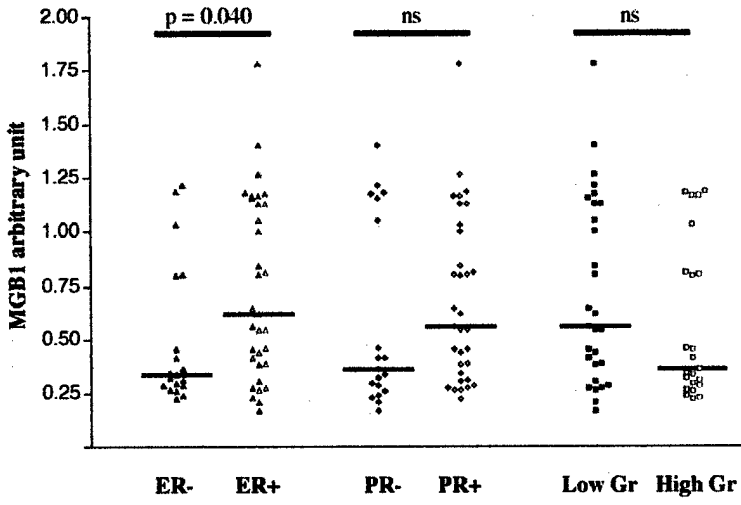


Figure 3

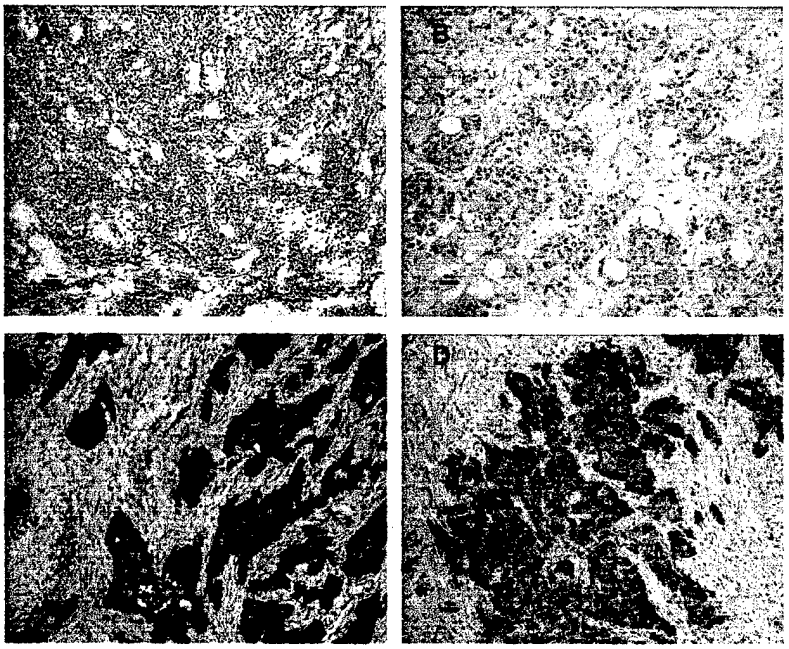
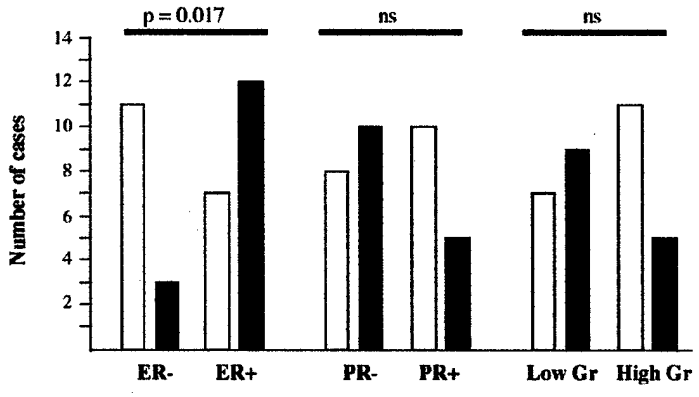


Figure 4



APPENDIX 2
Cancer Res. 62: 2736-2740, 2002

[CANCER RESEARCH 62, 2736–2740, May 15, 2002]

Advances in Brief

Identification of a Novel Breast- and Salivary Gland-specific, Mucin-like Gene Strongly Expressed in Normal and Tumor Human Mammary Epithelium[1]

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Abstract

Expression profiling using the public expressed sequence tag (EST) and serial analysis of gene expression (SAGE) databases resulted in the identification of a putative breast-specific mRNA that we have termed small breast epithelial mucin (SBEM). Hybridization analysis performed on 43 normal human tissues revealed that the *SBEM* gene was only expressed in mammary and salivary glands. Further reverse-transcription PCR analyses confirmed SBEM expression in most of established human breast epithelial cell lines analyzed (7 of 8) but not in cell lines of non-breast origin (0 of 6). *SBEM* mRNA expression was detected in >90% of invasive ductal carcinomas and correlated with the expression of a previously characterized breast-specific gene, mammaglobin-1 ($n = 54$; Spearman $r = 0.34$, $P = 0.011$). Interestingly, a higher SBEM:mammaglobin-1 ratio was observed in primary tumors with axillary lymph node metastasis than in node-negative tumors ($n = 46$; Mann-Whitney, $P = 0.04$). In a subset of 20 primary breast tumors and their matched axillary lymph nodes, a high concordance (Fisher's exact test, $P < 0.001$) was seen between PCR detection of *SBEM* mRNA in lymph node tissue and their histopathological status, indicating that SBEM mRNA expression is conserved in nodal metastasis. The *SBEM* gene is predicted to code for a putative low molecular weight, secreted sialoglycoprotein, potentially useful for the diagnosis of metastatic breast cancer.

Introduction

Early detection remains a central goal in breast cancer treatment to enable intervention at a localized and potentially curable stage and to maximize the opportunity for breast conservation. The 5-year survival rate for women with breast cancer increases dramatically when it can be diagnosed at an early stage, from >95% in patients with a localized tumor to ~75% with regional disease and <25% in women with disseminated cancer (1). Nevertheless, only 60% of all breast cancers are diagnosed at a local stage, and any improvement in early detection would have a significant impact on reducing overall breast cancer mortality.

Improving the diagnosis and clinical management of breast cancer requires access to a wider range of biomarkers able to reflect the molecular phenotype of breast tissue. A special need exists to identify novel genes whose expression is restricted to the mammary epithelium, because these genes have the greatest potential to enhance detection of micrometastatic disease and the potential to report on proliferative changes in the breast, analogous to the ability of elevated serum prostate-specific antigen levels to indicate the presence of hyperplasia or cancer of the prostate gland (2).

The identification of new tissue-specific markers has benefited especially from expansion of public and private databases for ESTs[4] (3, 4) and by large-scale efforts to profile patterns of gene expression using techniques such as serial analysis of gene expression (5). Using sequence analysis software and web-based tools developed for molecular profiling, we have identified a novel putative breast-specific gene, belonging to a recently regrouped cluster (UniGene identifier Hs.348419),[5] which represents an attractive candidate for a breast tumor marker with obvious potential for cancer diagnostics.

Materials and Methods

Database and Sequence Analysis. The cDNA xProfiler tool[6] was used to search for novel breast-specific ESTs. Protein sequence analysis used the SignalP algorithm[7] to search for the presence of a signal sequence (6) and the NetOGlyc algorithm[8] to predict sites of potential glycosylation (7).

RNA Hybridization Analysis for Tissue Specificity. A ^{32}P-labeled SBEM probe, generated using the cloned SBEM PCR product (396 bp) and the RadPrime DNA labeling system (Life Technologies, Inc., Burlington, Ontario, Canada), was hybridized to a commercially available RNA Master Blot (Clontech, Palo Alto, CA), containing poly(A)+ RNA (100–500 ng) isolated from a variety of adult and fetal human tissues, according to the manufacturer's instructions.

Cell Culture and RNA Preparation. Cell lines were obtained from the American Type Culture Collection or other sources and were cultured as follows: DMEM with 10% fetal bovine serum (MCF7, MCF10AT1, MCF10AT3c, SK-UT-1B, and HepG2); DMEM with 10% calf serum (MDA MB-231, Hec 1A, and HeLa); DMEM:Ham's F12 (1:1) with 10% fetal bovine serum (ZR-75-1 and RL95-2); RPMI 1640 with 10% fetal bovine serum (T-47D and LNCaP); or MSU-1 medium (8) with 5% fetal bovine serum (M13SV-1). All media were supplemented with penicillin (50 units/ml), streptomycin (50 μg/ml), HEPES (pH 7.4; 5 mM), and glutamine (2 mM). MCF7, T-47D, and ZR-75-1 cells also received bovine insulin (10 μg/ml). Media and sera were obtained from Life Technologies, Inc. (Life Technologies, Inc., Grand Island, NY). RNA was extracted from cultured cells using guanidinium isothiocyanate, followed by centrifugation through a 5.7 M cesium chloride cushion as described (9). RNA from cultured primary HMECs obtained by reduction mammoplasty was a kind gift from P. Ervin (Biotherapies, Inc., Ann Arbor, MI).

Breast Tumors and Axillary Lymph Nodes. Fifty-four invasive ductal carcinomas were selected from the Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). Cases spanned many ER (0–298 fmol/mg protein) and PR (0–1199 fmol/mg protein) levels, as determined by ligand binding assay. Tumors also spanned many grades (Nottingham grade scores from 5 to 9). For

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[2] These authors contributed equally to this work.

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[4] The abbreviations used are: EST, expressed sequence tag; SBEM, small breast epithelial mucin; HMEC, human mammary epithelial cell; ER, estrogen receptor-α; PR, progesterone receptor; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde phosphate dehydrogenase; MUC1, mucin 1.

[5] Internet address: http://www.ncbi.nlm.nih.gov/UniGene/. UniGene is a system for automatically partitioning GenBank sequences, including ESTs, into a nonredundant set of gene-oriented clusters.

[6] Internet address: http://cgap.nci.nih.gov/CGAP/Tissues/xProfiler.

[7] Internet address: http://www.cbs.dtu.dk/services/SignalP/.

[8] Internet address: http://www.cbs.dtu.dk/services/NetOGlyc/.

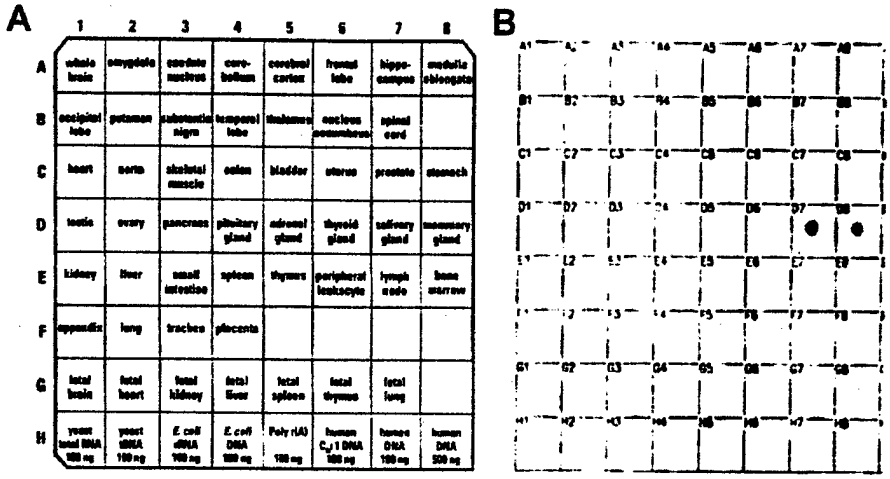

Fig. 1. Tissue expression of *SBEM* mRNA. An RNA Master Blot (Clontech Laboratories, Palo Alto, CA) containing poly(A)+ RNAs from different human tissues (*A*) was screened with ^{32}P-labeled SBEM probe as described in "Materials and Methods." The SBEM transcript (*B*) was expressed in the human salivary (*D7*) and mammary gland (*D8*).

46 tumors, pathological axillary lymph node status (presence or absence of metastasis) was known.

In a subset of cases (n = 20), frozen primary human breast tumor samples and their matched frozen lymph nodes containing (n = 14) or not (n = 6) histologically detectable metastatic cancer cells were available from the Manitoba Breast Tumor Bank. For the primary tumor samples, the ER levels, determined by ligand binding assays, ranged from 2.3 fmol/mg protein to 298 fmol/mg protein, whereas PR levels ranged from 10.1 fmol/mg protein to 112 fmol/mg protein.

RNA Analysis by RT-PCR. Total RNA was extracted from 20-μm frozen tissue sections (five sections/tumor) and reverse transcribed as described previously (10). The primers used for SBEM amplification consisted of SBEM-U (5′-CTTTGAAGCATTTTTGTCTGTG-3′; sense) and SBEM-L (5′-AAGGTAAGTAGTTGGATGAAAT-3′; antisense). PCR amplifications were performed, and PCR products were analyzed as described previously (11), with minor modifications. Briefly, aliquots of each reverse transcription mixture (2 μl for Fig. 2 and 0.8 μl for Fig. 3) were amplified in a final volume of 20 μl, in the presence of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, 5 ng/μl of each SBEM primer, and 0.5 unit of Taq DNA polymerase. Each PCR consisted of 35 cycles (15 s at 94°C, 30 s at 58°C, and 60 s at 74°C).

Primers used for mammaglobin-1 were: Mam-1 (5′-CCGACAGCAG-CAGCCTCAC-3′, sense strand) and Mam-2 (5′-TCCGTAGTTGGTTTCT-CAC-3′, antisense strand). Primers for the ubiquitously expressed *GAPDH* gene were GAP-1 (5′-ACCCACTCCTCCACCTTTG-3′, sense strand) and GAP-2 (5′-CTCTTGTGCTCTTGCTGGG-3′, antisense strand). To amplify cDNA corresponding to mammaglobin-1 and GAPDH, 30 cycles of PCR were used (30 s at 94°C, 30 s at 55°C, and 30 s at 72°C). All buffers were the same as for SBEM PCR, except that 2 mM $MgCl_2$ was used when amplifying mammaglobin-1 cDNA. PCR products were then separated on a 1.5% agarose gel. After electrophoresis, the gels were stained with ethidium bromide (0.5 μg/ml).

Quantification and Statistical Analysis. Three independent PCRs were performed for tumor specimens using SBEM, mammaglobin-1, and GAPDH primers and signals, visualized with UV irradiation on a GelDoc2000/Chemi-Doc System (Bio-Rad), were quantified by densitometry using the Quantity One software (version 4.2; Bio-Rad). SBEM and mammaglobin-1 expression was normalized to GAPDH expression as described previously (11). Correlation between SBEM expression and tumor characteristics or mammaglobin-1 expression was tested by calculation of the Spearman coefficient r. Differences between tumor subgroups were tested using the Mann-Whitney two-tailed test or Fisher's exact test.

***In Situ* Analysis of SBEM mRNA Expression.** *In situ* hybridization was performed on adjacent paraffin-embedded breast tumor tissue sections corresponding to a case shown to express high levels of *SBEM* mRNA by RT-PCR, using SBEM ^{32}P-labeled sense and antisense probes, as described previously (12).

Results

Identification of a Putative Novel Breast-specific Gene. The cDNA xProfiler tool (see "Materials and Methods") was used to identify tissue-restricted cDNAs with preferential representation in libraries prepared from normal breast tissue and breast tumors. This search identified a new cluster of ESTs now grouped under the UniGene identifier number Hs.348419. Of the 30 ESTs found in this cluster, 15 are ascribed to breast cDNA libraries, 9 were isolated from random activation of gene expression or pooled tissues, 5 were isolated from fetal sources (fetal heart and fetal skin), and 1 came from a head and neck tumor cDNA library. Alignment of these ESTs led to the construction of a 500-bp consensus cDNA sequence containing a 90-amino acid open reading frame in which the initiating methionine is framed by a nearly perfect consensus motif for translation initiation (5′-CCACCATGA-3′; Ref. 13). Further database analysis showed that this sequence, interrupted by three introns, is present on chromosome 12q13.2. Primers were designed to span the open reading frame, and we cloned a 396-bp fragment from both MCF-7 cells and breast tissue, which we called SBEM (GenBank accession number AF414087). The presence of a hydrophobic signal peptide (residues 1–19; Ref. 6) within the protein sequence (GenBank accession number AAL02119) suggests that SBEM is a secreted protein subject to proteolytic processing. The NetOGlyc glycosylation algorithm (7) further predicts this protein to be *O*-glycosylated on most of its 16 threonine residues. The SBEM protein contains three tandem copies of a neutral octapeptide core repeat (ThrThrAlaAlaXxxThrThrAla, where Xxx corresponds to Ala, Pro, or Ser). The NH_2 and COOH termini of the processed polypeptide are otherwise charged and fairly polar. These features suggest strong similarity to many sialomucins, although this protein lacks a transmembrane domain and is substantially shorter than most other known epithelial mucins (14, 15).

Expression of *SBEM* mRNA Is Restricted to the Mammary and Salivary Glands. Database searches suggested that SBEM expression was mainly restricted to breast tissue. To confirm this prediction, we performed hybridization analysis with an RNA MasterBlot containing highly purified polyadenylated RNA from 43 adult and 7 fetal human tissues arrayed on a nylon membrane. A *SBEM* cDNA probe hybridized exclusively to mRNA from the mammary and salivary glands (Fig. 1). Of note, no expression was observed in colon, lung, uterus, ovary, liver, pancreas, kidney, or prostate, all of which represent common primary tumor sites. Additionally, no hybridization to any of the fetal RNAs was observed.

***SBEM* mRNA Is Expressed in Breast Cancer Cell Lines but not in Cell Lines of Non-Breast Origin.** The profile of *SBEM* mRNA expression was further assessed using RT-PCR, followed by PCR amplification, in a panel of human breast and non-breast cell lines. A SBEM PCR product of the expected size (396 bp) was readily detected in MCF7 and ZR-75-1 breast tumor cells (data not shown). Lower but reproducible expression was also observed in primary HMECs and in several established breast epithelial cell lines including T-47D, M13SV-1 (8), MCF10AT1, and MCF10AT3c (16). MDA MB-231 breast tumor cells were negative for SBEM expression, as were six tumor cell lines of non-breast origin (uterus: RL95-2, SK-UT-1B, Hec 1A; cervix: HeLa; prostate: LNCaP; and liver: HepG2). As controls, we also examined the expression of a housekeeping gene (*GAPDH*) and mammaglobin-1, an established mammary-specific gene that is being independently investigated as a promising marker for breast tumor diagnosis and nodal metastasis (12, 17). Of the cell lines tested, only HMEC and ZR-75-1 cells expressed mammaglobin-1, consistent with published reports.

Analysis of *SBEM* mRNA in Human Breast Tumors. Northern blot analyses performed on a small series of 10 cases revealed that *SBEM* mRNA was 600 bp long and differentially expressed from one sample to another (data not shown). To determine whether *SBEM* mRNA was widely expressed in human breast tumor tissue, 54 human breast tumors, spanning many ER and PR levels as well as tumor grade and nodal status, were selected from the Manitoba Breast Tumor Bank. Total RNA was extracted from frozen tissue sections and reverse transcribed. PCR amplification of *GAPDH* (control), *mammaglobin-1*, and *SBEM* cDNA was then performed. A PCR product, 396-bp long was detected in all but three tumors (data not shown) when using SBEM-specific primers. After cloning and sequencing, this product was shown to correspond to *SBEM* cDNA. Quantification of the *SBEM* signal relative to the *GAPDH* signal was performed as described in "Materials and Methods." No correlation was found between SBEM expression and tumor characteristics such as ER ($n = 54$; Spearman $r = -0.01$, $P = 0.89$) and PR ($n = 54$; Spearman $r = -0.03$, $P = 0.77$) levels or tumor grade ($n = 44$; Spearman $r = -0.06$, $P = 0.68$). Interestingly, however, the *SBEM* signal correlated positively with *mammaglobin-1* expression ($n = 54$; Spearman $r = 0.340$, $P = 0.011$). Subgroup comparison of *SBEM* and *mammaglobin-1* expression confirmed our previous observation[9] that *mammaglobin-1* expression is higher in ER-positive and low-grade tumors (Table 1). Interestingly, although not statistically significant ($P = 0.09$), higher SBEM expression was found in lymph node-positive compared with node-negative tumors. Also of interest is the fact that the SBEM:mammaglobin-1 ratio is significantly ($n = 46$; Mann-Whitney, $P = 0.04$) higher in these lymph node-positive tumors.

[9] E. Leygue, L. C. Murphy, and P. H. Watson, unpublished results.

Table 1 *Median values (arbitrary units) of SBEM expression, mammaglobin-1 (Mam) expression, and SBEM:mammaglobin-1 ratio in different tumor subgroups*

Tumors	*n*	SBEM	*P*	Mam	*P*[a]	SBEM: Mam	*P*[a]
ER +	34	2.29	0.66	0.63	**0.04**	3.39	0.22
ER −	20	2.15		0.34		4.44	
PR +	34	2.30	0.63	0.59	0.27	3.56	0.50
PR −	20	1.97		0.38		3.58	
Node +	36	2.30	0.09	0.45	0.62	3.97	**0.04**
Node −	10	1.96		0.70		2.42	
Grades 5–7	24	2.05	0.84	0.59	**0.03**	3.33	0.10
Grades 8–9	20	2.24		0.34		4.02	

ER +, >3 fmol/mg of protein; ER −, ≤3 fmol/mg of protein; PR +, >10 fmol/mg of protein; PR −, ≤10 fmol/mg of protein, as determined by ligand binding assay. Node +, confirmed metastasis in axillary lymph nodes; Node −, absence of metastasis in analyzed axillary lymph nodes. Grade, Nottingham grading system. Subgroup comparison was performed using the Mann-Whitney two-tailed test.

[a] Bold face *P* values correspond to statistically significant differences between tumor subgroups ($P < 0.05$).

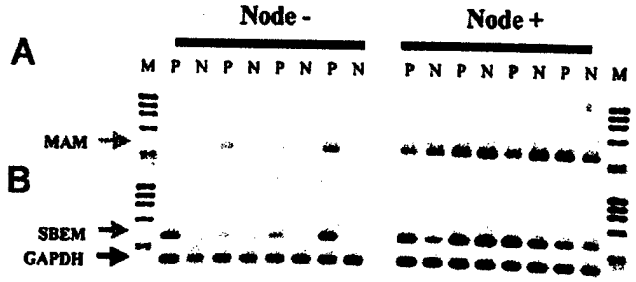

Fig. 2. RT-PCR analysis of *mammaglobin-1, SBEM,* and *GAPDH* mRNA expression in primary breast tumors (*P*) and their corresponding axillary lymph nodes (*N*), histologically shown to contain (*Node +*) or not to contain (*Node −*) metastases. *Mammaglobin-1* (*MAM*) PCR products were run separately (*A, dotted arrow*), whereas *SBEM* and *GAPDH* PCR products were mixed before separation on 2% agarose gels prestained with ethidium bromide (*B*). *Gray arrow,* product corresponding to SBEM; *black arrow,* product corresponding to *GAPDH. M,* molecular weight markers (Φ;x174 RF DNA/Hae III fragments; Life Technologies, Inc., Grand Island, NY). *N,* negative control, no cDNA added during the PCR reaction.

***SBEM* mRNA Expression in Primary Breast Tumors and Their Corresponding Axillary Nodes.** We next investigated the possibility that *SBEM* mRNA could be a tissue marker of axillary lymph node metastasis. Twenty independent cases were selected, including 14 tumors that were axillary lymph node positive and 6 that were node negative. Total RNA was extracted from frozen primary tumor sections and frozen node sections of corresponding axillary lymph nodes. The histological status of all tissues was confirmed in paraffin sections cut from adjacent mirror image paraffin tissue blocks that had been processed in parallel to the frozen blocks. These RNAs were reverse-transcribed and analyzed by RT-PCR using *SBEM*-specific primers. PCR was performed three times, giving the same result. A representative experiment is shown in Fig. 2. A signal corresponding to *SBEM* was detected in all lymph nodes containing metastatic cells by histopathological assessment (14 of 14 cases). In contrast, no signal was detectable in lymph nodes from cases without histologically detectable tumor cells (0 of 6 cases). RT-PCR detection of *SBEM* mRNA in axillary lymph nodes is therefore strongly associated (Fisher's exact test, $P < 0.001$) with the histopathological detection of lymph node metastases. The higher sensitivity afforded by RT-PCR detection therefore indicates that *SBEM,* perhaps together with *mammaglobin-1,* represents an excellent marker for the detection or confirmation of occult breast tumor metastasis, where histopathology may not be definitive.

***SBEM* mRNA Is Expressed in Mammary Epithelial Tumor Cells *in Vivo*.** To further establish whether *SBEM* was expressed by mammary epithelial cells *in vivo*, paraffin breast tumor tissue sections corresponding to a case shown to strongly express *SBEM* mRNA by RT-PCR were studied by *in situ* hybridization. No signal was detectable when using a sense probe (Fig. 3*A*). In contrast, a signal was observed in epithelial tumor cells when using an antisense probe (Fig. 3*B*). *SBEM* mRNA was not detected in stromal or inflammatory cells in any of the sections studied.

Discussion

This article reports the identification, cloning, and preliminary characterization of a cDNA encoding a novel mucin-like protein that displays an unusually narrow pattern of expression. Hybridization analysis revealed that *SBEM* mRNA was only detectable in two normal tissues, breast and salivary gland. Interestingly, the tissue-

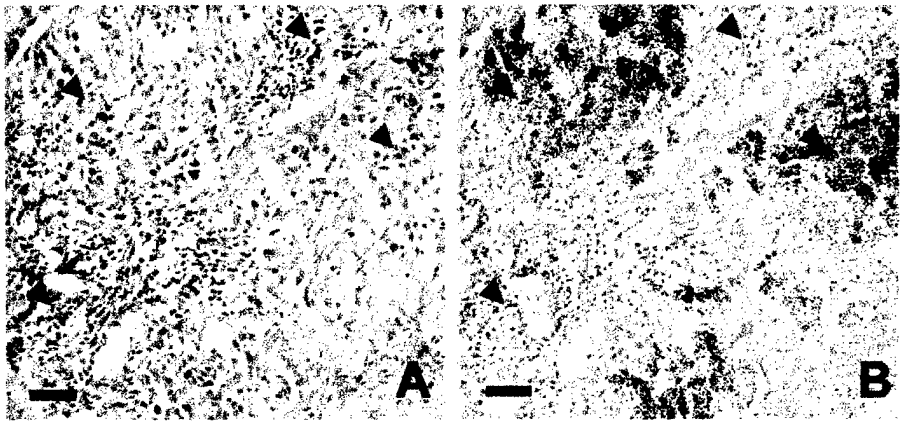

Fig. 3. Expression of *SBEM* mRNA in a primary breast tumor studied by *in situ* hybridization. These plates illustrate consecutive sections from a single breast tumor and show H&E-stained paraffin section treated with a sense probe (*A*) and *SBEM* mRNA expression in epithelial cells detected using an antisense probe (*B*). *Mauve* and *black arrowheads* show tumor epithelial cells and regions of stroma with inflammation, respectively. *Bar*, 30 μm.

specific expression that we observed experimentally directly reflects the distribution of ESTs within the Hs.348419 cluster. Indeed, as mentioned above, only two adult tissues (breast and head/neck tumors) have been shown to express SBEM-related ESTs. The fact that SBEM is also expressed in salivary tissue does not undermine the possible use of SBEM as a marker of breast cancer, because tumors of the salivary gland are less common and can readily be distinguished clinically.

Among the primary breast tumors examined in this study (representing mostly invasive ductal carcinoma), *SBEM* mRNA was observed by RT-PCR analysis in the majority (>90%) of cases. Despite a significant overall correlation between the expression of *SBEM* and *mammaglobin-1* mRNA, a significantly higher SBEM:mammaglobin-1 ratio was observed in primary tumors associated with positive axillary lymph nodes as compared with node-negative tumors. This was mostly attributable to a trend toward higher SBEM expression in node-positive tumors. Although further analysis of a larger number of tumors will be required to confirm these observations, this may suggest differences in the biology of these tumors and also a possible role of SBEM and mammaglobin-1 in the mechanisms involved in tumor metastasis. Our findings indicate, however, that SBEM expression is a common feature of breast cancer and can furthermore serve as a useful marker for breast nodal metastasis, both for detection of micrometastatic cells within lymph nodes as well as in the differential diagnosis of the primary origin of an unknown metastasis. This potential is enhanced by the conserved SBEM expression in high grade and ER/PR-negative tumors that are most likely to metastasize.

The potential diagnostic relevance of SBEM is also increased by its predicted biochemical structure. The *SBEM* cDNA sequence codes for a 90-amino acid polypeptide that contains a distinctive tandem repeat, rich in alanine and threonine residues, that represents a probable target for *O*-glycosylation. Consistent with such posttranslational modification is the presence of a well-defined signal peptide, leading us to predict that SBEM is likely to be processed at the apical surface of luminal epithelial cells and to be secreted into the alveolar or ductal lumen. Further study is needed to ascertain whether higher SBEM expression occurs in association with tumors.

Secreted (or transmembrane) proteins that contain internally repeated, densely glycosylated neutral core motifs such as this are characteristic of mucins, which are typically expressed by the surface epithelium of secretory mucosae and by exocrine glands (14, 15). The role of mucins is primarily one of hydrating and lubricating epithelial linings, although several mucins have been implicated in modulating both cell adhesion and growth factor signaling (18, 19). Furthermore, mucins have a well-established link to cancer, best illustrated by the product of the *MUC1* gene. *MUC1* is overexpressed in a variety of epithelial tumors including breast cancer and gives rise to several well-characterized tumor antigens including CA15.3 and CA27.29 (20, 21). Combined with the loss of cell polarity and changes in glycosylation patterns observed in transformed epithelial cells, overexpression of *MUC1* results in the appearance of mucin-derived tumor antigens in the sera of cancer patients that are not seen in normal controls (14, 22). We hypothesize that a similar situation may hold for SBEM expression in the human mammary epithelium and in human breast tumors. However, *MUC1* displays relatively broad expression among epithelial tissues including the colon, breast, pancreas, ovary, prostate, tracheobronchial tree, stomach, and uterus. For this reason, *MUC1*-derived tumor antigens have relatively poor specificity for individual tumor types, and their clinical utility is limited to monitoring the efficacy of cancer therapy and warning of tumor relapse or malignant spread (21–23).

Parallels between SBEM and known epithelial mucins such as *MUC1*, together with its more narrowly restricted pattern of expression, suggest that this novel gene represents an attractive candidate for a breast biomarker with potential for cancer diagnostics, as well as being a possible future target for the development of a breast tumor vaccine. Moreover, the absence of *SBEM* expression in normal lymph node tissue suggests that this gene could also be used to detect breast micrometastases in axillary lymph nodes.

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APPENDIX 3
Submitted, Endocrinology

PUTATIVE FUNCTIONAL CHARACTERISTICS OF HUMAN ESTROGEN RECEPTOR-BETA ISOFORMS.

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Abstract.

Estrogen receptors (ERα and ERβ) are clearly multifaceted in terms of structure and function. Several relatively abundant ERβ isoforms have been identified, which can be differentially expressed in various tissues. In order to provide insight into the possible role of the ERβ family in breast tissue a study of the putative functions of the hERβ1, hERβ2 and hERβ5 isoforms was undertaken. Only hERβ1 was found to bind ligand, which induced conformational changes as determined by protease digestion assays. All ERβ isoforms could bind to and bend DNA although the relative efficiency with which they bound DNA differed with hERα > hERβ1> hERβ2 >>hERβ5 . All ERβ isoforms inhibited ERα transcriptional activity on an ERE-reporter gene. The relative activities were hERβ1>hERβ2> hERβ5, however only hERβ1 had transcripitonal activity alone. Both LY117018-hERα and LY117018-hERβ1 complexes alone could activate transcription on a TGF-β3- reporter gene. Although hERβ2 and hERβ5 had no activity alone, they inhibited ERα but not hERβ1 transcriptional activity of TGF-β3-CAT. In marked contrast to activity on an ERE-CAT reporter gene, hERβ1 did not modulate ERα transcriptional activity on a TGF-β3-CAT reporter gene. These data support promoter-specific differential activities of ERβ isoforms with respect to models of ERα regulated gene expression, and suggest that they may have a role in differentially modulating estrogen action.

Introduction.

The estrogen receptor family of steroid hormone receptors is clearly multifaceted (1) and more complex than originally thought. There are two genes which encode estrogen receptors (ER), ERα and ERβ. Both are ligand regulated transcription factors which classically modulate target gene transcription by binding as homo- and/or hetero-dimers to estrogen responsive sequences in target gene promoters (2). These receptors likely have distinct roles in estrogen action, independent of each other (3) when they are expressed separately but can also have direct interactions due to heterodimerization when the receptors are expressed together in the same target cell (4). In addition both ERs may encode variant isoforms generated by alternative splicing mechanisms (5, 6). In particular there are data to support variant isoforms of ERβ at the protein level (7, 8) Furthermore, we have shown in human breast tissues that variant forms of ERβ are more abundant than the wild-type at least at the RNA level (9).

Human ERβ2 (also called hERβbx (10)) and ERβ5 variant mRNAs are missing the wild type exon 8 sequences and contain extra sequences which are distinct from each other, followed by sequences that are then identical with each other (see Fig 1). They are predicted to encode C-terminally truncated ERβ like proteins identical to wild-type until amino acid residue 468 (by reference to the long form of hERβ1 (11). After amino acid 468 hERβ2 is predicted to encode 28 novel amino acids, with the full-length protein having a predicted molecular mass of 55.5 kDa. In contrast, after amino acid 468 hERβ5 is predicted to encode only 5 novel amino acids with the full-length protein having a predicted molecular mass of 53 kDa.

Although total ERβ expression appears to decrease between normal breast and ER+ breast tumors (12, 13), the relative expression of the variant ERβ isoforms to the wild-type ERβ can also change during breast tumorigenesis, at least at the RNA level (9). This suggests that the expression and/or the activity of the ERβ family of receptors changes during breast tumorigenesis and may have a role in this process as well as have a role in altered estrogen action that occurs during breast tumorigenesis. In order to provide insight into the possible role of the ERβ family in breast tissue we

have undertaken a study of the putative functions of the hERβ1, hERβ2 and hERβ5 isoforms.

MATERIALS AND METHODS.

Materials. 17β-estradiol (E2), 4-hydroxytamoxifen (4-OH-TAM) and CAPS (3-cyclohexylamino-1-propanesulfonic acid) were from Sigma Chemical Co (St Louis, MO). ICI 182,780 was a gift from Dr AE Wakeling (AstraZeneca Pharmaceuticals, England). LY117018 was a gift from Eli Lilly Co. (Indianapolis, IN). (^{3}H)-17β-estradiol (^{3}H-E_2), (^{14}C)-chloramphenicol, and (^{35}S)-methionine were from New England Nuclear (Boston, MA). (α-^{32}P)-dCTP, (γ-^{32}P)-ATP, and (^{35}S)-ATP were from ICN Pharmaceuticals (Irvine, CA). All cell culture reagents were obtained from GIBCO/BRL (Burlington, Ontario).

***In vitro* transcription and translation.** *In vitro* transcription/translation reactions were performed using a coupled transcription/translation system (TnT coupled Reticulocyte Lysate System, Promega, Madison, WI). Reactions were performed according to the manufacturer's instructions.

Scatchard Analysis. Human ERα (pcDNA3.1/wild-type human ERα from HEGO, (14)), human ERβ1 (pcDNA3.1hERβ1, long form of 530 amino acids (11, 15)), human ERβ2 (pcDNA3.1 hERβ2, long form (10)) and human ERβ5 (pcDNA3.1 hERβ5, long form) proteins were synthesized by *in vitro* transcription-translation as described above. Ligand binding studies were conducted as previously described (16). *In vitro* generated receptor was diluted 10-fold in buffer (10 mM Tris-Cl, pH 7.5, 1.5 mM EDTA, 10 mg/ml BSA, 10% glycerol) and kept on ice until use. One hundred µl of the diluted protein were used in each binding reaction, that contained varying concentrations of (^{3}H)-E_2 (0.01 - 100 nM), followed by overnight incubation at 4^{0}C. Non-specific binding was determined by parallel incubations containing a 200-fold excess of unlabelled E_2. Unbound steroid was removed by addition of 500 µl of 0.5% charcoal-0.05% dextran in the above dilution buffer for 30 mins at 4^{0}C followed by centrifugation at 10000 x g for 10 mins at 4^{0}C. Radioactivity was determined in an aliquot of the supernatant and in aliquots of total (^{3}H)-E_2 solutions using a scintillation counter. The ratio of specifically bound/unbound steroid and the

concentration of specifically bound steroid were used for Scatchard analysis, from which was determined the equilibrium dissociation constant, K_d.

Limited proteolytic digestion analysis. Conformational studies were performed as described previously (17).*In vitro* synthesized ERs were incubated with agonist (E2, DES) and antagonists (TAM, LY) for overnight at 4^0 C. The liganded receptors were then diluted 1: 10 (v/v) in TE buffer, then 20 ul of these ER solution was treated with increased concentration of trypsin (0.2 to 5 ug) for 20 min at room temperature and stopped loading buffer was added. The samples were boiled and were directly analyzed by SDS-polyacrylamide gel electrophoresis (10% w/v). The gel was dried and the digested bands were visualized by autoradiography.

Electrophoretic Mobility Gel-Shift Assay (EMSA). *In vitro* synthesized human ERs were used for EMSA. Typically 1 μl of programmed lysates containing equal amounts of each receptor as determined by polyacrylamide gel electrophoresis of ^{35}S-methionine labelled protein generated in parallel *in vitro* transcription-translation assays was assayed in EMSA. One μl of lysate was incubated in a final volume of 20 μl, and the reaction solution was 5 mM Tris-Cl, pH 7.5, 100 mM KCl, 2 mM DTT, 5% v/v glycerol and contained 2 mg polyd(I-C). The binding reaction was initiated by adding 1 μl (approx. 10 fmol) of 5'-(^{32}P)-end-labeled, double stranded ERE oligonucleotide (35mer, 5'-AACTTTGATCAGGTCACTGTGACCTGACTTTGGAC -3' containing the vitellogenin A2 ERE sequence), and the mix was incubated at 20^0C for 30 min. DNA-bound complexes were electrophoretically separated on a 4.5% loosely cross-linked acrylamide gel (1:29 bis:acrylamide) at 150V for 90 min at 20^0C temperature in 0.5xTBE buffer. Gels were then vacuum dried and autoradiographed. To identify immunoreactive ER within retarded DNA-bound complexes, parallel incubations containing 1μg of ER antibody (usually 1 μl of H222 for ERα, or 1 μl of PAI-310 for ERβ1 and ERβ2) were run to determine the presence of super-shifted antibody-bound ER-ERE complexes (data not shown). Reticulocyte lysates containing *in vitro* translated ER proteins were incubated with or without saturating concentrations of ligand (estrogen or antiestrogen) at 4^0C overnight to allow receptors to bind ligand, followed by EMSA.

DNA Bending Assay. The DNA bending vector ERE Bend I (kindly provided by Dr A Nardulli, University of Illinois, Urbana, IL) (18) was digested with EcoRI and EcoRV to produce a 430 bp DNA fragment with a single consensus ERE either at the end (EcoRI fragment) or in the middle (EcoRV fragment). The fragments were then gel purified, labeled by incubation with polynucleotide kinase in the presence of (γ-^{32}P)-ATP, and purified on a G50 Sephadex column. Gel mobility shift assays were carried out essentially as described above. Aliquots of the binding reactions were run on 8% non-denaturing acrylamide gels, dried and exposed to X-ray film. The degree of DNA-bending was determined using the method of Thompson and Landy (19).

Cells, Cell Culture and Transient Transfection. For transient transfection analysis tagged ER expression vectors were generated. Human ERα, ERβ1, ERβ2 and ERβ5 were tagged at their N-terminus with a polyhistidine and an Xpress™ epitope tag using the pcDNA4/HisMax(A) plasmid (Invitrogen Canada Inc, Burlington, ON). Cos-1 and Cos-7 cells were obtained from the ATCC (Manassas,VA). The cells were routinely cultured in DMEM containing 5% v/v fetal calf serum (FBS), 1% w/v glucose, glutamine and penicillin-streptomycin (5%CM). To obtain estrogen depleted cells, the culture medium of stock cells was changed to phenol red-free DMEM supplemented with 5% charcoal dextran-treated FBS, 1% w/v glucose, glutamine and penicillin-streptomycin (5%CS) and replaced every 2 days. Six days later the medium was replaced by 10%CS until required for experiments. For transient transfection experiments, the cells were transfected using the Effectene transfection reagent according to the manufacturer's instruction (QIAGEN, Mississauga, Ontario). Briefly, the day before transfection, the estrogen depleted cells were seeded in six-well plates at 2.5 x 10^5 cells per well in 2 ml of 5%CS and left overnight. The plates were 70-80% confluent on the day of transfection. The transfection mixture was prepared according to the manufacturer's protocol, then fresh medium (5%CS) was added to the transfection mixture and 0.6 ml per well of the above mixture either ERE-II-TCO-CAT (gift from P Webb, (20)) or TGF-β3-CAT-reporter plasmid DNA (21). ER expression plasmid (50-450 ng) or empty vector and 100 ng β-gal pCH110 plasmid DNA (Pharmacia canda, Mississauga, ON) was added drop-wise into the medium (CS) and the plates were gently swirled to ensure uniform distribution of the DNA-

Effectene complexes. Vehicle (ethanol), estradiol-17β or LY117018 was then added 20 to 30 minutes later. The cells were left for 48 hr and then harvested. Cell extracts were prepared by freeze/thawing and used to determine CAT and β-galactosidase activity as previously described (22)

Western Blot Analysis. For Western blot analysis, 2.5 x 10^5 Cos-1 cells were set up in 6 well plates, then transiently transfected with plasmids and treated with estrogen or antiesotrgen under the same conditions as for the CAT assay described above. Cells were harvested 48 hour after transfection, washed once with Isoton II and then the washed cell pellets were resuspended in 200 µl Isoton II. Aliquots of cell suspension (150 µl) were extracted and used for Western blots and the remainder was used for determination of β–galactosidase activity. For Western blotting, the cells were pelleted and then extracted using 40 µl of hot (95°C) extraction buffer J with shaking for 20 min at 95°C as previously described by Joel *et al.*, (23). The entire extract was subjected to 10% SDS-polyacrylamide gel electrophoresis as previously described (24). The separated proteins were transferred to nitrocellulose membranes and processed as previously described (24). Detection of the tagged estrogen receptor proteins was by incubation of blots with anti-Xpress antibody (1/5000 in TBST, Cat#R910-25, Invitrogen Canada Inc, Burlington, ON) overnight at 4°C, followed by washing and incubation with secondary antibody (horseradish peroxidase conjugated goat anti-mouse antibody, 1/5000 in TBST, Jackson Immuno Research Labs Inc, PA) at room temperature for 2 hours. Visualization was carried out using the SuperSignal West Dura Extended Duration Substrate kit (Pierce, IL) according to the manufacturer's instructions.

Statistical Analysis. Differences in variance were tested using ANOVA where appropriate. Differences between individual mean values were then determined using students t-tests. All tests were performed using GraphPad Prism statistical analysis software (GraphPad Software Inc).

Results.

Identification and Organization of hERβ1, hERβ2 and hERβ5 cDNA Sequences at the hERβ Locus on Chromosome 14.

The estrogen beta gene has been localized to human chromosome 14q22-24 and the genomic structure of the 8 exons comprising hERβ1 has been previously published (4). However, the previously described variant hERβ2 (also called hERβcx) and hERβ5 mRNA only contain sequences corresponding to exons 1 to 7 of hERβ1 and then diverge (see Figure 1A). They do not contain exon 8 sequences of hERβ1, but contain sequences termed exon 9, which are located downstream of exon 8 on chromosome 14 (Figure 1B), identified using database sequences of chromosome 14 (Accession numbers CNSO1RHJ and AF215937) and the Human Genome Working Draft. It should be noted that neither of these genomic sequences contain an extra A 5' of the start site of translation for hERβ, that would place another upstream ATG in frame with the known coding region and introduce 18 amino acids to the N-terminal of the known coding region as recently described (25). Interestingly, hERβ5 mRNA also contains sequences between exon 7 and part of exon 9 which are not present in either hERβ1 or hERβ2 mRNA. These hERβ5 mRNA specific sequences can be found immediately following exon 7 sequences in intron 7 of the human ERβ gene (Figure 1B), suggesting that the normal splice donor site is not recognized and a cryptic splice donor site is present in intron 7. Furthermore the exon 9 sequences present in hERβ5 cDNA start 28 nucleotides downstream of those present in hERβ2, suggesting a cryptic splice acceptor site is present within exon 9. There are also multiple non-coding exons 5' to exon 1 as previously identified (4), since several hERβ cDNAs contain sequences in their 5' UTR which are found further upstream of exon 1 on chromosome 14 (Figure 1B, 1H-1C seen in AB006589, 1B seen in NM_001437, AX234658, AF05428, AF060555, AB006589 references). The sequences of a recently characterized promoter region of hERβ (26) are found immediately upstream and overlapping with exon 1^{B}. However, the presence of hERβ mRNAs whose 5'UTR contain exonic sequences found upstream of this documented promoter suggest that there are alternative promoters for the hERβ gene. This is similar to the hERα gene and suggests that regulation of expression of these genes is complex (27).

The predicted open reading frames for hERβ1, hERβ2 and hERβ5 are shown in Figure 2. hERβ2 contains amino acids 1-468 which are identical to hERβ1 and then

diverges with another 28 novel amino acids encoded in the open reading frame. hERβ5 was isolated as a partial cDNA but is likely to also be identical to hERβ1 from amino acids 1-468 and then diverges containing another 5 novel amino acids. Both these variant hERβ proteins would be truncated at the C-terminus, disrupted in helix 11 and missing helix 12 and therefore unlikely to bind ligand or have AF2 mediated transcriptional activity. Lack of ligand binding has been confirmed using *in vitro* generation of these proteins as outlined below.

Ligand Binding Activity of hERβ1 and variant isoforms hERβ2 and hERβ5 Proteins.

Human ERβ1 has been shown previously (4) to bind E2 with high affinity and specificity, and our data confirm these findings. Figure 3A shows specific saturable binding of $(^3H)E_2$ to *in vitro* translated hERβ1 with a calculated K_d = 0.11 nM. However, the open reading frames of hERβ2 and hERβ5 cDNA predict for proteins C-terminally truncated compared to hERβ1 and predicted not to bind ligand. As shown in Figure 3 B and C no saturable binding of $(^3H)E_2$ to *in vitro* translated hERβ2 or 5 was observed.

Human Estrogen Receptor Isoform Conformational Status and Ligand Induced Changes.

To determine the possible conformational status of variant hERβ isoforms, a previously used limited trypsin digestion assay (17, 28) was employed to compare the proteolytic digestion patterns of variant S^{35}-methionine labelled hERα, hERβ1, hERβ2 and hERβ5 in the presence and absence of estrogens and antiestrogens (4-OH-tamoxifen and LY117018). The results are shown in Figure 4. In the absence of any ligand all ER isoforms were sensitive to proteolysis, with hERα and hERβ1 being relatively more resistant than hERβ2 and hERβ5. In the presence of estradiol both hERα and hERβ1 become more resistant to digestion, and a 32.5 kDa resistant band (shown by * in Fig 4) was observed. Addition of antiestrogens 4-OH-tam and LY117018 increased slightly the sensitivity of hERα and hERβ1 to trypsin digestion compared to the receptors in the absence of ligand. The sensitivity of the variant isoforms hERβ2 and hERβ5 was not effected by ligand, consistent with their inability to bind ligand. Furthermore, the variant isoforms were more sensitive to trypsin

digestion than either the unliganded hERα or hERβ1, suggesting that the variant isoforms are unlikely to be in an activated conformation.

DNA Binding and Bending Activity of hERβ1 and variant isoforms hERβ2 and hERβ5 Proteins.

As previously demonstrated hERβ1and hERβ2 can bind to an ERE in a gel mobility shift assay (Figure 5), although the efficiency of hERβ2 DNA binding was less than hERβ1(6, 10). hERβ5 also has the ability to bind an ERE in gel mobility shift assays (Figure 5), but was less efficient than hERβ2. The specificity of the binding was determined by competition with excess unlabelled ERE whereas no competition was seen with an excess of unlabelled nonspecific 33 mer oligonucleotide.

DNA bending assays demonstrated that hERα, hERβ1 and hERβ2 were all able to bend DNA as demonstrated by the reduced mobility of complexes when the ERE is in the middle of the DNA fragment (M) in comparison to the mobility of complexes when the ERE is at the end of the DNA fragment (16, 29). The calculated bending angle for hERα was 64.8 ± 1 (mean ± SEM, n = 3), for hERβ1 was 53.6 ± 0.5 and for hERβ2 was 54.6 ± 0.7. A lower overall signal of the retarded complexes were seen with hERβ1 and hERβ2 compared to hERαwhich likely reflects the lower efficiency of the hERβ1 isoforms compared to the hERα of binding to an ERE. Furthermore, the DNA binding ability of hERβ5 was too low to obtain accurate data for DNA bending calculations. No effect of ligand was observed (data not shown).

Transcriptional Activity of hERβ1, hERβ2 and hERβ5.

The ability of tagged ERs to activate transcription was initially investigated using Cos-1 cells and an ERE containing reporter gene, ERE_{45}2-delta-TCO-CAT, which has two vitellogenin A2 (-333/-288) EREs upstream of a CAT reporter (20). Epitope tagged receptors were used so that relative expression of all the ERs could be measured using antibodies to the epitope tag, and preliminary experiments demonstrated that the tagged ERα and ERβ1 were similar to their untagged counterparts in activating transcription with and without ligand (data not shown). Preliminary studies showed that transfection of 50 ng ERαexpression vector gave maximal estradiol induced transactivation of this reporter gene. hERαand hERβ1 activated transcription in a ligand inducible manner (Figure 6), but hERβ1 was

overall less active than hERα ($P < 0.0001$, $n = 5$), and increased expression of hERβ1 did not alter this relationship. These data are consistent with previous findings. As shown in Figure 6, low doses of estradiol (0.1 nM) which significantly activated hERα did not activate hERβ1 ($P < 0.0001$, $n = 5$), and the apparent ligand independent activity (zero ligand added) of hERα was significantly higher than that of hERβ1 ($P = 0.024$, $n = 5$). This ligand-independent activity was inhibited by 0.1 and 100 nM of the antiestrogen LY117018 (a raloxifene analog) as well as by hERβ1 (50 and 450 ng) and hERβ2 (450 ng)(data not shown). These results were not due to different levels of expression of hERαand hERβ1 since Western blot analysis showed similar levels of expression of the two receptors when similar amounts of plasmid were transfected (Figure 7). The expression of the variant isoforms hERβ2 and hERβ5 alone demonstrated little if any transcriptional activity under these conditions (Figure 6).

ERα and hERβ isoforms can heterodimerize (2), which may underlie the functional interactions between ER isoforms. All hERβ isoforms tested inhibited the transcriptional activity of hERα on an ERE containing promoter (Figure 8A and 8B) but the various hERβ isoforms had different efficiencies with hERβ1 > hERβ2 > hERβ5. Ligand activation of hERβ1 did not effect its ability to decrease the activity of hERα, since under conditions when it was not activated (0 or 0.1 nM estradiol, see Figure 6) hERβ1 activity was similar to that under conditions where it was activated (100 nM estradiol). Variant isoforms of hERβ had little if any effect on hERβ1 activity on ERE-containing promoters (data not shown).

The transcriptional activity of ER isoforms was next examined on the non-ERE-containing promoter, TGFβ-3-CAT where the DNA binding domain of ERα is not required for activity (21). This promoter was shown to be preferentially activated by the raloxifene bound hERα compared to estradiol in cultured cells (21), and we have previously shown differential abilities of murine ERβ isoforms to effect this promoter compared to ERE-containing promoters (16). Therefore, the activity of hERβ isoforms on TGFβ-3-CAT was examined (Figure 9). Optimal activity for hERα was obtained with transfection of 50 ng of expression plasmid (data not shown). A

significant increase in transcription was obtained with 0.1 nM of LY117018 that was not further increased with 100nM LY117018 treatment (P = 0.0061, n = 3). LY117018 significantly increased the transcriptional activity of hERβ1 on the TGFβ-3-CAT reporter gene at the lower levels of hERβ1 expression (50ng, P = 0.008; 150 ng, P = 0.02, n = 3) but at high levels of hERβ1 expression (450 ng), a significant increase in ligand-independent activity was seen, and no further increase was seen due to ligand. Overall, hERβ1 was significantly less active than hERα in inducing TGFβ-3-CAT (P<0.0001, n = 3). Although there was a trend towards inhibition of TGFβ-3-CAT with increasing expression of hERβ2 or hERβ5 (data not shown), this was not statistically significant.

When the ability of hERβ isoforms to effect hERα activity was investigated at the TGF-β3 promoter, differences between the wild-type and variant isoforms were observed. The wild-type hERβ1 did not significantly effect hERα transcriptional activity at any level of expression tested (Figure 10A and 10B). However, under the same conditions hERβ2significantly inhibited hERα transcriptional activity on TGFβ-3-CAT (P= 0.0002, n = 3), and as expected the effect was not influenced by LY117018, since hERβ2 does not bind ligand. However, hERβ2 inhibits both the ligand activated and the non-ligand activated (data not shown) hERα (P = 0.017, n = 3) at the TGFβ-3-CAT promoter. hERβ5 also inhibited hERα transcriptional activity on TGFβ-3-CAT but only at the highest expression of hERβ5 (Figure 10B, P = 0.038, n = 3). Similar to their action at an ERE containing promoter, the truncated ERβ variants 2 and 5 do not modulate wild-type hERβ1 transcriptional activity on TGFβ-3-CAT (data not shown).

Discussion.

There is a growing body of evidence that ERα and ERβ can be expressed together in some cell types and independently expressed in others (30-32). If expressed together they form heterodimers, which under experimental conditions are preferred over homodimerization (2). Further, transient coexpression of ERα and ERβ in cell lines, results in ERβ1 induced reduction of ERα activity at low ligand concentrations, as measured using ERE-regulated reporters (33). A conclusion from these data is that

ERβ can directly modulate ERα activity. This has significance since many reports exist of differential expression of the two receptors under conditions of altered estrogen sensitivity. For example, ERβ expression is significantly downregulated and ERα expression upregulated during human breast tumorigenesis, suggesting that ERβ's ability to modulate ERα is significantly altered during breast tumorigenesis (12, 13). In addition, current data show that in normal and neoplastic breast tissues, the level of expression of the C-terminally truncated ERβ variants, ERβ2and ERβ5, is markedly higher than the ligand binding ERβ1 . These data suggest that the variant ERβ isoforms may also have a role in modulating estrogen and possibly antiestrogen action in human breast cells. The experiments described in this manuscript were undertaken to gain insight into the possible role of the truncated ERβ variants.

Our data show that only hERβ1 is able to bind ligand. Steroid hormone receptors are known to undergo conformational changes during the process of activation especially due to ligand binding and differences are seen between agonist and antagonist binding (17, 28). Recent structural analyses of the Ligand Binding Domain (LBD) of several nuclear receptors suggest that the LBD contains common structural motifs that generate a conserved ligand binding pocket, and that agonist and antagonists bind to the same site but induce different conformational changes that are now known to affect transcriptional function, providing structural evidence for antagonism (34). The variant hERβ isoforms, while not binding ligand, may exist in an activated state in the absence and presence of ligand, however, our data suggest that hERβ2 and hERβ5 are unlikely to be in an activated conformation, and this is consistent with their inability to activate transcription of either a 'classical' or a'non-classical' estrogen receptor regulated reporter gene.

All ERβ isoforms examined (ERβ1, 2, and 5) inhibit the transcriptional activity of ERα on ERE containing promoters, while only ERβ1 has any activity alone. This confirms and extends previous data and demonstrates the relative inhibitory activity of the ERβ isoforms is ERβ1 >ERβ2 >ERβ5. This correlates with the relative efficiencies with which ERβ homodimers bind to DNA and may suggest a competition of the beta isoform homodimers with ERα homodimers for DNA

binding. However, since heterodimers are preferred under these conditions, it is likely that these predominant under our experimental conditions and the intrinsically lower transcriptional activity of the heterodimers predominants. Cowley *et al.*, (2) demonstrated that when hERα and hERβ1 are expressed at both a 1:1 and 1:2 ratio the ERα/ERβ1 heterodimer was predominant. This heterodimer had a DNA binding affinity similar to that of the ERα homodimer, and was capable of recruiting SRC-1. However, the heterodimer was less transcriptional activity than the ERα/ERα homodimer, suggesting that it may be less efficient in recruiting coactivators than the ERαhomodimer. In contrast, the C-terminally truncated hERβ2 has markedly reduced ability to bind to DNA and likely the ERα/ERβ2 heterodimer also binds less well than ERα/ERα homodimers to an ERE (6, 10). But in contrast to hERβ1, hERβ2 does not recruit coactivators (10). Our data show that hERβ5 is less efficient than hERβ2 in binding to DNA, and is also unlikely to recruit coactivators. However, at an ERE the wild-type hERβ1 is more potent than either of the two variants in inhibiting the ability of ERα to activate transcription. So it appears that the inability to recruit coactivators is not correlated with the ability of ERβ isoforms to inhibit ERα activity. Since DNA binding activity is also a reflection of efficiency of dimerization, it is speculated that the truncated ERβ isoforms have reduced ability to dimerize with ERα and form stable heterodimers than the wild-type ERβ1. Together with our Western blot data it seems that significant inhibition of ERα transcription occurs at levels of ERβ1 expression that are less than or equivalent to ERα. Therefore our data would be consistent with the mechanism of inhibition being related to a high efficiency of dimerization and reduced efficiency in recruiting coactivators, but not the inability to recruit coactivators.

Interestingly, marked differences in the ability of the ERβ isoforms to affect ERα activity are seen at an estrogen receptor responsive site where the mechanism of transcriptional regulation is quite distinct from that operating at a classical ERE, e.g. the so-called raloxifene responsive element in the TGF-β3 promoter (21). This is in marked contrast to the results seen at an ERE regulated reporter gene. The ER responsive site in the TGF-β3 promoter is poorly activated by the estradiol-ERα

complex, but strongly activated by the raloxifene-ERα complex. In addition the DNA binding domain of the ER is not required for this activation. It is assumed that protein:protein interactions between ERα and other transcription factors bound to this promoter are involved in regulation. However, the identity of these 'other' transcription factors is unknown. Using an analogue of raloxifene, LY117018 (16), we have confirmed that this promoter is poorly activated by the estradiol-ERα complex (and this was not altered in our hands by treatment of the transfected Cos-1 cells with EGF (35), data not shown) but was significantly activated by the LY117018-ERα complex. Similarly, the LY117018- hERβ1 complex was found to activate transcription from the TGF-β3 promoter, but in contrast to the murine ERβ1 (16), is less active than the LY117018-hERα complex. Human ERβ2 and hERβ5 alone could not activate this promoter. This is in contrast to the murine ERβ2 variant (16) which is structurally quite different to the hERβ2 Furthermore, no murine equivalent to either hERβ2 or hERβ5 isoforms that are frequently expressed in human tissues, have as yet been identified. However, coexpression of increasing amounts of hERβ2 and hERβ5 with ERα resulted in inhibition of LY117018-ERα transcriptional activity but not LY117018-ERβ1 activity from the TGF-β3 promoter. In contrast to an ERE containing promoter is the observation that the wild-type hERβ1 did not significantly inhibit the transcriptional activity of the LY117018-ERα complex at the TGF-β3 promoter. At this promoter the differences in the hERβ isoform activity on LY117018-ERα complexes, was correlated to the ability to recruit coregulatory factors. Significant effects of hERβ2 on hERα were seen under conditions of equimolar expression, as determined by Western blot analysis of the similarly tagged proteins, but hERβ5was less active than hERβ2 and this is consistent with a reduced efficiency of dimerization. There appears to be differential expression of hERβ isoforms at least at the RNA level in different human tissues, as well as altered relative expression during breast tumorigenesis (9, 36), and altered levels of hERβcx (hERβ2) as well as hERβ1 during prostate cancer progression (8). Therefore, it is possible that the differential activities of hERβ isoforms on some genes may have both physiologically and pathophysiologically importance.

In conclusion we have characterized some potential functions of several commonly expressed hERβ isoforms. Generally, the ligand binding wild-type hERβ1 has transcriptional activity alone on both 'classical' and 'non-classical' estrogen responsive promoters, although it is less efficient than ERα. Furthermore, the hERβ family of receptors generally negatively modulate ERα transcriptional activity when coexpressed, at 'classical' as well as 'non-classical' ER responsive promoters. However, promoter specific differential activity of the various hERβ isoforms was found, in particular between the wild-type hERβ1 and its C-terminally truncated variants hERβ2 and hERβ5. The possibility that there is differential expression of the hERβ isoforms suggests that they may have a role in differentially modulating estrogen action.

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Legends to Figures.

Figure 1.

A. cDNA structure of human ERβ1, human ERβ2 and human ERβ5.
B. Genomic structure of the human estrogen receptor β locus on chromosome 14. Human ERβ cDNA, ESTs and published promoter sequences (accession numbers in the text) where aligned with sequences from two genomic clones of human chromosome 14 (AL162756/CNS01RHJ and AF215937). Exons 1C to 1H are found only in one cDNA, accession number AB006589).

Figure 2.

Predicted proteins of the human ERβ isoform cDNAs.

Figure 3.

Determination of estradiol binding to hERβ1 (A), hERβ2 (B), and hERβ5 (C). Increasing amounts of ^{3}H-estradiol-17β (0.01-100 nM) were incubated with a constant amount of each *in vitro* transcribed/translated hERβ protein. Two separate experiments were performed. Total binding is shown by the closed circles and non-specific binding is shown by the empty circles.

Figure 4.

Sensitivity of human Estrogen Receptor Isoforms to Protease Digestion. Radiolabelled ER was made *in vitro* as described in the Material and Methods section, and digested with increasing levels of trypsin, with and without ligand. The products were visualized by autoradiography after SDS-PAGE. Resistant bands are shown by arrows. The asterisk shows agonist induced resistant 32.5 kDa band.

Figure 5.

Determination of the ability of hERα, hERβ1, hERβ2 and hERβ5 to bind to DNA. Autoradiograph of an electrophoretic mobility gel shift analysis of *in vitro* transcribed/translated hERα, hERβ1, hERβ2 and hERβ5 proteins binding to a 35 mer double stranded ERE oligonucleotide containing the vitellogenin A2 ERE sequence. Free ERE and the shifted complexes are indicated. The presence of the appropriate ER isoform in the shifted complex was determined by the ability of a specific antibody (H222 for ERα; PA1 for the ERβ proteins) to super-shift the complex, data not shown. Specificity of the complexes was determined by the ability of a 200 fold excess of the unlabelled ERE (specific competitor) to compete for the shifted complex and non-specific interactions were determined using a 200 fold excess of unlabelled nonspecific 33 mer oligonucleotide (nonspecific competitor).

Figure 6.
Transcriptional activity of hERα, hERβ1, hERβ2 and hERβ5 on ERE regulated CAT reporter gene. Increasing amounts of hER isoform expression vectors were cotransfected with an ERE-CAT reporter gene into Cos-1 cells. The transfected cells were treated for 48 hours with or without estradiol-17β. Results show the mean CAT activity after correction for β–galactosidase activity (transfection efficiency) ± SEM of 5 independent experiments for hERα, hERβ1 and hERβ2, and 3 independent experiments for hERβ5. See text for statistical analysis.

Figure 7.

Western blot analysis of Cos-1 cell extracts 48 hours after transfection of the indicated amounts of tagged - hERα, - hERβ1 and - hERβ2 expression vectors, as detailed in Materials and Methods. Lane 1 is extract of Cos-1 cells transfected with empty expression vector alone; lane 2 is tagged - hERα expression vector; lane 3 is tagged - hERα (50ng) + tagged - hERβ1 (50 ng) expression vectors; lane 4 is tagged - hERα (50 ng) + tagged - hERβ1 (150 ng) expression vector; lane 5 is tagged - hERα (50ng) + tagged - hERβ2 (50 ng) expression vectors; lane 6 is tagged - hERα (50ng) + tagged - hERβ2 (450 ng) expression vectors. The tagged proteins were visualized with anti-Xpress antibody as described in Materials and Methods.

Figure 8.

Effect of increasing amounts of coexpressed hERβ isoforms on the ability of hERα (50 ng) to activate transcription from an ERE (vitellogenin A2) regulated CAT reporter gene in the presence and absence of ligand following co-transfection into Cos-1 cells. The results show the mean CAT activity after correction for β–galactosidase activity (transfection efficiency) ± SEM of 3 independent experiments. A. Effect of hERβ1 and hERβ2 on hERα; B. Effect of hERβ5 on hERα. See text for statistical analysis.

Figure 9.

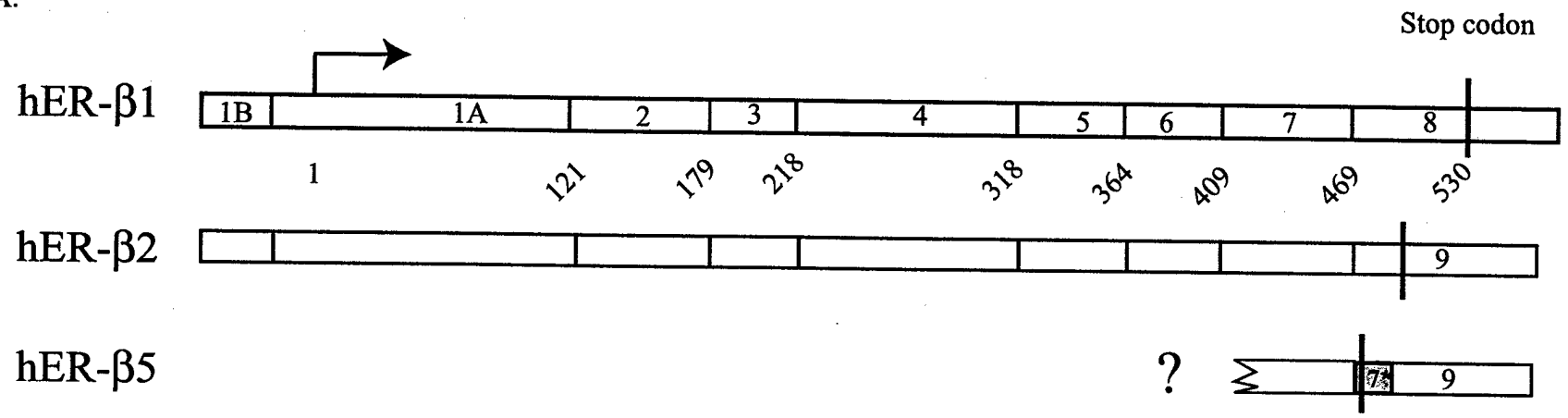
Transcriptional activity of hERα, hERβ1 and hERβ2 on a novel raloxifene responsive element regulated reporter gene, TGF-β3-CAT. Increasing amounts of hER isoform expression vectors were cotransfected with a TGF-β3-CAT reporter gene into Cos-1 cells. The transfected cells were treated for 48 hours with or without or the raloxifene analog LY117018. The results show the mean CAT activity after correction for β–galactosidase activity (transfection efficiency) ± SEM of 3 independent experiments. See text for statistical analysis.

Figure 10.

Effect of increasing amounts of coexpressed hERβ isoforms on the ability of hERα (50 ng) to activate transcription from a TGFβ3-CAT reporter gene in the presence and absence of the raloxifene analog LY117018 following co-transfection into Cos-1 cells. The results show the mean CAT activity after correction for β–galactosidase activity (transfection efficiency) ± SEM of 3 independent experiments. A. Effect of hERβ1 and hERβ2 on hERα; B. Effect of hERβ5 on hERα. See text for statistical analysis.

Figure 1

A.


Stop codon
hER-β1
1B
1A
2
3
4
5
6
7
8
1
121
179
218
318
364
409
469
530
hER-β2
9
hER-β5
?
7*
9

B.

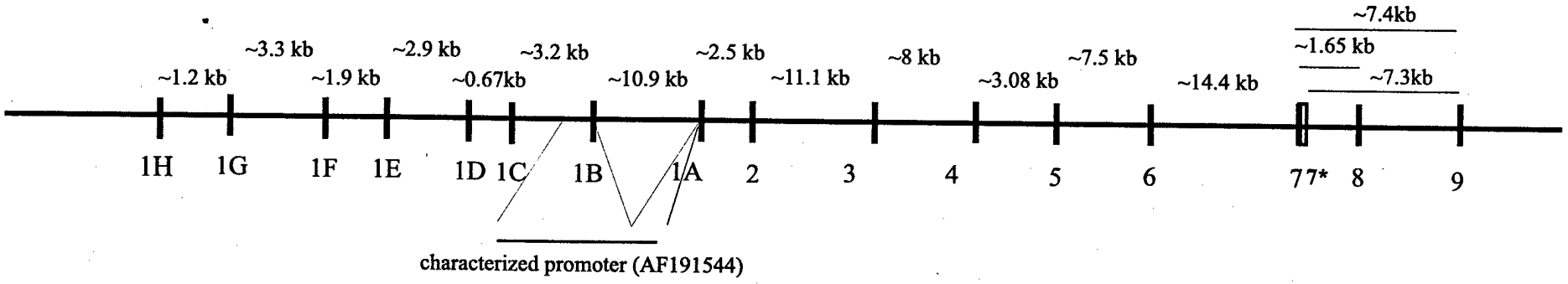

~7.4kb
~1.65 kb
~7.3kb
~3.3 kb
~2.9 kb
~3.2 kb
~2.5 kb
~8 kb
~7.5 kb
~1.2 kb
~1.9 kb
~0.67kb
~10.9 kb
~11.1 kb
~3.08 kb
~14.4 kb
1H
1G
1F
1E
1D
1C
1B
1A
2
3
4
5
6
7*
8
9
characterized promoter (AF191544)

Figure 2

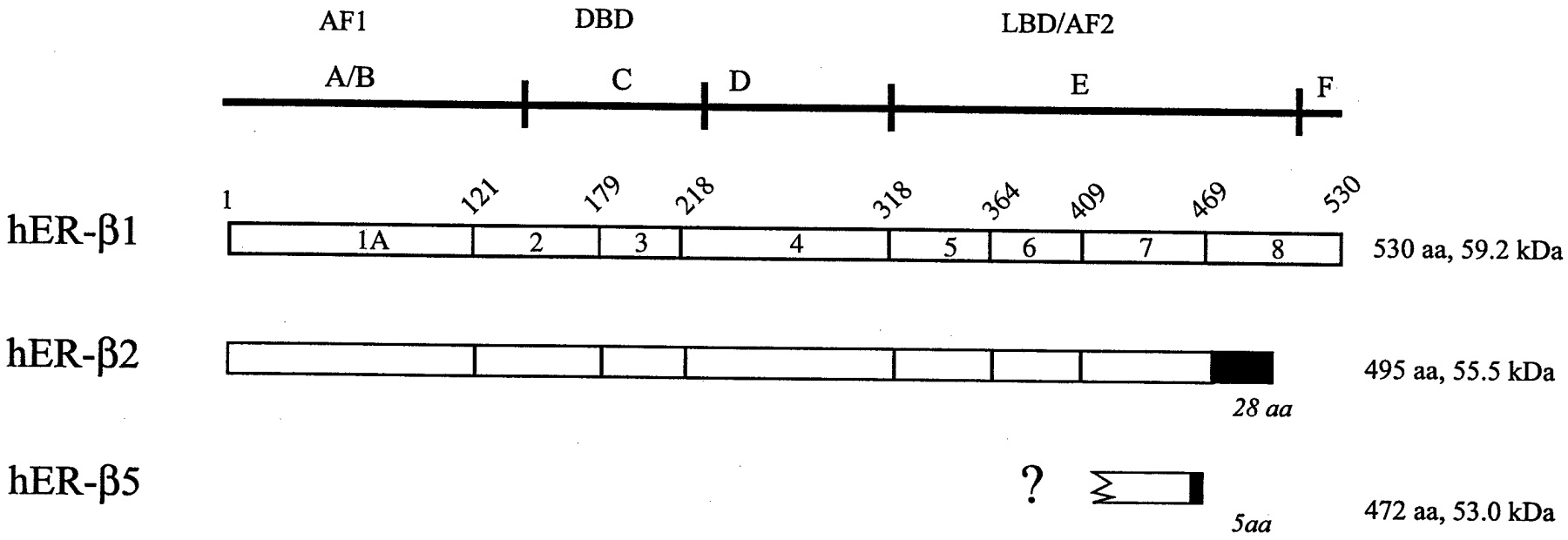
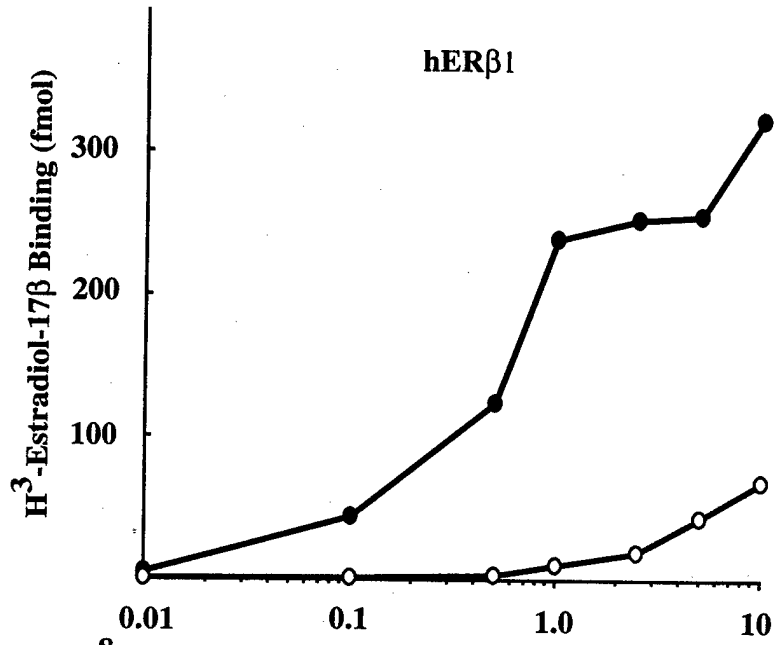
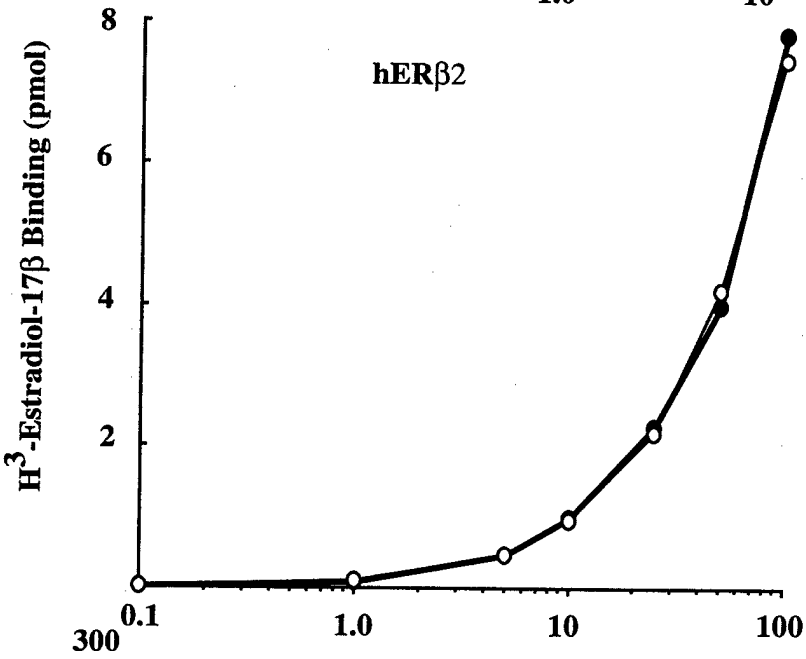

AF1
DBD
LBD/AF2
A/B
C
D
E
F
1
121
179
218
318
364
409
469
530
hER-β1
1A
2
3
4
5
6
7
8
530 aa, 59.2 kDa
hER-β2
495 aa, 55.5 kDa
28 aa
hER-β5
?
5aa
472 aa, 53.0 kDa

Figure 3

A.


hERβ1
H3-Estradiol-17β Binding (fmol)
300
200
100
0.01
0.1
1.0
10

B.


hERβ2
H3-Estradiol-17β Binding (pmol)
8
6
4
2
300
0.1
1.0
10
100

C.

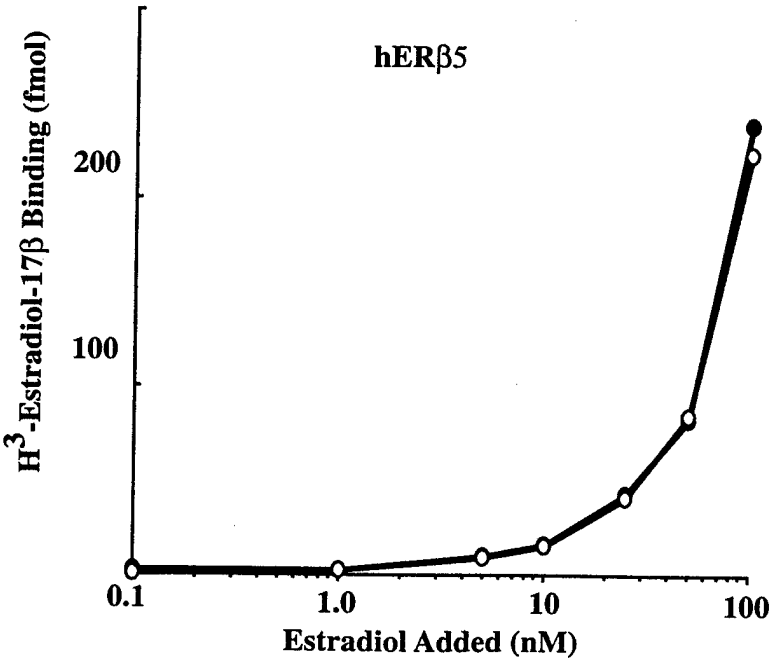

hERβ5
H3-Estradiol-17β Binding (fmol)
200
100
0.1
1.0
10
100
Estradiol Added (nM)

Figure 4

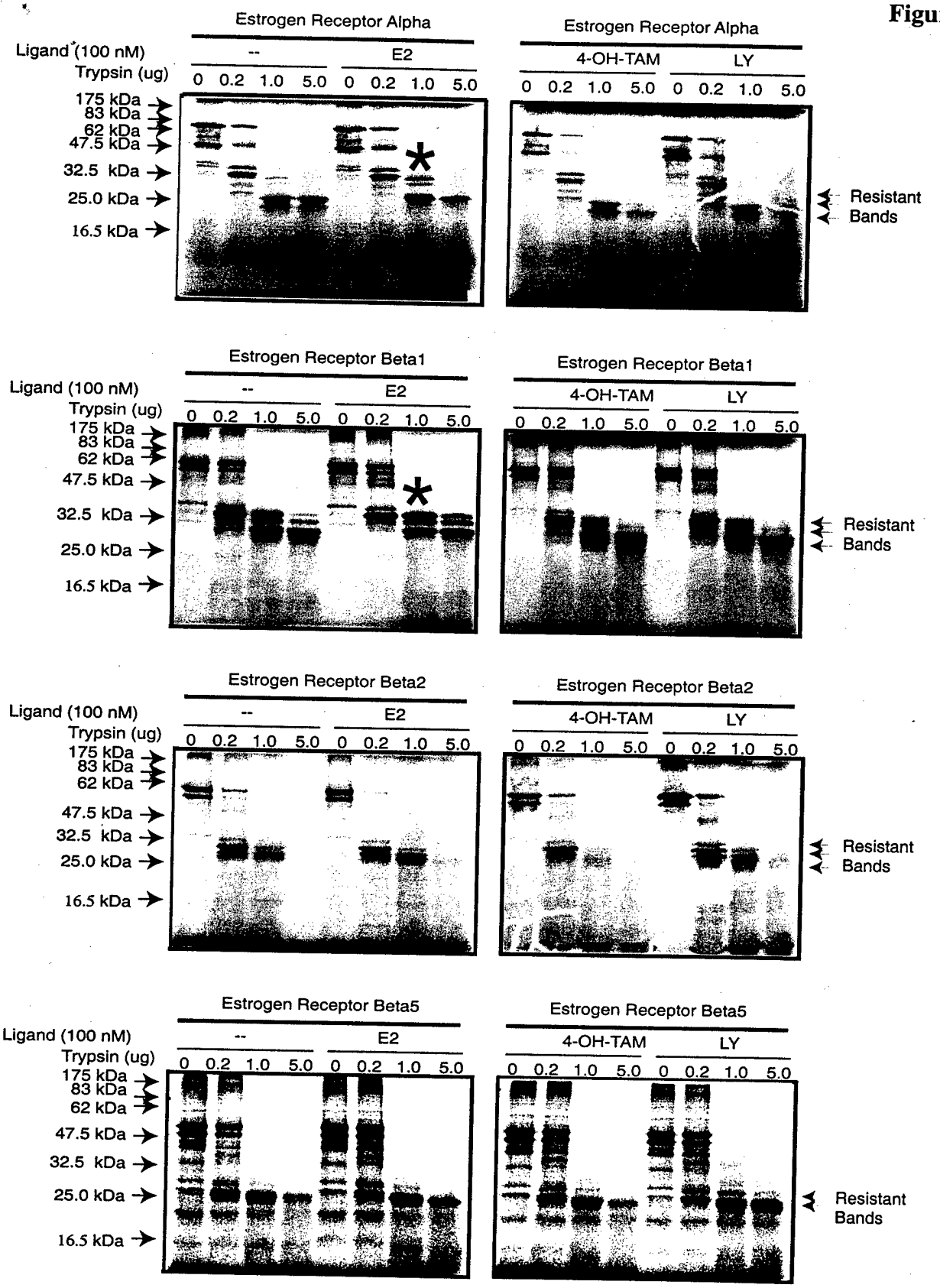

Estrogen Receptor Alpha
Ligand (100 nM)
--
E2
Trypsin (ug)
0 0.2 1.0 5.0 0 0.2 1.0 5.0
175 kDa
83 kDa
62 kDa
47.5 kDa
32.5 kDa
25.0 kDa
16.5 kDa
*
Estrogen Receptor Alpha
4-OH-TAM
LY
0 0.2 1.0 5.0 0 0.2 1.0 5.0
Resistant Bands
Estrogen Receptor Beta1
Ligand (100 nM)
--
E2
Trypsin (ug)
0 0.2 1.0 5.0 0 0.2 1.0 5.0
175 kDa
83 kDa
62 kDa
47.5 kDa
32.5 kDa
25.0 kDa
16.5 kDa
*
Estrogen Receptor Beta1
4-OH-TAM
LY
0 0.2 1.0 5.0 0 0.2 1.0 5.0
Resistant Bands
Estrogen Receptor Beta2
Ligand (100 nM)
--
E2
Trypsin (ug)
0 0.2 1.0 5.0 0 0.2 1.0 5.0
175 kDa
83 kDa
62 kDa
47.5 kDa
32.5 kDa
25.0 kDa
16.5 kDa
Estrogen Receptor Beta2
4-OH-TAM
LY
0 0.2 1.0 5.0 0 0.2 1.0 5.0
Resistant Bands
Estrogen Receptor Beta5
Ligand (100 nM)
--
E2
Trypsin (ug)
0 0.2 1.0 5.0 0 0.2 1.0 5.0
175 kDa
83 kDa
62 kDa
47.5 kDa
32.5 kDa
25.0 kDa
16.5 kDa
Estrogen Receptor Beta5
4-OH-TAM
LY
0 0.2 1.0 5.0 0 0.2 1.0 5.0
Resistant Bands

Figure 5

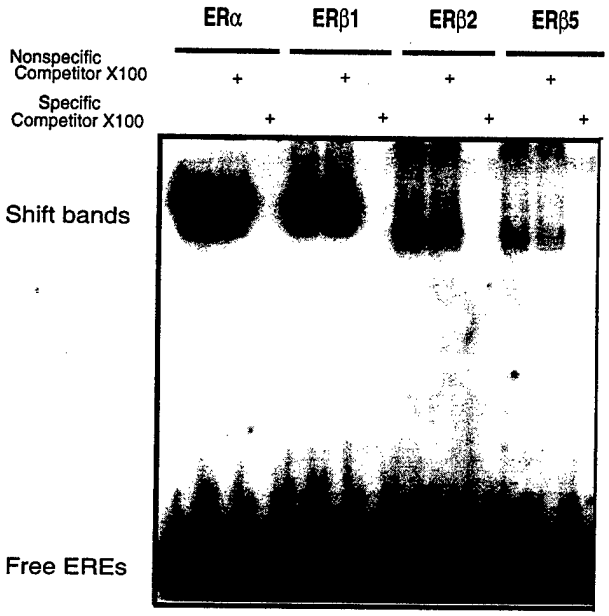

ERα
ERβ1
ERβ2
ERβ5
Nonspecific Competitor X100
Specific Competitor X100
+
+
+
+
+
+
+
+
Shift bands
Free EREs

Figure 6

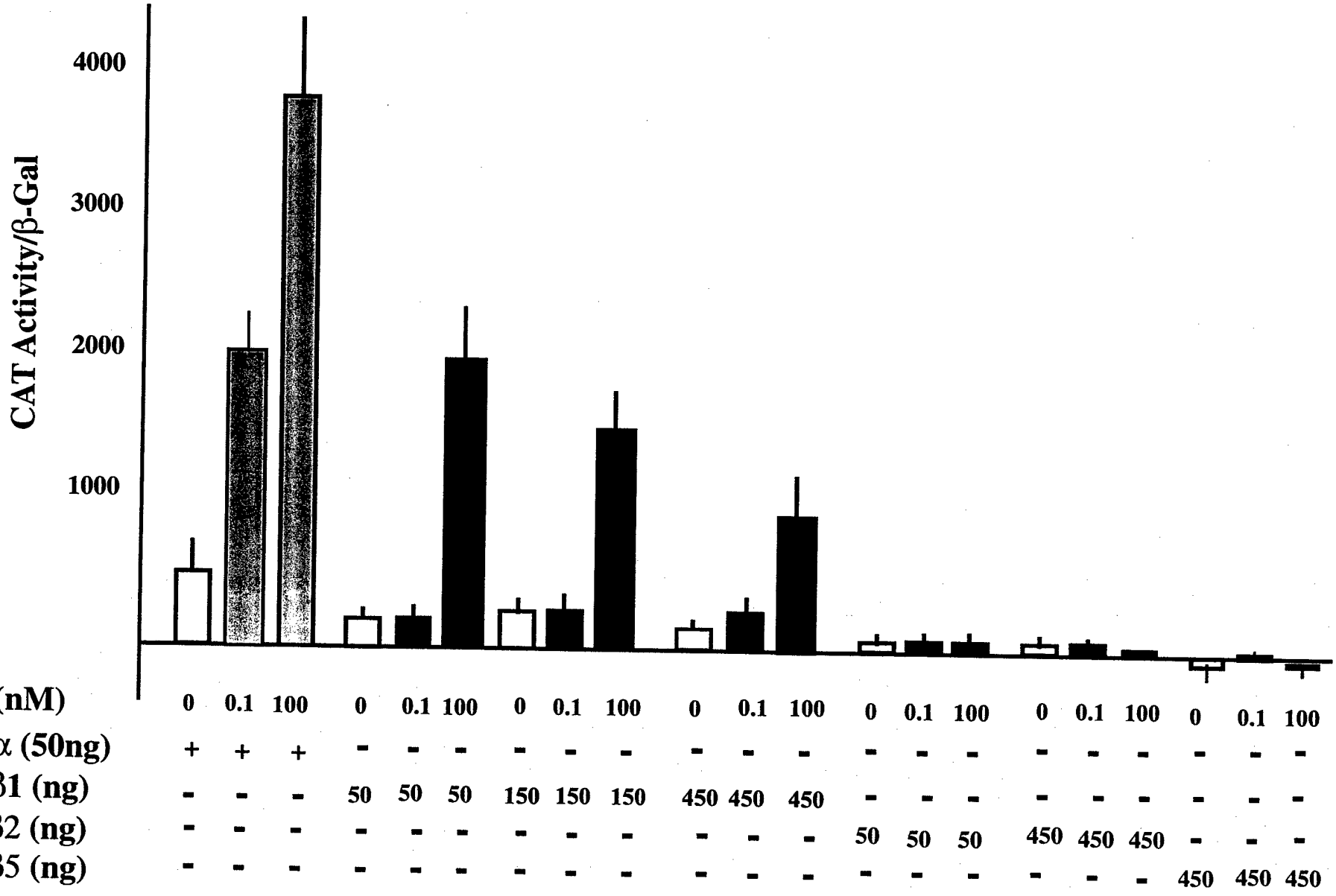

CAT Activity/β-Gal
4000
3000
2000
1000
E2 (nM)
0 0.1 100 0 0.1 100 0 0.1 100 0 0.1 100 0 0.1 100 0 0.1 100 0 0.1 100
ERα (50ng)
+ + + - - - - - - - - - - - - - - - - - -
ERβ1 (ng)
- - - 50 50 50 150 150 150 450 450 450 - - - - - - - - -
ERβ2 (ng)
- - - - - - - - - - - - 50 50 50 450 450 450 - - -
ERβ5 (ng)
- - - - - - - - - - - - - - - - - - 450 450 450

Figure 7.

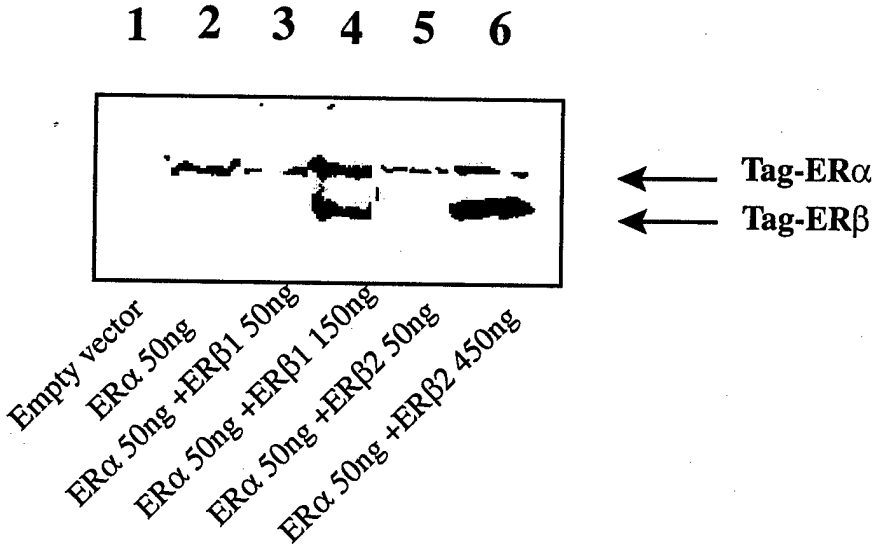

1 2 3 4 5 6
Tag-ERα
Tag-ERβ
Empty vector
ERα 50ng
ERα 50ng +ERβ1 50ng
ERα 50ng +ERβ1 150ng
ERα 50ng +ERβ2 50ng
ERα 50ng +ERβ2 450ng

Figure 8A.

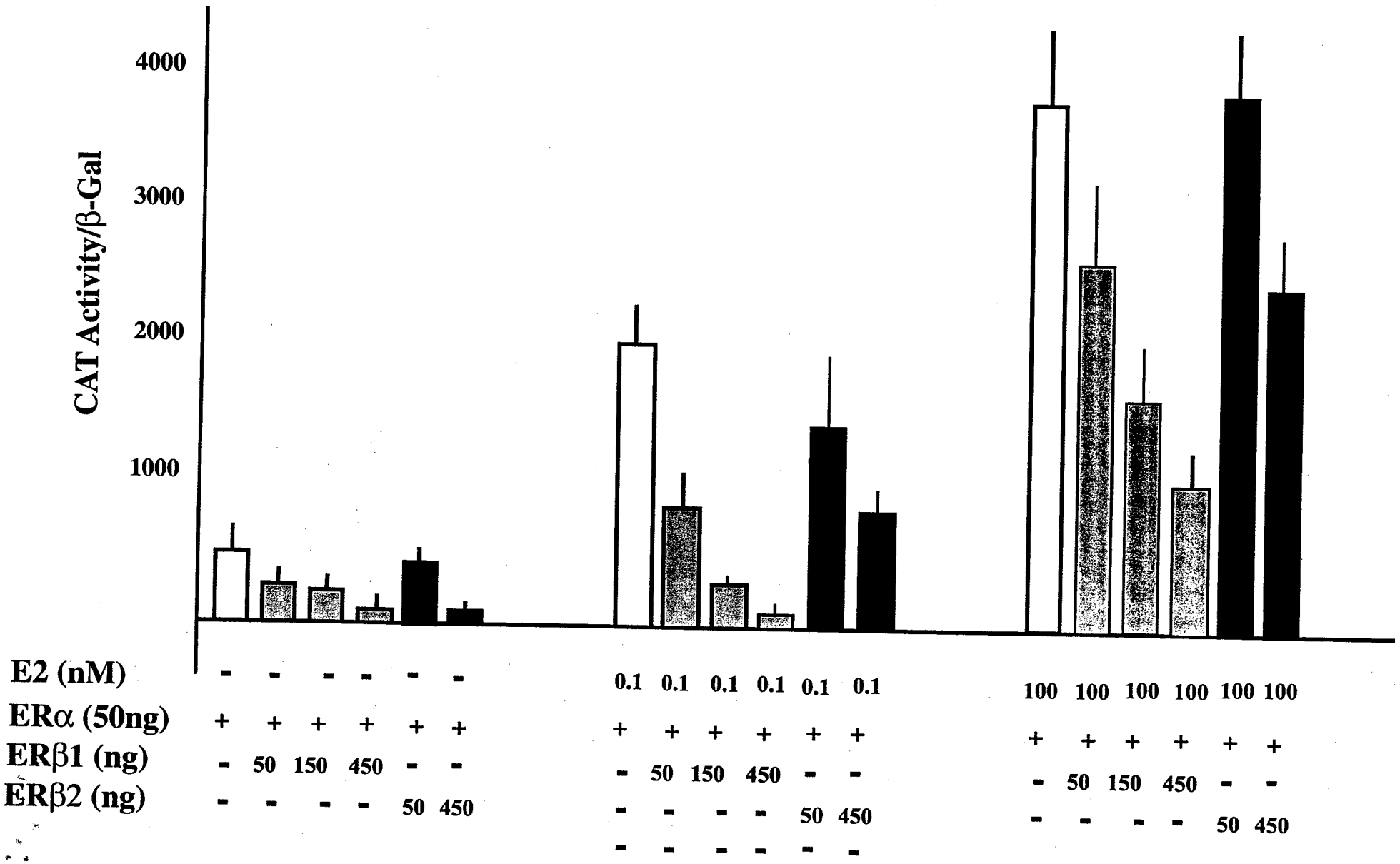

CAT Activity/β-Gal
4000
3000
2000
1000
E2 (nM)
ERα (50ng)
ERβ1 (ng)
ERβ2 (ng)
- - - - - -
+ + + + + +
- 50 150 450 - -
- - - - 50 450
0.1 0.1 0.1 0.1 0.1 0.1
+ + + + + +
- 50 150 450 - -
- - - - 50 450
100 100 100 100 100 100
+ + + + + +
- 50 150 450 - -
- - - - 50 450

Figure 8B

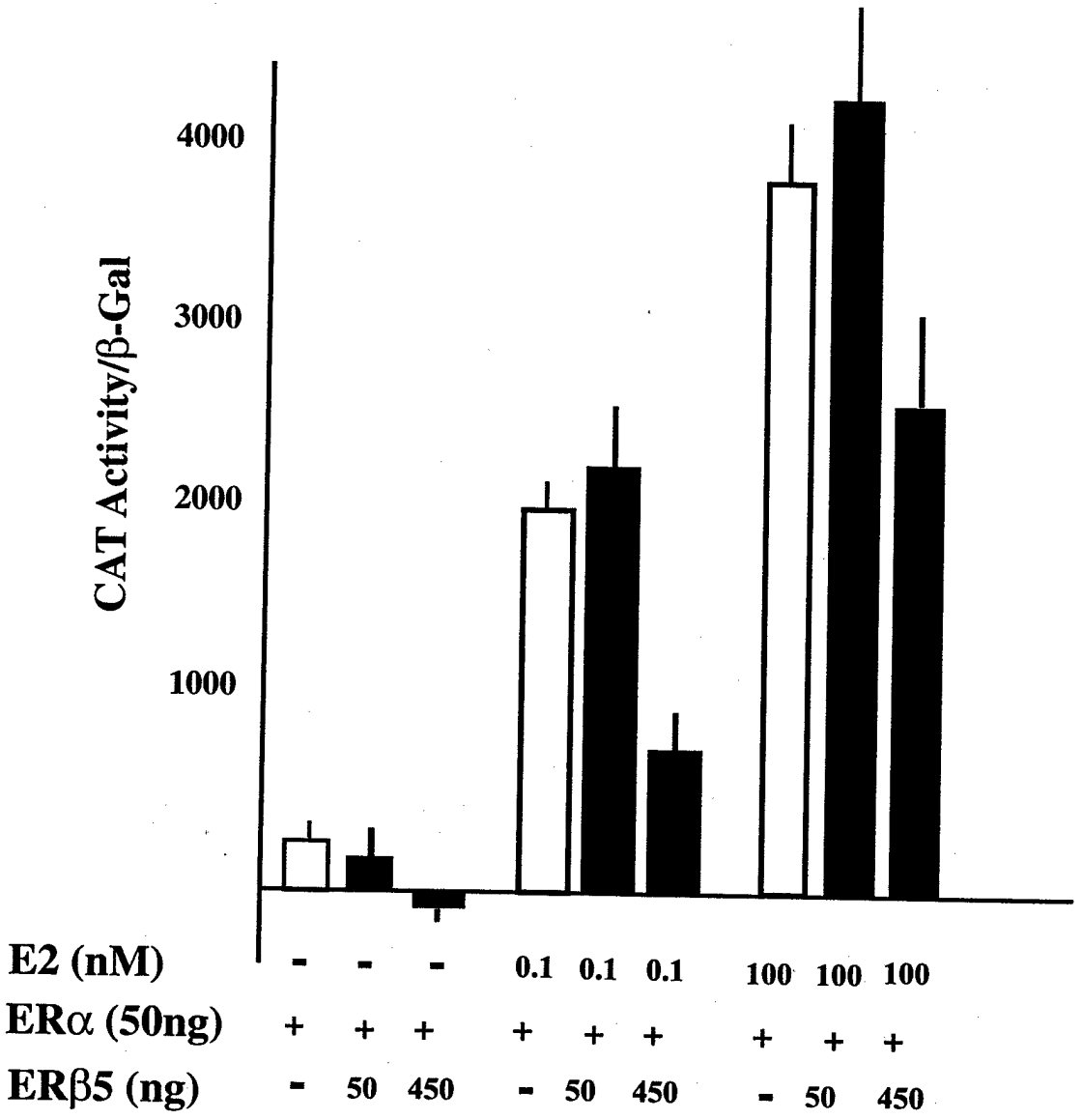

CAT Activity/β-Gal
4000
3000
2000
1000
E2 (nM) - - - 0.1 0.1 0.1 100 100 100
ERα (50ng) + + + + + + + + +
ERβ5 (ng) - 50 450 - 50 450 - 50 450

Figure 9

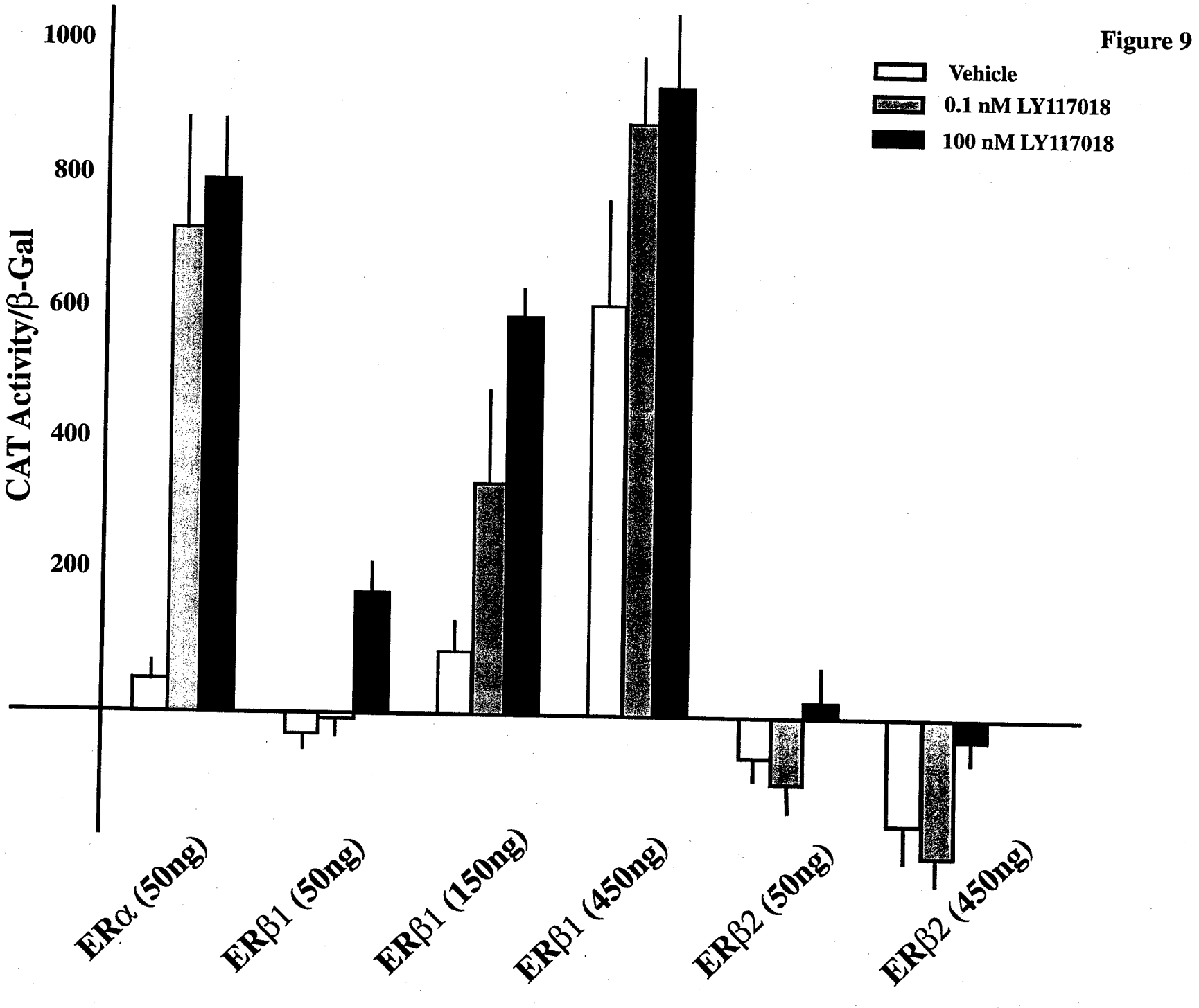

Vehicle
0.1 nM LY117018
100 nM LY117018
CAT Activity/β-Gal
1000
800
600
400
200
ERα (50ng)
ERβ1 (50ng)
ERβ1 (150ng)
ERβ1 (450ng)
ERβ2 (50ng)
ERβ2 (450ng)

Figure 10A

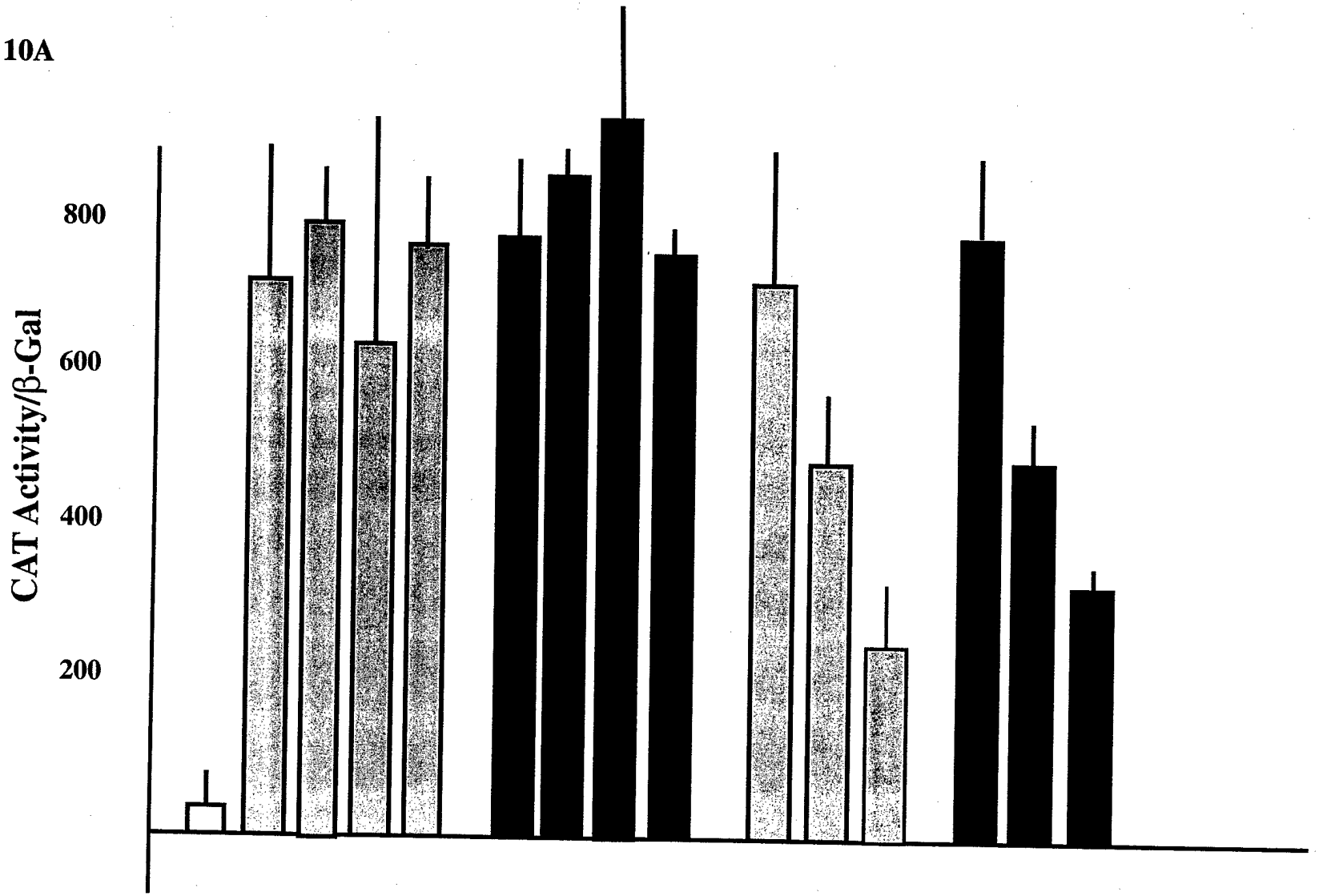
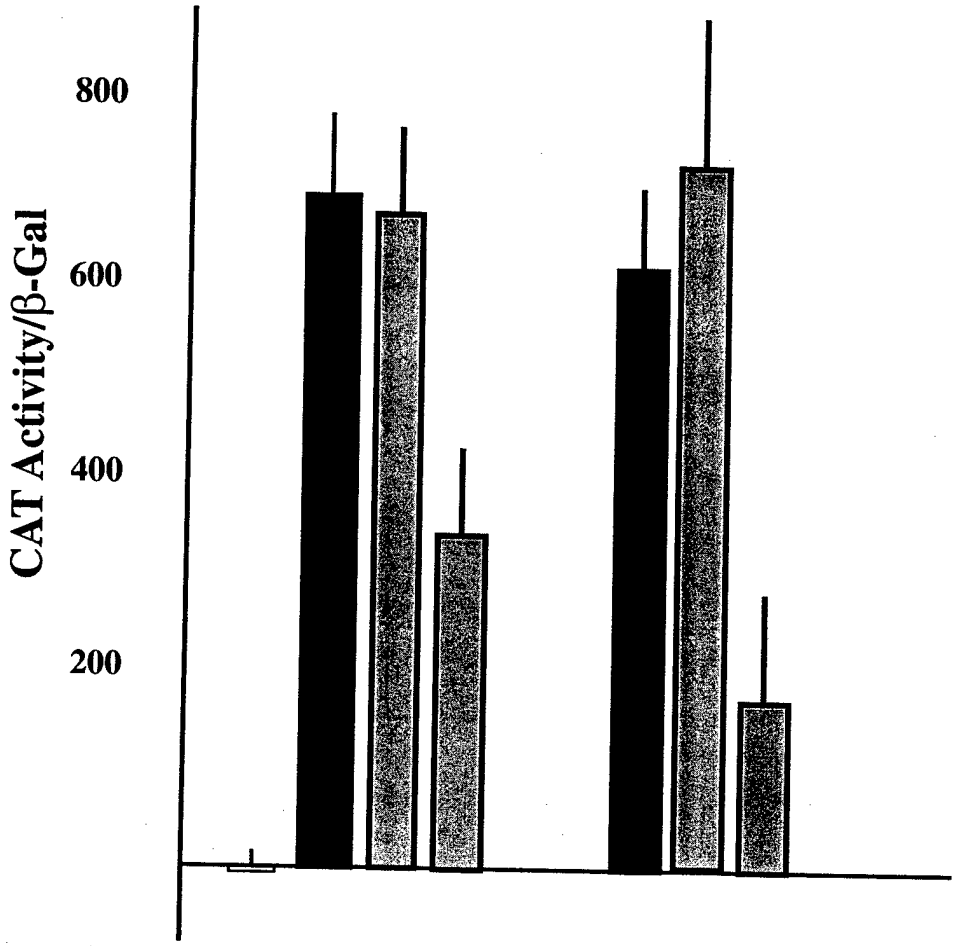

CAT Activity/β-Gal
800
600
400
200
LY (nM) 0 0.1 0.1 0.1 0.1 100 100 100 100 0.1 0.1 0.1 100 100 100
ERα (50ng) + + + + + + + + + + + + + + +
ERβ1 (ng) - - 50 150 450 - 50 150 450 - - - - - -
ERβ2 (ng) - - - - - - - - - - 50 450 - 50 450

Figure 10B


800
600
400
200
CAT Activity/β-Gal
LY (nM) 0 0.1 0.1 0.1 100 100 100
ERα (50ng) + + + + + + +
ERβ5 (ng) - - 50 450 - 50 450

APPENDIX 4
in press, Brit. J. Cancer

Relationship of co-regulator and estrogen receptor isoform expression to *de novo* tamoxifen resistance in human breast cancer.[1]

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and Peter H Watson.

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Running title: ER isoform and coregulator expression in primary breast tumors

Key words: SRA, AIB1, REA, co-regulators, ER isoforms, human breast cancer, tamoxifen.

Footnotes:

[1] This work was supported by grants from the Canadian Breast Cancer Research Initiative (CBCRI), the Canadian Institutes for Health Research (CIHR) and the U.S. Army Medical Research and Materiel Command (USAMRMC). P. H. W. is a recipient of a CIHR Scientist Award and a US Army Academic Award. E.L. has a USAMRMC Career Development Award.

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[3] The abbreviations used are: ER, estrogen receptor; PR, progesterone receptor; SRA, steroid receptor RNA activator; AIB1, amplified in breast cancer 1; REA, repressor of estrogen receptor activity.

Abstract

This study addresses the hypothesis that altered expression of estrogen receptor-beta and/or altered relative expression of coactivators and corepressors of estrogen receptors are associated with and may be mechanisms of *de novo* tamoxifen resistance in estrogen receptor positive breast tumors. All cases were ER+, node negative, primary breast tumors from patients who later had no disease progression (tamoxifen sensitive) or whose disease progressed while on tamoxifen (tamoxifen resistant). Using an antibody to estrogen receptor-beta that detects multiple forms of this protein (total) but not an antibody that detects only wild-type estrogen receptor-beta 1, it was found that high total ER beta protein expressors were more frequently observed in tamoxifen sensitive tumors than resistant tumors (Fishers exact test, P=0.046). However, no significant differences in the relative expression of ERβ2, ERβ5 and wild-type ERβ1 RNA in the tamoxifen sensitive and resistant groups were found. As well, when the relative expression of two known coactivators SRA and AIB1 RNA to the known corepressor, REA RNA, was examined, no significant differences between the tamoxifen sensitive and resistant groups was found. Altogether, there is little evidence for altered coregulators expression in breast tumors that are *de novo* tamoxifen resistant. However, our data provide preliminary evidence that the expression of ERβ protein isoforms may differ in primary tumors of breast cancer patients who prove to have differential sensitivity to tamoxifen therapy.

Introduction

The ability of anti-estrogens such as tamoxifen to compete with estrogens for binding to ER and to antagonize their mitogenic action provides the basic rationale for endocrine therapy and prevention (for a review see (1)) in breast cancer. Adjuvant tamoxifen post-operative therapy reduces the number of recurrences and prolongs survival in women whose primary tumors are ER positive (2). However, even though ER level is considered a marker for predicting the likelihood of responding to adjuvant hormonal therapies, some patients, whose primary tumors are ER positive do not respond to tamoxifen treatment. Such apparent *de novo* tamoxifen resistance does not depend upon the level of ER within the primary tumor. As well many of those patients whose disease initially responds to tamoxifen, progress under tamoxifen having acquired resistance to tamoxifen and this occurs despite continued expression of ER. Thus suggesting other components of the

estrogen signaling pathway may be altered. Recent observations using laboratory models (3-6) have demonstrated that altered levels of ER isoforms and/or alteration of expression of co-activators and co-repressors can result in altered estrogen and antiestrogen activity in target cells, suggesting the hypothesis that altered levels of ER isoforms and/or co-regulators *in vivo* could be a mechanism of tamoxifen resistance. Previously we have demonstrated that the relative expression of ERα/ERβ as well as the relative expression of some ER coactivators to corepressors is significantly altered during breast tumorigenesis *in vivo* (7, 8). Furthermore, since these alterations parallel the marked changes in estrogen action that accompany breast tumorigenesis, they may have a role in this process. To explore the hypothesis that such changes could underlie *de novo* tamoxifen resistance *in vivo* the expression of ER isoforms, two known co-activators (steroid receptor RNA activator, SRA, (4) and amplified in breast cancer-1, AIB1 (9)) and one co-repressor (repressor of estrogen receptor activity, REA (5)) of ER activity have been investigated in primary breast tumors from node negative patients whose tumors were ER positive and that subsequently responded or had disease progression while on adjuvant tamoxifen therapy.

Materials and methods

Human breast tumors.

All breast tumor cases used for this study were selected from the NCIC-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). As previously described (10), tissues are accrued to the Bank from cases at multiple centers within Manitoba, rapidly collected and processed to create matched formalin-fixed-embedded and frozen tissue blocks for each case with the mirror image surfaces oriented by colored inks. The histology of every sample in the Bank is uniformly interpreted by a pathologist in Hematoxylin/Eosin (H&E) stained sections from the face of the paraffin tissue block. This information is available in a computerized database along with relevant pathological and clinical information and was used as a guide for selection of specific paraffin and frozen blocks from cases for this study. For each case, interpretations included an estimate of the cellular composition (including the percentage of invasive epithelial tumor cells and stroma), tumor type and tumor grade (Nottingham score). Steroid receptor status was determined for all cases by ligand binding assay performed on an adjacent portion of tumor tissue. Tumors with estrogen receptor levels above 3 fmol/mg of

total protein were considered ER positive.

To identify cases that responded divergently to tamoxifen, review of approximately 1000 consecutive cases was undertaken to identify cases that were ER positive, node negative and that had been treated with adjuvant tamoxifen following surgery +/- local radiation. From these the first cohort of 27 cases was selected to include a subset (n=13) that had shown progression of disease (either died or alive with recurrent disease, referred to as tamoxifen resistant cases) and a similar control subset (n=14) specifically selected to comprise cases with similar lengths of follow-up (resistant 34 versus sensitive 39 months), ER status, tumor grade and tumor histology, but that had shown no progression of disease (referred to as tamoxifen sensitive cases). The tumor characteristics were: 1) 'Tam Sensitive' group median ER was 60.5 fmol/mg protein (range 6 –146 fmol/mg protein), median PR was 32 fmol/mg protein (range 8 – 216 fmol/mg protein); median grade was 5 (range 4-8); median age at biopsy was 69 years (range 35-87 years); median follow-up time was 56 months (range 18- 79); 2) Tam Resistant' group median ER was 57 fmol/mg protein (range 4 – 136 fmol/mg protein); median PR was 14 fmol/mg protein (range 4 – 288 fmol/mg protein); median grade was 6 (range 4-9); median age at biopsy was 67 years (range 49-83 years); median follow-up time was 56 months (range 9 - 85).

For the RNA studies, frozen tissue corresponding to the blocks for several of the first cohort of older cases used above, were not available. Therefore a second study cohort was selected that had frozen tissue available. The relevant patient/tumor characteristics were similar to the above cohort, although the follow-up time was shorter: 1) 'Tam Sensitive' group (n=16) median ER was 37.5 fmol/mg protein (range 4.4 –146 fmol/mg protein), median PR was 44 fmol/mg protein (range 13.1 – 216 fmol/mg protein); median grade was 6 (range 4-9); median age at biopsy was 72 years (range 47-87 years); median follow-up time was 39 months (range 13- 76); 2) Tam Resistant' group (n=16) median ER was 21.5 fmol/mg protein (range 5.6 – 107 fmol/mg protein); median PR was 14.3 fmol/mg protein (range 7.8 – 288 fmol/mg protein); median grade was 6 (range 4-9); median age at biopsy was 71 years (range 60-89 years); median follow-up time was 34 months (range 9 - 63).

Immunohistochemistry. Immunohistochemistry was performed on serial 5 μm sections from a representative, formalin fixed paraffin embedded archival tissue block from each tumor. Immunohistochemical

staining for ERβ was performed using two different primary antibodies. IgYERB503 (a gift from Dr Jan-Ake Gustafson) detects total ERβ isoforms (11, 12) and GC17 (a gift from Dr Shuk-Mei Ho) detects only the wild-type ERβ (13). Antibodies were applied using an automated tissue immunostainer (Discovery module, Ventana Medical Systems, Phoenix, AZ, U.S.A.), DAB immunohistochemistry kit and bulk reagents that were supplied by the manufacturer. Briefly, the Discovery staining protocol was set to "Standard Cell Conditioning" procedure, followed by 12 hours incubation with primary antibody and 32 minutes incubation with secondary antibody. Concentrations of primary antibodies initially applied to the Ventana instrument were 1:200 for IgYERB503 and 1:50 for GC17, which translates into final concentrations of 1:600 and 1:150 after a 1:3 dilution with buffer dispensed onto the slide with the primary antibody. Levels of nuclear ERβ expression were scored semi-quantitatively, under the light microscope. Scores were obtained by estimating average signal intensity (on a scale of 0 to 300) and the proportion of epithelial cells showing a positive signal (0, none; 0.1, less than one tenth; 0.5, less than one half; 1.0 greater than one half). The intensity and proportion scores were then multiplied to give an overall H-score. Cases with a score lower than or equal to 100 were considered negative or weakly positive, whereas tumors with scores higher than 100 were classified as positive for ERβ expression (14).

RNA Extraction and RT-PCR conditions. Total RNA was extracted from 20 μm frozen tissue sections (20 sections per tumor) using Trizol™ reagent (Life Technologies, NY) according to the manufacturer's instructions and quantified spectrophotometrically. One μg of total RNA was reverse transcribed in a final volume of 25 μl as previously described (15).

Primers and PCR conditions.

Coregulators: The primers used were: SRAcoreU primer (5'- AGGAACGCGGCTGGAACGA -3'; sense; positions 35-53, Genbank accession number AF092038) and SRAcoreL primer (5'- AGTCTGGGGAACCGAGGAT -3'; antisense; position 696-678, Genbank accession number AF092038); AIB1-U primer (5'- ATACTTGCTGGATGGTGGACT - 3'; sense; positions 110-130, Genbank accession number AF012108) and AIB1-L primer (5'- TCCTTGCTCTTTTATTTG ACG -3'; antisense; positions 458 - 438, Genbank accession number AF012108);

REA-U primer (5'- CGAAAAATCTCCTCCCCTACA - 3'; sense; positions 385-405, Genbank accession number AF150962) and REA-L primer (5'- CCTGCTTTGCTTTTTCTACCA -3'; antisense; positions 781-761, Genbank accession number AF150962).

Radioactive PCR amplifications for SRA were performed and PCR products analyzed as previously described (16) with minor modifications. Briefly, 1 μl of reverse transcription mixture was amplified in a final volume of 15 μl, in the presence of 1.5 μCi of (α-^{32}P) dCTP (3000 Ci/mmol), 4 ng/μl of each primer and 0.3 unit of Taq DNA polymerase (Gibco BRL, Grand Island, NY). For SRA each PCR consisted of 30 cycles (30 sec at 94°C, 30 sec at 60°C and 30 sec at 72°C). PCR products were then separated on 6% polyacrylamide gels containing 7M urea. Following electrophoresis, the gels were dried and exposed 2 hours to a Molecular Imager™-FX Imaging screen (Bio-Rad, Hercules, CA).

PCR amplifications for AIB1 and REA were performed and PCR products analyzed as previously described (15) with minor modifications. Briefly, 1 μl of reverse transcription mixture was amplified in a final volume of 20 μl, in the presence of 4 ng/μl of each primer and 0.3 unit of Taq DNA polymerase (Gibco BRL, Grand Island, NY). For AIB1, each PCR consisted of 30 cycles (30 sec at 94°C, 30 sec at 55°C and 30 sec at 72°C). For REA each PCR consisted of 30 cycles (30 sec at 94°C, 30 sec at 57°C and 30 sec at 72°C). PCR products were then separated on agarose gels stained with ethidium bromide as previously described (15).

Primers for ER isoforms:

ERα–U primer (5'-TGTGCAATGACTATGCTTCA-3'; sense; located in ERα 792-811) and ERα–L primer (5'-GCTCTTCCTCCTGTTTTTA-3'; antisense; located in ERα 940-922). Nucleotide positions given correspond to published sequences of the human ERα cDNA (17). PCR amplifications were performed and PCR products analyzed as previously described with minor modifications (18). Briefly, 1 μl of reverse transcription mixture was amplified in a final volume of 15 μl, in the presence of 1 μCi (α-^{32}P) dCTP (3000 Ci/mmol), 2 ng/μl of ERα–U/ERα–L and 0.3 unit of Taq DNA polymerase (Gibco BRL, Grand Island, NY). Each PCR consisted of 30 cycles (30 sec at 94°C, 30 sec at 60°C and 30 sec at 72°C).

A previously validated triple primer assay was used to determine the relative expression of ERβ1 and its variant isoforms ERβ2 and ERβ5 (19). Briefly, 1 μl of reverse transcription mixture was amplified in a final

volume of 15 µl, in the presence of 1 µCi of (α-^{32}P) dCTP (3000 Ci/mmol), 4 ng/µl of each primer (ERβ1U, ERβ1L and ERβ2L) and 0.3 unit of Taq DNA polymerase (Gibco BRL, Grand Island, NY).

All ER PCRs consisted of 30 cycles (30 sec at 94°C, 30 sec at 60°C, and 30 sec at 72°C). PCR products were then separated on 6% polyacrylamide gels containing 7M urea. Following electrophoresis, the gels were dried and autoradiographed. Three independent PCRs were performed.

Quantification of SRA and ER RNA expression. Exposed screens were scanned using a Molecular Imager™-FX (Bio-Rad, Hercules, CA) and the intensity of the signal corresponding to SRA or the appropriate ER isoform PCR fragments was measured using Quantity One™ software (Bio-Rad, Hercules, CA). Three independent PCRs were performed. In order to control for variations between experiments, a value of 1 was arbitrarily assigned to the signal of one particular tumor measured in each set of PCR experiments (always the same tumor) and all signals were expressed relative to this signal. Levels of SRA was expressed relative to REA (SRA/REA), AIB1 (SRA/AIB1) or ERα (SRA/ERα) in each individual tumor sample. Levels of ERβ isoforms were expressed relative to other ERβ isoforms shown under statistical analysis and as previously described (19).

Quantification of the relative expression of the deleted SRA variant RNA. It has previously been shown that the co-amplification of a wild-type and a deleted variant SRA (SRA-Del) cDNA resulted in the amplification of two PCR products, the relative signal intensity of which provided a reliable measurement of the relative expression of the deleted variant (16). For each sample, the signal corresponding to the SRA-Del was measured using Quantity One™ software (Bio-Rad, Hercules, CA) and expressed as a percentage of the corresponding core SRA signal. For each case, 3 independent assays were performed and the mean determined.

Quantification of REA and AIB1 RNA expression. Following analysis of PCR products on prestained agarose gels, signals were quantified by scanning using MultiAnalyst™ (Bio-Rad, Hercules, CA). At least, three independent PCRs were performed. A value of 1 was arbitrarily assigned to the REA or AIB1 signal of the one particular tumor and is the same tumor as described above and all signals were expressed relative to

this signal. Levels of AIB1 were expressed relative to REA (AIB1/REA) and ERα (AIB1/ERα), and levels of REA were expressed relative to ERα (REA/ERα).

Statistical analysis. Differences between tamoxifen sensitive and tamoxifen resistant cases were tested using the Mann-Whitney rank sum test, two-tailed. Potential differences in expression between the two groups with respect to each ERβ isoform RNA relative to other ERβ isoform RNA expression (e.g. log ERβ1/total ERβ; log ERβ2/total ERβ; log ERβ5/total ERβ, as previously described (19)) , and the relative expressions of co-regulators (i.e. logAIB1/REA; logSRA/REA; logSRA/AIB1; logAIB1/ERα ; logSRA/ERα ; logREA/ERα) were determined.

Tumors were classified as low (scores between 0 and 100) and high (150 to 300) ERβ expressors, and differences between tamoxifen sensitive and tamoxifen resistant cases were tested using Fisher's exact test. Correlation between ERβ protein expression (H-score) and tumor characteristics was tested by calculation of the Spearman coefficient r.

Results.

Expression of ERβ protein in primary human breast tumors from patients who later progressed on tamoxifen treatment or showed no progression on tamoxifen treatment. ERβ protein was determined immunohistochemically on adjacent sections from each tumor, using two different antibodies. GC-17 is an antibody recognizing an epitope in the C-terminus of full-length ERβ1 (13). Normal breast tissue was used as a positive control and is shown in Figure 1A. Examples of staining in human breast tumor sections are shown in Figure 1B to D. Some tumor sections showed no (Figure 1B, wild-type ERβ score = 0) or low (Figure 1C, wild-type ERβ score = 100), while others showed strong wild-type ERβ signals (Figure 1D, wild-type ERβ score = 300). Tumors were classified as low (scores between 0 and 100) and high (150 to 300) wild-type ERβ protein expressors, and differences between between tamoxifen sensitive and resistant tumors determined by Fishers exact test. No significant differences were found.

IgYERbeta503 is an antibody that recognizes all known putative ERβ protein isoforms (11, 12) and which we refer to as total ERβ protein. Normal breast tissue was used as a positive control and is shown in

Figure 2A. Examples of staining with this antibody in human breast tumor sections are shown in Figure 2 B to D. Some sections showed no (Figure 2B, total ERβ score = 0) or low (Figure 2C, total ERβ score = 100) total ERβ expression whereas others had strong total ERβ protein signal (Figure 2D, total ERβ score = 300). Tumors were classified as low and high total ERβ protein expressors, and there was a statistically significant difference in high total ERβ protein between the Tamoxifen sensitive and resistant groups (Fishers exact test, P=0.046). High total ERβ protein expressors were more frequently observed in tamoxifen sensitive tumors than resistant tumors.

Correlation between ERβ protein expression and tumor characteristics was tested by calculation of the Spearman coefficient. A positive correlation between ERβ1 (GC17) protein and PR levels (Spearman r=0.44, P=0.022) was found when each was examined as continuous variables. When tumors were divided into PR+ (> 10 fmol/mg protein) or PR- (≤ 10 fmol/mg protein) groups was used there was a significantly higher level of ERβ1 (GC17) protein in PR+ tumors compared to PR- tumors (Mann-Whitney test, P = 0.0268; median for PR+ tumors = 55, range 5 to 150 and median for PR- tumors = 10, range 0 to 75). As well, there was also a significantly higher level of total ERβ (IgY503) protein in PR+ tumors compared to PR- tumors (Mann-Whitney test, P = 0.0085; median for PR+ tumors = 125, range 25 to 270 and median for PR- tumors = 50 range 0 to 100).

Relative expression of ERβ isoform RNA in primary human breast tumors from patients who later progressed on tamoxifen treatment or showed no progression on adjuvant tamoxifen. To determine if the differences described above in ERβ protein expression were correlated with differences in ERβ variant isoform RNA expression, we compared the relative expression of ERβ variant RNA to wild-type ERβ RNA in the tamoxifen sensitive and resistant groups. Unfortunately, frozen tissue samples corresponding to some of the paraffin blocks from patients in the cohort used for immunohistochemistry were not available. The tumor bank was queried and additional cases selected as described in Materials and Methods. Using previously validated assays (7, 19) the relative expression of ERβ2, ERβ5 and wild-type ERβ1 RNA in the tamoxifen sensitive and resistant groups was not significantly different.

Relative expression of coregulators in primary human breast tumors from patients who later progressed on tamoxifen treatment or showed no progression on tamoxifen treatment. To address the hypothesis that altered relative expression of steroid receptor coactivators and corepressors could underly altered tamoxifen sensitivity in human breast tumors, and since we previously showed that the relative expression of two coactivators (SRA and AIB1) to a corepressor (REA) is altered in ER+ breast tumors compared their adjacent normal breast tissue, we chose these coregulators to study. They were measured by RT-PCR in the above tumor cohorts. SRA, AIB1, and REA mRNAs were detectable in most samples, even though their level of expression differed from one sample to another. Consistent with our previous results (16), an additional fragment, migrating at an apparent size of 459 bp was also observed in most tumors when using SRA specific primers. Sequencing analysis revealed that this band corresponded to a variant form of SRA (referred to as SRA-Del) deleted in 203 bp between positions 155 and 357 (positions given correspond to Genbank accession number AF092038). There were no significant differences between the tamoxifen sensitive and the *de novo* tamoxifen resistant breast cancers in the relative expression of any of the coactivators to corepressor RNA, or in the relative expression of SRA/AIB1 RNA, or in expression of any of these coregulator RNAs relative to ERα or total ERβ RNA expression. As well, there was no significant difference in the relative expression of variant SRA/wild-type SRA between the groups either.

Tumor Characteristics. No statistically significant differences were found between the tamoxifen sensitive and tamoxifen resistant cohorts in any of the tumor characteristics described in the Materials and methods section except for PR. PR levels were statistically significantly different (P=0.044) between the two groups using a Mann-Whitney rank sum test (two sided). PR levels were higher (median PR was 32 fmol/mg protein; range 8 – 216 fmol/mg protein) in the tamoxifen 'sensitive' group compared to the tamoxifen '*de novo* resistant' group (median PR was 14 fmol/mg protein; range 4 – 288 fmol/mg protein). This was a consistent finding in both selected cohorts (that used for immunohistochemistry and that used for the RNA study), and provides strong support for differences in estrogen signaling pathways in these two groups since PR is a marker of ER signal transduction (20, 21).

Discussion

We and others have shown that the relative expression of ERα and ERβ is significantly altered during breast tumorigenesis (7, 22), and a similar mechanism has been proposed to underlie tamoxifen resistance in breast cancers (23). The current study shows no significant differences in expression of wild-type ERβ (ERβ1) between tamoxifen sensitive and resistant tumors. Interestingly, in this small cohort of tumors when total ERβ expression was examined, there were significantly more high total ERβ expressors in the tamoxifen 'sensitive' compared to the 'resistant' group. The data suggest the possibility that increased and altered ERβ isoform protein expression may have a role in *de novo* tamoxifen resistance, or at least together with other parameters may provide better markers of endocrine sensitivity. The increased expression of ERβ proteins in the tamoxifen sensitive group is also consistent with recently published data where patients with ERβ positive tumors (determined using an antibody to an N-terminal epitope of the ERβ protein, and defined as nuclear staining in > 10% of cancer cells) had a significantly better overall survival than patients with ERβ negative tumors while receiving adjuvant tamoxifen therapy (24). Both these latter data and those presented currently in this manuscript are in contrast to data showing increased ERβ RNA expression in tamoxifen resistant tumors versus tamoxifen sensitive tumors previously published (25). Together these studies suggest that the ERβ status and the nature of ERβ isoforms together with ERα status in human breast cancers may be important biomarkers on endocrine sensitivity, and warrants further study, in larger, clinical trials cohorts. The association of increased ERβ isoform expression with tamoxifen sensitivity, suggests a possible mechanistic role, and one possible mechanism may be suggested by several publications which have shown that ERβ isoforms have a modulatory effect on ERα, both in normal tissues (26) as well as in cell culture models (6, 27).

Interestingly, if one examines previous studies in which ERβ protein expression was investigated in breast tumors immunohistochemically, marked differences in correlations of ERβ protein with various clinical and pathological parameters have been documented (24, 28-30). Much of the difference seen, can be related to the antibody used and often if only the wild-type ERβ was detected or if total (wild-type plus variant) ERβ isoforms were likely detected. In our hands measurement of wild-type ERβ protein expression was

positively correlated with PR levels when measured as continuous variables as well as when a cut-off value for PR positivity was used. Total ERβ protein expression was correlated with PR levels only when a cut-off values for PR positivity was used. This is a reasonably consistent finding in the literature usually when only wild-type ERβ is measured (24, 28, 30, 31), although recently the same relationship has been observed using an antibody recognizing an N-terminal epitope and therefore total ERβ protein expression (30), as well as in the current study. Indeed most of these studies also find that wild-type ERβ expression is correlated with both ER and PR. The current study did not find a correlation with ER levels, which is likely due to the selection criteria for our tumor cohorts, retrospectively, to be node negative and ER+ tumors and then to have either no disease recurrence while on tamoxifen treatment or to have died or had disease recurrence/progression while on tamoxifen treatment. Indeed the tumors in the two cohorts had no significant differences in level of ER expression as determined by ligand binding. The data in this current study are in contrast with our previous observations (32) that total ERβ RNA expression is inversely correlated with PR expression and progestins downregulate total ERβ RNA levels (32). However, our previous data were measured in tumors that ranged widely in ER/PR status and included ER-/PR+ and ER-/PR- tumors (32), whereas all the tumors in the current study are ER+, the majority are PR+, as well and none are ER-/PR-.

The potential differences between tamoxifen sensitive and resistant groups with respect to ERβ–like proteins, was not correlated with differences in the relative expression of wild type ERβ and two known variants ERβ2 and ERβ5 at the RNA level between the tamoxifen 'sensitive' versus the tamoxifen 'resistant' groups, however. This may due to differential regulation of protein versus RNA level or the likelihood that there are other potential ERβ isoforms (known and unknown) expressed in breast tissues in addition to ERβ1, ERβ2 and ERβ5 (33, 34), whose cognate proteins would be detected by the antibody but not measure in the triple primer RT-PCR assay.

Another mechanism for differential tamoxifen sensitivity in ER+ breast tumors could be altered coregulator expression. Since we previously had shown differential expression of some coregulators during breast tumorigenesis in ER+ tumors, a semi-quantitative reverse-transcription polymerase chain reaction (RT-

PCR) assay was used to determine the relative expression of ER co-regulators SRA, AIB1 and REA in the primary breast tumors of patients who, following tamoxifen adjuvant therapy, relapsed or remained disease free. To our knowledge, no study has previously compared the expression of these co-regulators (SRA, AIB1 and REA) in such cohorts. No significant differences were observed in the ratios of any of the coactivators /corepressors or any of the ratios of these coregulators to ERα RNA levels between the two breast tumor groups, from patients who were later found to be disease free (sensitive) or have disease progression (resistant) while on adjuvant tamoxifen treatment. These data suggest that altered relative expression of these coregulators is unlikely to be a marker of tamoxifen sensitivity in ER+, node negative, primary breast tumors, and unlikely to have a functional role in *de novo* tamoxifen resistance. Although SRA is functional as an RNA molecule, REA and AIB1 are functional as proteins. Furthermore, other factors can affect protein activity for example phosphorylation in the case of AIB1 (35) or sequestration by other proteins such as prothymosin-alpha in the case of REA (36). Our studies do not exclude differences at the protein and/or activity levels of REA and AIB1 being involved in *de novo* tamoxifen resistance, nor do they exclude altered expression of these factors having a role in acquired tamoxifen resistance. The possible involvement of altered coregulators in acquired resistance has support from previously published data where it was demonstrated that decreased levels of the nuclear receptor co-repressor (N-CoR) complexes, that play a role in the mediation of the inhibitory effects of tamoxifen, correlate with the acquisition of tamoxifen resistance in a mouse model system of acquired tamoxifen resistance for human breast cancer (37). One should also note that Berns et al. (38) recently reported that a high expression of SRC-1 co-activator indicated a favorable response to tamoxifen of patients with recurrent breast cancer, and more recently Chan et al.(39) reported no significant differences compared to control groups in expression of the coactivators TIF-1, SUG-1, RIP140 and the corepressor SMRT RNAs in ER+ human breast tumors from patients that were *de novo* tamoxifen resistant or in recurrent tumors obtained from patients who acquired tamoxifen resistance. Altogether, there is little evidence for altered coregulators expression in breast tumors that are *de novo* tamoxifen resistant. However, our data provide preliminary evidence that the expression of ERβ protein isoforms may differ in primary tumors of breast cancer patients who prove to have differential sensitivity to tamoxifen therapy. As

well our data support distinct differences in the ER signaling pathways between these two groups of patients since the expression of a known estrogen responsive gene PR is significantly different between the two groups, the precise mechanisms underlying these differences remain to be elucidated.

Legends to Figures.

Figure 1. Examples of immunohistochemistry using the GC-17 antibody which will only recognized the wild-type ERβ1: A, normal human breast tissue; B, ERβ1 negative human breast tumor, H-score = 0; C, ERβ1 low expressing human breast tumor, H-score = 100; D, ERβ1 high expressing human breast tumor, H-score = 150.

Figure 2. Examples of immunohistochemistry using the IgYERβ503 antibody which recognizes most ERβ isoforms: A, normal human breast tissue; B, ERβ negative human breast tumor, H-score = 25; C, ERβ low expressing human breast tumor, H-score = 100; D, ERβ high expressing human breast tumor, H-score = 225.

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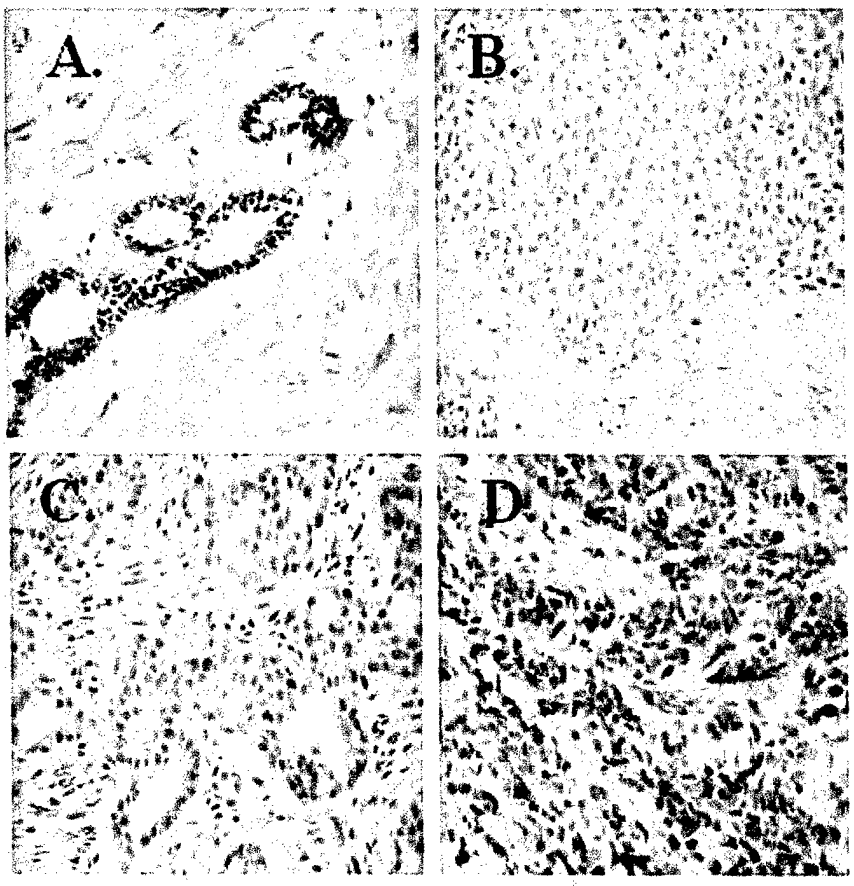

Figure 1

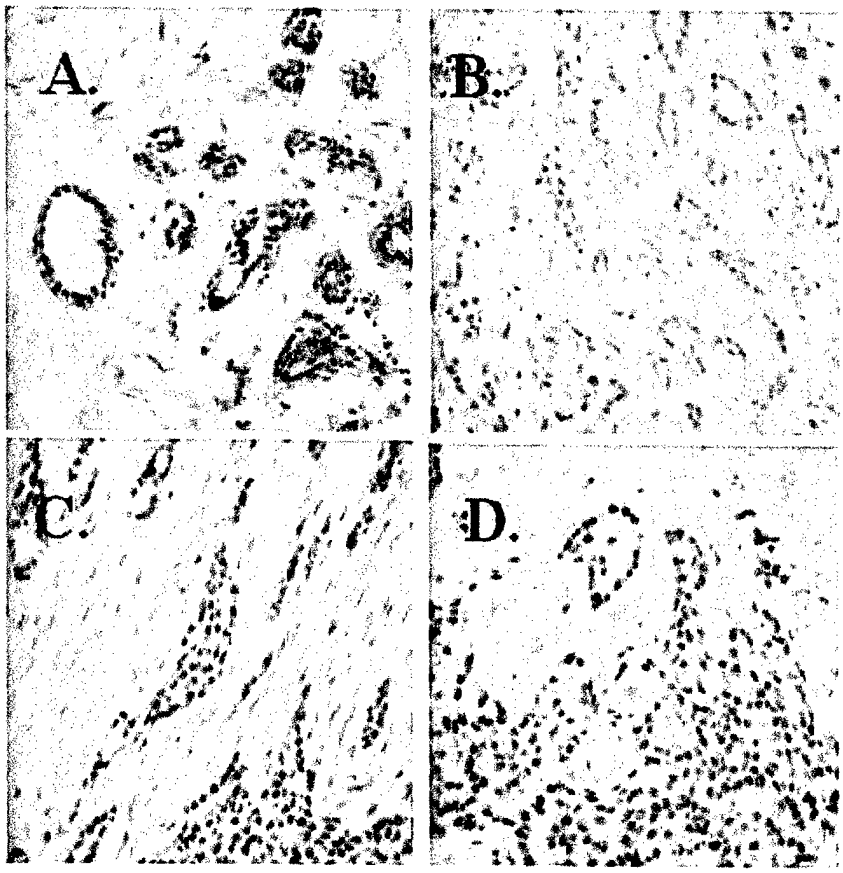

Figure 2

APPENDIX 5
Sumitted, Biochem. Biophys. Res. Commun.

Identification of new coding steroid receptor RNA activator (SRA) isoforms

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[2] Abbreviations used: SRA, steroid receptor RNA activator; ER, estrogen receptor; PR, progesterone receptor.

Abstract

Recently, Lanz et al. (Cell 97, 17-27, 1999) identified and characterized a new transcriptional co-activator, called steroid receptor RNA activator (SRA), which was found to function as an RNA transcript and to be specific for steroid receptors. Lanz et al. reported that none of the different SRA transcript isoforms isolated encoded detectable levels of SRA protein following *in vitro* translation experiments. We have identified three new SRA isoforms encoding *in vitro* stable SRA proteins. One of these isoforms contains a point mutation followed by a codon insertion that is found in both normal and tumor breast tissue as well as in some breast tumor cell lines. This insertion is present at the DNA level and is likely to represent a genetic polymorphism. We hypothesize that wild-type and inserted SRA mRNA and protein could play a role in the mechanisms underlying breast tumorigenesis and tumor progression.

Key words: SRA, steroid receptor co-activator, normal and tumor human breast tissue, polymorphism, tumorigenesis, PCR.

Introduction

Endogenous hormones such as estrogen and progesterone, known to regulate the growth and the development of female reproductive organs, also play key roles in other target tissues such as bone, central nervous system and cardiovascular systems. Estrogen and progesterone actions are mainly mediated through specific receptors that belong to the steroid/thyroid/retinoic acid receptor super-family and act as ligand-dependent transcription factors (1-3). The transcription of target genes by hormone-liganded receptors depends upon interactions between these receptors and several members of a complex co-regulator population (see 2 and references herein). To an already long list of nuclear receptor co-activators (3), Lanz et al. (4) recently added the steroid receptor RNA activator (SRA).[2] *SRA* differs from other co-activators in two main features. Firstly, *SRA* transcripts do not appear to be translated and therefore, this co-activator acts as an RNA and not as a protein. Indeed, Lanz et al. were unsuccessful when trying to generate *in vitro* or *in vivo* stable SRA encoded protein and showed that *SRA* exists in a ribonucleoprotein complex that contains SRC-1 and is recruited by steroid receptors (4). Secondly, *SRA*

appears to be actually specific for steroid receptors. Indeed, most of the receptor-interacting factors interact with and co-activate both class I and class II nuclear receptors (4).

Our recent data suggest that the expression of SRA and AIB1 (another steroid receptor co-activator) is higher in estrogen receptor (ER) positive breast tumors than in matched adjacent normal breast tissues (5 and unpublished data). As well, we showed that SRA expression correlated with estrogen receptor and progesterone receptor levels in particular tumor subgroups (6). A variant form of SRA, containing a deletion of 203 bases within the SRA core sequence, was also observed in breast tumor tissues. The relative expression of this new *SRA* isoform correlated with tumor grade (6). Altogether, these data suggested that changes in the expression of SRA related molecules occur during breast tumorigenesis and breast tumor progression. We therefore speculated that changes in SRA expression could be involved in the mechanisms underlying these phenomena.

SRA sequence (Genbank AF092038) is fully contained in a genomic sequence of chromosome 5 (Genbank AC005214), within five separated exon-like regions (FIG.1). Within this latter clone we have found, using Gene finder (http://dot.imgen.bcm.tmc.edu:9331/gene-finder/gf.html), the sequence of a hypothetical mRNA which could encode a 236 amino-acid protein, that corresponds exactly to the published SRA sequence, except for an additional 37 nucleotides in the 5' region (FIG.1). This 5' region now contains an AUG codon encoding the first methionine of a novel, putative 236 amino-acid SRA protein in contrast to the previously predicted 162 amino-acids which was unstable. We have investigated the expression of this hypothetical mRNA in normal and tumor breast tissues.

Materials and methods

Human breast tissues and cell lines.

14 cases were selected from the NCIC-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). For each case, matched adjacent normal and tumor frozen tissue blocks were available. The quality of each block was determined by the histo-pathological assessment of sections from adjacent mirror image paraffin embedded tissue blocks, as previously described (7). The presence of normal ducts and lobules as well as the absence of any atypical lesion were confirmed in all normal tissue specimens. Breast epithelial cell lines (BT-20, MDA-MB-468, MDA-MB-231, MCF10AT1, MCF10A1, MCF10AT3B, ZR-75, T47D, T5, MCF7, HBL100) were grown, harvested and cell pellets stored at -70°C, as previously described (8). Total RNA was extracted from 20

µm frozen tissue sections (15 and 5 sections for normal and tumor breast tissue, respectively) or cell pellets using Trizol™ reagent (Gibco BRL, Grand Island, NY) according to the manufacturer's instructions.

Primers and RT-PCR conditions.

Detection of the hypothetical coding SRA RNA: primers used consisted of SRAU1 primer (5'-TCCTTTGGTGCCTTGTGAC-3'; sense; position 36132-36149, Genbank accession number AC005214), and SRAL1 primer (5'-AGTCTGGGGAACCGAGGAT-3'; antisense; position 43128-43110, Genbank accession number AC005214).

Pilot study: 200 ng of total RNA was reverse transcribed in a final volume of 25 µl using ThermoScript™ RT-PCR system (Gibco BRL, Grand Island, NY) and oligo-dT according to the manufacturer's instructions. PCR amplifications were performed as previously described (10). Briefly, 1 µl of RT mixture was amplified in a final volume of 30 µl, in the presence of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dGTP, 0.2 mM dCTP, 4 ng/µl of each primer and 1 unit of Taq DNA polymerase (GIBCO-BRL). Each PCR consisted of a 2 minute pre-incubation step at 94°C followed by 30 cycles of amplification (30 sec at 94°C, 2 minutes at 72°C and 30 seconds at 55 °C) using a Thermocycler (Perkin Elmer). PCR products were then separated on 2% agarose gels before staining with ethidium bromide (15 µg/ml). PCR products were sub-cloned using TOPO TA cloning ® kit for sequencing (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction and sequenced as previously described (10).

Optimized conditions: With further experimentation, we found that the above conditions were not optimal: we used the PCR Optimizer™ kit (Invitrogen, Carlsbad, CA) to set-up optimal RT-PCR amplification conditions. One µg of total RNA was reverse transcribed in a final volume of 25 µl using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase and random hexamers as previously described (10). One µl of RT mixture was amplified in a final volume of 30 µl, in the presence of 60 mM Tris-HCl (pH 8.5), 15 mM $[NH_4]^2SO_4$, 1.5 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dGTP, 0.2 mM dCTP, 4 ng/µl of each primer and 1 unit of Taq DNA polymerase (GIBCO-BRL). Each PCR consisted of a 5 minutes pre-incubation step at 94°C followed by 30 cycles of amplification (30 sec at 94°C, 30 seconds at 55 °C, and 2 minutes at 72°C).

Detection of* SRA isoform 3 *isoform: primers used consisted of SRAU2 primer (5'-GGGCCTCCACCTCCTTCAAGTA-3'; sense; position 41664-41685, Genbank accession number AC005214), SRAL2 primer (5'-GCAGTCTTCCAATGCCTG-3'; antisense; position 41813-41796, Genbank accession number AC005214) and SRAL-**GTCG** primer (5'-CACATCCTCCATCA**GTCG**-3'; antisense; position 41780-41767, Genbank accession number AC005214 plus **GTCG** sequence). Radioactive PCR amplifications were performed and PCR products analyzed as previously described (11) with minor modifications. Briefly, 1 µl of reverse transcription mixture was amplified in a final volume of 15 µl, in the presence of 1.5 µCi of (α-^{32}P) dCTP (3000 Ci/mmol), 4 ng/µl of each primer (SRAU2/SRAL2 or SRAU2/ SRAL-**GTCG** primers) and 0.3 unit of Taq DNA polymerase (Gibco BRL, Grand Island, NY). Each PCR consisted of a 2 minutes pre-incubation step at 94°C followed by 30 cycles (30 sec at 60°C, 30 sec at 72°C and 30 sec at 94°C). PCR products were then separated on 6% polyacrylamide gels containing 7M urea. Following electrophoresis, the gels were dried and exposed 30 minutes to a Molecular Imager™-FX Imaging screen (Bio-Rad, Hercules, CA). Exposed screen was then scanned using a Molecular Imager™-FX (Bio-Rad, Hercules, CA). As positive control, we amplified 10 ng of sequenced plasmids previously shown to correspond to SRAcod1, SRAcod2 and SRAcod1 inserted.

In vitro synthesis of SRA protein

In vitro transcription/translation reactions were performed using the TnT coupled Reticulocyte Lysate System (Promega, Madison, EI) according to the manufacturer's instructions and using SRAcod1, SRAcod2 and SRAcod1 inserted corresponding plasmids. The programmed/un-programmed lysates were then subjected to SDS-PAGE and [^{35}S]-methionine labelled protein bands visualized after exposing overnight to a Molecular Imager™-FX Imaging screen (Bio-Rad, Hercules, CA) subsequently scanned using a Molecular Imager™-FX (Bio-Rad, Hercules, CA).

Results

Detection of hypothetical coding SRA isoforms in normal and tumor breast tissues.

In order to determine if indeed our hypothetical coding SRA mRNA was expressed *in vivo*, primers were designed corresponding to sequences upstream of the putative first AUG codon (SRAU1, contained in the

genomic sequence of chromosome 5, Genbank AC005214) and dowstream of the putative stop codon (SRAL1). See Materials and Methods and FIG. 1 for the exact primer positions.

Initially, total RNA was extracted from two normal and one tumor breast tissues. Reverse transcription was performed using Thermoscript and PCR amplification performed as described in Materials and Methods (*pilot study*). Using these primers, we obtained the predicted 920 bp PCR product in both normal and tumor components (data not shown). Cloning and sequencing of this fragment revealed that it essentially corresponded to the hypothetical SRA coding sequence. Interestingly, three different SRA cDNAs were identified: SRA isoform 1 (Genbank AF293024), SRA isoform 2 (Genbank AF293025) and SRA isoform 3 (Genbank AF293026). These all migrated with a similar apparent size of 920 bp (see FIG. 2). SRA isoform 1 contained the full coding hypothetical SRA, whereas SRA isoform 2 contained two point mutations at positions 338 (C→T) and 348 (A→C), and SRA isoform 3 contained a point mutation followed by an insertion of three nucleotides at position 520 (G→CGAC). All these sequences contained a potential open reading frame able to encode two 236 aa and one 237 aa protein from SRA isoform 1, 2 and 3 respectively (FIG.3).

***In vitro* translation of three new SRA isoforms: SRA isoforms 1, 2 and 3.**

Previously cloned SRA cDNAs, in which only the third ATG codon (encoding Met3, FIG.2) was present, were unable to support detectable protein synthesis *in vitro* (4). To determine if the three new SRA cDNAs isolated in our laboratory could be translated in vitro, vectors containing SRA isoform 1, 2 and 3 sequences downstream of a T7 polymerase promoter were used in a TnT coupled Reticulocyte Lysate system as described in Materials and Methods. Results are shown FIG. 4. The three different SRA isoforms encoded stable SRA proteins under these conditions. It should however be noted that two bands were observed for each construction, consistent with the use of two different initiating methionine codons (FIG.2, Met1 and Met2). Moreover, the observed molecular masses (31 and 30 kDa for SRA isoforms 1-2 and 3, respectively) were slightly higher that those predicted (25.7 and 25.8 kDa for SRA isoform 1-2 and 3, respectively). Possible reasons for such the discrepancy between observed and theoretical molecular weight are under investigation.

Detection of hypothetically coding SRA isoforms in breast epithelial cell lines.

To determine if the SRA putatively coding sequences (that will be referred to as hypothetical coding SRAs) were expressed in breast cancer cells, total RNA was extracted from different cancer cell lines and amplified as

described above. It should be noted that the conditions used in the pilot study failed to give reproducible results, even on the control plasmid DNA preparations (data not shown). Optimized conditions were therefore determined (see Materials and Methods), and used to obtain the results presented in the following sections. The predicted PCR product corresponding to the hypothetical coding SRAs was observed in all cell lines (FIG.5A), confirming that these transcripts are indeed expressed in tumorigenic and non tumorigenic human breast epithelial cells.

Blast searches of human EST databases (http://www.ncbi.nlm.nih.gov/blast/) revealed that the specific sequence of SRA isoform 3 (i.e G→CGAC at position 520) had already been cloned by others (Genbank accession number: AW954396, AW957456, AW630779, AA305793, AA410852, AA353911). This confirmed our own data resulting from independent RT-PCRs, cloning and sequencing and underlined that the SRA isoform 3 was not the result of a technical artifact. Therefore, it was of interest to investigate the expression of this isoform in breast cancer cells. PCR primers were designed to specifically amplify a fragment overlapping the putatively inserted region (SRAU2, SRAL2) or to specifically anneal with the inserted sequence (SRAL-**GTCG**) (see FIG.2 for primer positions). PCR products amplified using the former set of primers (SRAU2, SRAL2) were expected to migrate at an apparent size of 150 bp and 153 bp for SRA isoforms 1-2 and 3 cDNA, respectively. Using the latter set of primers (SRAU2, SRAL-**GTGC**), a PCR product 117 bp long was expected only in samples expressing SRA isoform 3 mRNA. Results obtained using SRAU2/SRAL2 and SRAU2/SRAL-**GTGC** are shown FIG.5B and FIG.5C, respectively. Interestingly, some cell lines expressed only SRA isoform 3 specific fragment (MDA-MB-231, MCF-7) whereas others expressed both SRA isoforms 1-2 and 3 (T47D, T5).

DNA extracted from these cell lines was also amplified using SRAU2 and SRAL2. As shown in FIG.5D a perfect correspondence existed between SRA isoform expression and DNA sequence, suggesting the possible existence of a genetic polymorphism and therefore of at least two alleles of the SRA gene.

Detection of similar SRA isoforms in matched normal and tumor breast tissues.

Total RNA was extracted from matched normal and tumor breast tissues from twelve different patients, reverse transcribed and amplified using the different pairs of primers mentioned earlier. Results corresponding to 4 different patients are shown FIG.6. Hypothetical coding SRAs were detectable in matched normal and tumor

components (FIG.6A). Interestingly, even though the cohort studied was too small to look for statistical significance, it was noticed that the expression of the hypothetical coding SRAs was higher in tumor than in matched normal breast tissues (data not shown). Using primers overlapping the inserted region of SRA isoform 3 (FIG.6B) or specifically amplifying this isoform (FIG.6C), it appeared that, as observed for breast epithelial cells grown in culture, some patients expressed either one (patients 2-4) or two (patient 1) isoforms. Further, the pattern of expression (either one or two isoforms expressed) was conserved between matched normal and tumor components.

Discussion

Three new SRA isoforms have been identified, that are able to encode stable SRA proteins in vitro. Interestingly, using "classical" RT-PCR conditions (Cf. *pilot study*) it was not possible to reproducibly amplify the PCR fragments corresponding to these isoforms. The reasons for this lack of reproducibility are unknown but RNA or cDNA secondary structures might be involved. Indeed, changing the set of primers during the PCR was not sufficient to overcome this problem (our unpublished data) suggesting inherent properties of these particular nucleotide sequences. The technical difficulties we have encountered associated with the reverse-transcription and/or amplification of these isoforms together with the possible tissue specificity of their expression could underlie the lack of detection of these isoforms in previous studies.

Indeed, database searches revealed that although many partial SRA-like sequences isolated from various normal and tumor tissues have been entered in the human EST sequence database, only one appears to correspond to a full length hypothetical coding SRA. This particular EST (Genbank R51952), 1500 bp long, was isolated from normal breast tissue, but had only been sequenced in to its first 460 5' nucleotides. This partial sequence contained exactly the 5' terminal extremity of the hypothetical SRA mRNA we had predicted from the genomic clone. We have fully sequenced this EST and found that it indeed corresponded to the SRA isoform 2 which we had isolated from human breast tissue. Also of interest is the fact that the Northern blot analysis performed by Lanz et al. has shown an apparently less abundant SRA transcript of similar length ~1500 bases, in addition to the 0.7-0.8 transcript, in some normal tissues (e.g. muscle), but not all (4). Such data suggest that the expression of the hypothetical coding SRA isoforms may be tissue specific.

Our data are the first to show that naturally expressed SRA RNAs can generate a cDNA from which a detectable SRA protein is generated *in vitro*. Indeed, Lanz *et al.*, who reported the identification of three different SRA isoforms, concluded that their cloned SRA cDNA sequences (i.e. predicted to encode a 162 a.a. protein) did not encode a detectable translation product (4). However, when engineered to form a fusion product e.g. with GST, Gal4 or HSV-thymidine kinase initiation sequences attached to the N-terminal region, the appropriate fusion SRA-like product was detected. This suggests the possibility that the extra 74 N-terminal amino acids predicted in the hypothetical coding SRA mRNAs could provide sequences which would result in a translation product detectable *in vivo*.

We observed that one or two SRA isoforms can be expressed in breast epithelial cells as well as in normal and tumor breast tissues. It should be noted that our PCR assays (using SRAU2/SRAL2 and SRAU2/SRAL-**GTCG**) do not establish whether SRA isoform 1 and/or 2 are expressed. Indeed, these assays only establish whether or not SRA isoform 3 is expressed, alone or together with one of the other isoforms (SRA isoform 1 or 2). Interestingly, we also found that in breast cancer cells the pattern of expression was directly related to the detection of these particular isoforms within the genomic DNA. Moreover, when normal breast tissue expressed specific isoforms, the adjacent tumor tissue also expressed the same isoforms. Altogether, these data suggest the existence of a genetic polymorphism within the SRA gene. Moreover, some patients appeared to be homozygotes or heterozygotes for this gene. Whether or not this polymorphism contributes to a predisposition to breast tumorigenesis or to development of breast tumors with a particular phenotype (i.e steroid receptor status, resistance to tamoxifen, grade etc...) is under investigation.

In our previous studies, we showed that the expression of SRA (Genbank AF092038) correlated with estrogen receptor and progesterone receptor levels in particular tumor subgroups. We also determined that the expression of SRA was higher in tumor that in adjacent normal human breast tissues (5 and unpublished observation). It is therefore reasonable to speculate that the different SRA isoforms, at the RNA and/or at the protein levels, could be involved in the mechanisms underlying breast tumorigenesis and tumor progression. The role of the different SRA isoforms and their putative protein products on ER and PR signaling pathway remains to be determined.

In conclusion, we have identified several SRA mRNA isoforms, which are able to encode stable proteins *in vitro* and are expressed in normal and neoplastic breast tissues. We therefore speculate that SRA proteins, encoded

by these mRNA isoforms, are present in vivo. We also hypothesize that these isoforms and their translation products could be involved in the mechanisms underlying breast tumorigenesis and tumor progression.

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Figures legend

FIG. 1 Schematic representation of SRA mRNA and gene structure. Human SRA isoforms identified to date differ in their 5' and 3' terminal regions (white circles) but present an identical nucleotide sequence (dark gray boxes) in between. The only SRA sequence entered in Genbank (AF092038) is fully contained within 10,000 bp of Bac 5 genomic sequence (AC005214). Numbers indicate positions relative to this latter sequence. Hyp coding SRA: hypothetical coding SRA sequence found using Gene Finder. Position of SRAU1 and SRAL1 primers are indicated by arrows.

FIG. 2 Alignment of SRA isoforms and primer positions. Previously (4) cloned SRA sequence (AF092038) is aligned with new SRA isoforms: SRA 1 (AF293024), SRA 2 (AF293025) and SRA 3 (AF293026). Differences between sequences are circled. Positions of two new putative starting ATG codons are indicated (Met 1 and Met 2), together with that of the initially predicted starting codon (Met 3, reference 4) and the common stop codon. The positions of SRAU1, SRAU2, SRAL-**GTCG**, SRAL2 and SRAL1 primers are also depicted.

FIG. 3 Alignment of proteins putatively encoded by SRA isoforms. SRA, SRA 1, SRA 2, and SRA 3: Proteins putatively encoded by open reading frames contained in AF092038, AF293024, AF293025 and AF293026, respectively. Positions of two putative first methionine (Met 1 and Met 2), together with initially predicted first methione (Met 3, reference 4) are indicated. Differences between sequences are circled.

FIG.4 *In vitro* translation of SRA isoform 1, 2 and 3 cDNAs. *In vitro* transcription/translation reactions were performed using SRA isoform 1, 2 and 3 corresponding plasmids and programmed / un-programmed (UPL) lysates analyzed as described in the Materials and Methods section. Size in kDa, corresponding to the prestained marker, broad range (Premixed format, BioLabs), are shown on the left.

FIG.5 Detection of SRA isoforms in breast epithelial cell lines. Total RNA was extracted from breast epithelial cell lines (BT-20, MDA-MB-468, MDA-MB-231, MCF10AT1, MCF10A1, MCF10AT3B, HBL-100, ZR-75, T47D, T5, MCF-7), reverse transcribed, PCR amplified using SRAU1/SRAL1 (A), SRAU2/SRAL2 (B) or SRAU2/SRAL-GTCG (C) primers and PCR products were separated on agarose (A,

C) or acrylamide (B) gels, as described in the Materials and Methods section. (D) genomic DNA corresponding to these cell lines was amplified using SRAU2/SRAL2 and PCR products separated on acrylamide gel as described in the Materials and Methods section.

FIG.6. Detection of SRA isoforms in matched normal and tumor breast tissues. Total RNA was extracted from matched normal (N) and tumor (T) breast tissues from different patients (1-4), reverse transcribed, amplified using SRAU1/SRAL1 (A), SRAU2/SRAL2 (B) or SRAU2/SRAL-GTCG (C) primers and PCR products were separated on agarose (A) or acrylamide (B, C) gels, as described in the Materials and Methods section. SRA iso1, SRA iso3, and SRA iso1/iso3: positive controls amplified in parallel consisting of 50 pg of plasmid containing SRA isoform 1, SRA isoform 3 cDNA, and a mix of these plasmids respectively.

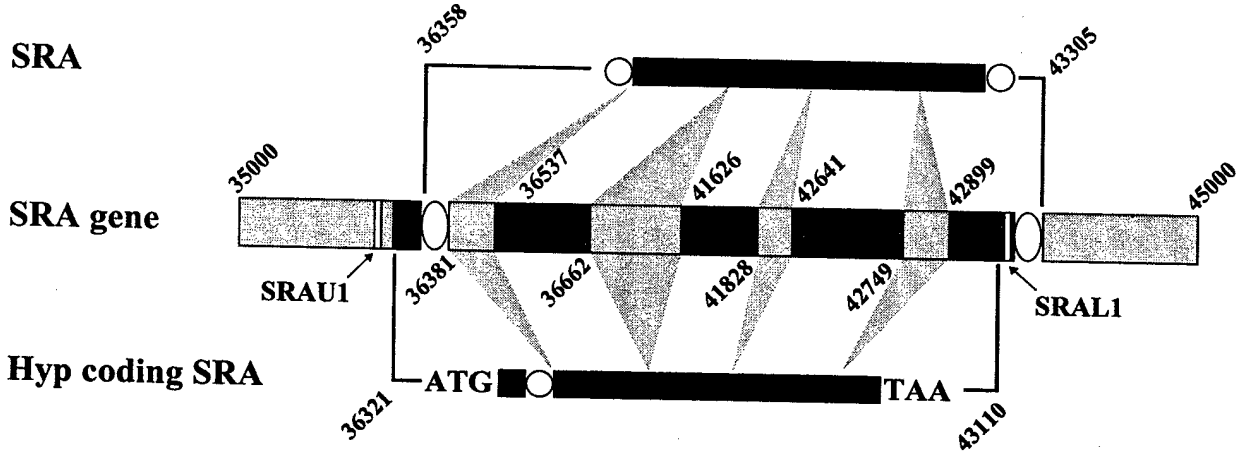
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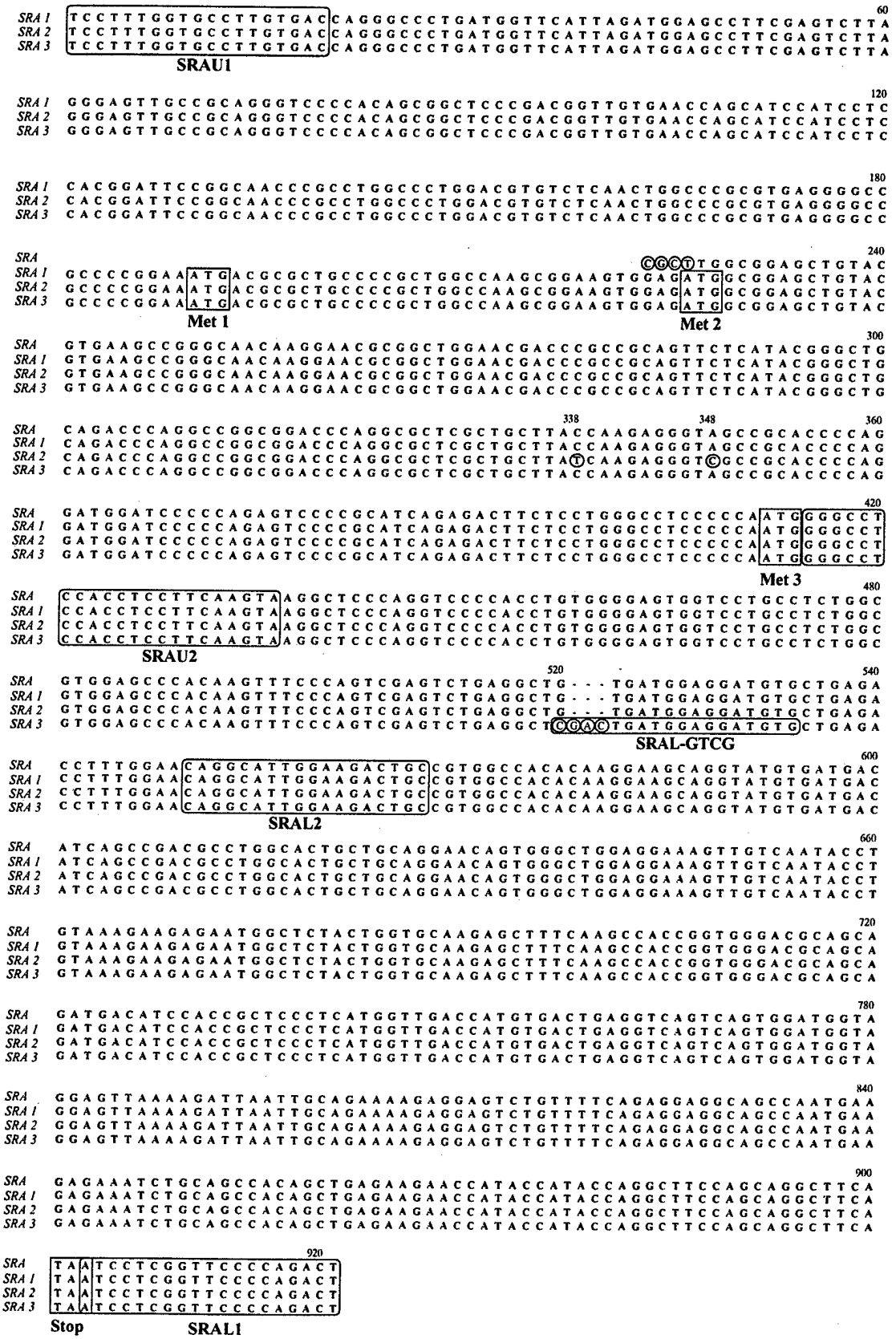
Figure 1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Met 2
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SRAL2
Stop
SRAL1

Figure 2

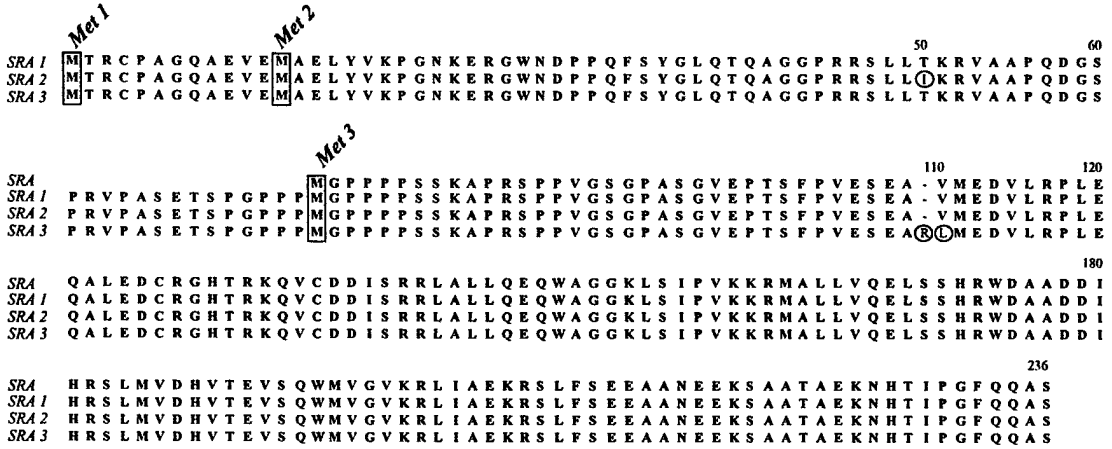


Figure 3

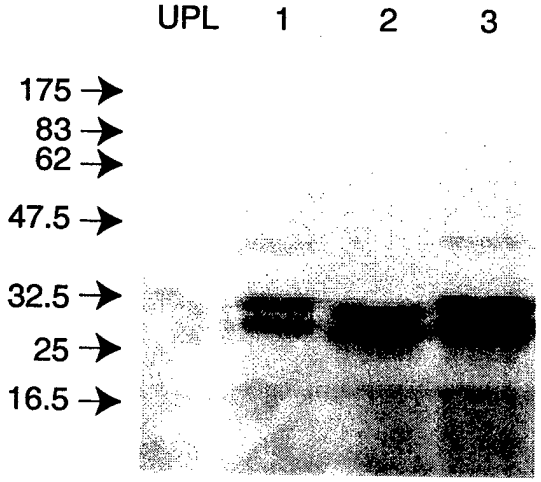
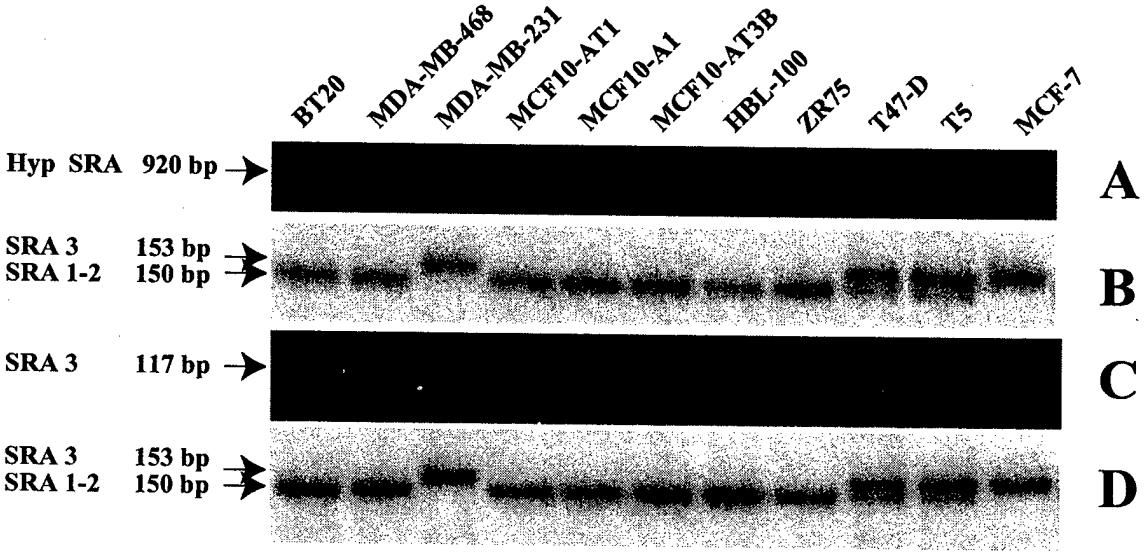


Figure 4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MCF-7
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SRA 1-2 150 bp
A
B
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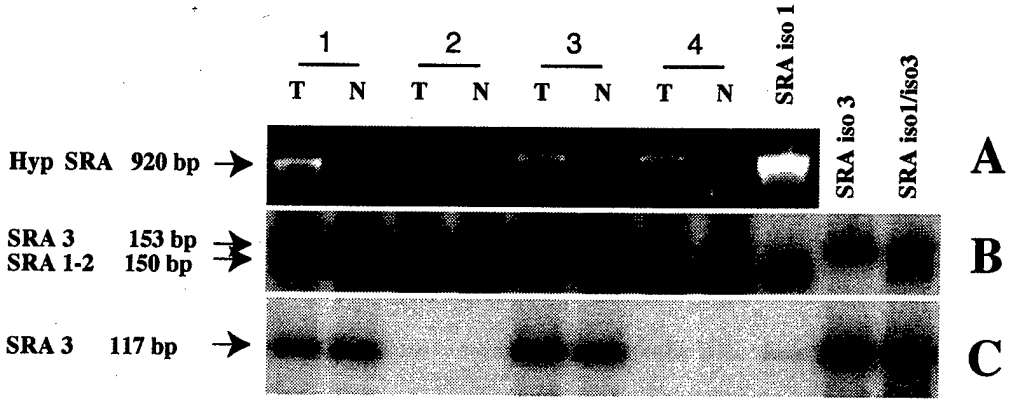
D

Figure 5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3
4
T N T N T N T N
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SRA iso 3
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SRA 1-2 150 bp
SRA 3 117 bp
A
B
C

Figure 6