

Estrogen Receptor- β Messenger RNA Expression in Human Breast Tumor Biopsies: Relationship to Steroid Receptor Status and Regulation by Progestins¹

Helmut Dotzlaw, Etienne Leygue, Peter H. Watson, and Leigh C. Murphy²

Departments of Biochemistry and Molecular Biology [H. D., E. L., L. C. M.] and Pathology [P. H. W.], Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, R3E 0W3 Canada

Abstract

When the level of estrogen receptor (ER)- β mRNA in tumors, determined by reverse transcription-PCR, was assessed according to either ER status or PR status alone, determined by ligand binding assays, the level of ER- β mRNA was significantly lower in PR+ tumors compared with PR- tumors ($P = 0.036$), and no association with ER status was found. Subgroup analysis showed that ER- β mRNA expression in ER+/PR+ breast tumors was significantly less than in ER+/PR- ($P = 0.009$), ER-/PR+ ($P = 0.029$), and ER-/PR- ($P = 0.023$) groups. Interestingly, the ER- β mRNA expression was specifically decreased by progestin in T-47D breast cancer cells. The data suggest the possibility that expression of ER- β in human breast tumors is a marker of endocrine therapy responsiveness.

Introduction

Both estrogen and antiestrogen can mediate transcriptional activity via the recently identified ER³- β (1–3). Recently, we have shown the presence of ER- β mRNA in both normal and neoplastic human breast tissues (4, 5). Furthermore, the relative expression of ER- α and ER- β mRNA changes between normal human breast tissues and their concurrent matched ER+ breast tumors (6), suggesting that altered expression of ER- α and ER- β occurs and may be functionally involved in breast tumorigenesis. Interestingly, it also seemed that the level of ER- β mRNA varied among breast tumors but was not correlated with the expression of ER- α (4), although the two receptor mRNAs were often coexpressed in the same tumor. These observations raised the question of whether the expression of ER- β in breast tumors was correlated with known prognostic and endocrine treatment response markers. In this study, the relationship of ER- β mRNA expression to ER and PR status, as determined by ligand binding analysis, was investigated.

Materials and Methods

Materials. All cell culture reagents were obtained from Life Technologies, Inc. (Burlington, Ontario). MPA and dexamethasone were purchased from Sigma Chemical Co. (St. Louis, MO). R5020 and Org 2058 were purchased from Amersham Corp. (Oakville, Canada). RU 486 was a gift from Roussel

Uclaf (Romainville, France). [α -³²P]dCTP was purchased from ICN (Montreal, Quebec).

Human Breast Tumors. Forty invasive ductal carcinomas were selected from the National Cancer Institute of Canada-Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). The cases were selected for ER and PR status as determined by ligand binding assays. Ten tumors were classified as ER+/PR+ (ER range, 50–127 fmol/mg protein; PR range, 105–285 fmol/mg protein); 10 tumors were classified as ER+/PR- (ER range, 59–156 fmol/mg protein; PR range, 5–10 fmol/mg protein); 10 tumors were ER-/PR- (ER range, 0–2 fmol/mg protein; PR range, 0–10 fmol/mg protein); and 10 tumors were classified as ER-/PR+ (ER range, 5–9 fmol/mg protein; PR range, 51–271 fmol/mg protein). These tumors spanned a wide range of grade (grade 4–9), determined using the Nottingham grading system.

Cell Culture. T-47D human breast cancer cells were obtained from Dr. D. Edwards (University of Colorado, Denver, CO). The cells were grown in DMEM supplemented with 5% fetal bovine serum, 100 nM glutamine, 0.3% (v/v) glucose, and penicillin/streptomycin, as previously described (7). Cells were plated at 1 times 10⁶ in 100-mm dishes and 2 days later were treated as indicated in the text. The steroids and other compounds were added directly from 1000 times stock solutions in ethanol to achieve the concentrations indicated. The cells were harvested at the times indicated by scraping with a rubber policeman. After centrifugation, the cell pellet was frozen and stored at -70°C until RNA was isolated.

RNA Extraction and RT-PCR Conditions. Total RNA was extracted from 20- μ m frozen tissue sections (5 sections/tumor) or frozen cell pellets using Trizol reagent (Life Technologies, Inc., Grand Island, NY), according to the manufacturer's instructions. Total RNA (1 μ g) was reverse transcribed in a final volume of 25 μ l, as previously described (4).

The primers used consisted of ER- β -U primer (5'-GTCCATCGCCAGT-TATCACATC-3'; sense; located in ER- β 130-151) and ER- β -L primer (5'-GCCTTACATCCTTACACGA-3'; antisense; located in ER- β 371-352). Nucleotide positions given correspond to published sequences of the human ER- β cDNA (2). PCR amplifications were performed, and PCR products were analyzed as previously described, with minor modifications (4). Briefly, 1 μ l of reverse transcription mixture was amplified in a final volume of 15 μ l, in the presence of 1.5 μ Ci [α -³²P]dCTP (3000 Ci/mmol), 4 ng/ μ l ER- β -U/ER- β -L, and 0.3 units of Taq DNA polymerase (Life Technologies, Inc.). Each PCR consisted of 30 cycles (30 s at 94°C, 30 s at 60°C, and 30 s at 72°C). PCR products were then separated on 6% polyacrylamide gels containing 7M urea. After electrophoresis, the gels were dried and autoradiographed. Amplification of the ubiquitously expressed *GAPDH* cDNA was performed in parallel, and PCR products were separated on agarose gels stained with ethidium bromide, as previously described (4). PCR products were subcloned and sequenced, as previously described (4).

Quantification and Statistical Analysis. Quantification of signals was carried out by excision of the band corresponding to ER- β cDNA, addition of scintillant, and scintillation counting. Three independent PCRs were performed. To control for variations between experiments, a value of 100% was assigned to the case exhibiting the highest signal measured, and all signals were expressed as a percentage of this signal. In parallel, *GAPDH* cDNA was amplified and, after analysis of PCR products on prestained agarose gels, signals were quantified by scanning using NIH Image 161/ppc software. Each *GAPDH* signal was also expressed as a percentage of the highest signal observed in the experiment. Two independent PCRs were performed. For each sample, the average of the ER- β signal was then expressed as a percentage of the average *GAPDH* signal. The statistical significance of any differences of

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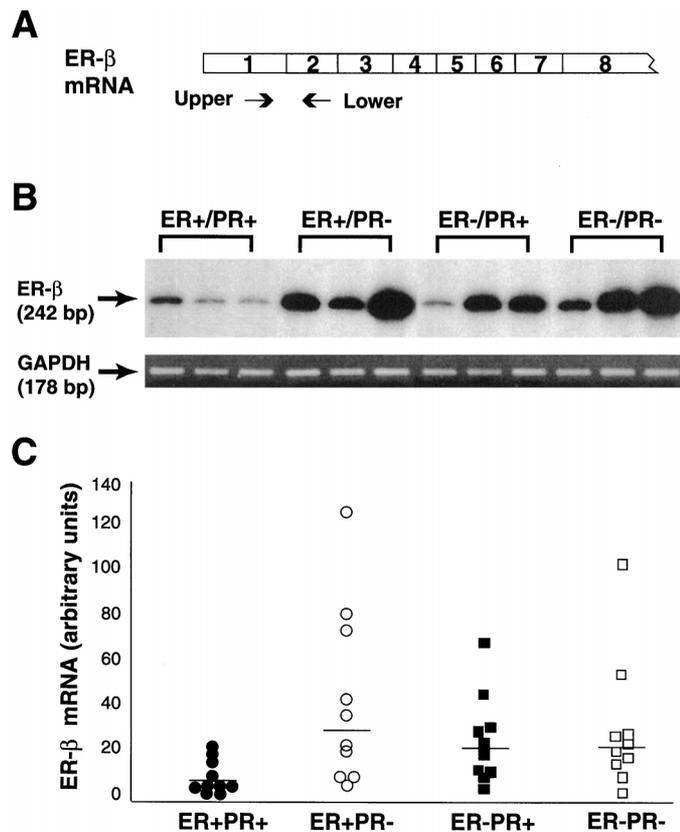
² To whom requests for reprints should be addressed, at The University of Manitoba, Department of Biochemistry and Molecular Biology, 770 Bannatyne Avenue, Winnipeg, Manitoba, R3E 0W3 Canada. Phone: 204-789-3233; Fax: 204-789-3900; E-mail: lcmurph@cc.umanitoba.ca.

³ The abbreviations used are: ER, estrogen receptor; MPA, medroxyprogesterone acetate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PR, progesterone receptor; RT-PCR, reverse transcription-PCR.

the mean ER- β mRNA level between groups was determined using the Mann-Whitney test (two-tailed).

Results

Measurement of ER- β mRNA Expression in Primary Human Breast Tumors with Different ER and PR Status. Previous data have suggested that the level of ER- β mRNA varied widely in human breast tumor samples (4), which raised the question of whether the expression of ER- β in breast tumors was correlated with the known prognostic and treatment response variables, ER and PR status. Four groups, containing 10 breast tumor samples each, were identified according to their ER/PR status, as defined by ligand binding analysis (see "Materials and Methods"). ER- β mRNA levels were measured by RT-PCR and normalized to the *GAPDH* mRNA level, as measured in parallel by RT-PCR. The primers used in this analysis are located in exons 1 and 2 (Fig. 1A) of the human ER- β gene (2, 8) and would, therefore, measure the wild-type human ER- β mRNA and all ER- β mRNA variants so far documented (5, 9, 10). Examples of the results obtained are shown in Fig. 1B. The results obtained for all tumors assayed are shown in Fig. 1C, arranged in groups according to the ER/PR status of the tumor, as measured by ligand binding analysis.



The level of ER- β mRNA in ER+/PR+ breast tumors was significantly less than in all other groups (see Table 1), with no significant differences seen among the ER+/PR-, ER-/PR+, or ER-/PR- groups. When the level of ER- β mRNA in tumors was assessed according to either ER status or PR status alone, as defined by ligand binding analysis, the level of ER- β mRNA was significantly lower in PR+ tumors compared with PR- tumors (Table 1, *G versus H*; $P = 0.036$), with no significant differences associated with ER status alone (Table 1, *E versus F*; $P = 0.323$).

Spearman correlation analysis showed no significant correlations of the level of ER- β mRNA with grade, age, nodal status, or the percentage of normal duct and lobular epithelium, stromal or fat cell content within the tissue section analyzed. However, an inverse relationship was found when the level of ER- β mRNA was correlated with the absolute level of PR, as measured by ligand binding analysis ($r = -0.31$; $P = 0.052$), consistent with the data when analyzed using clinically relevant cut-off values for both ER and PR status as shown above.

Regulation of Steady-state Levels of ER- β mRNA by Progestins in T-47D Human Breast Cancer Cells. The relationship of the level of ER- β mRNA with PR status in human breast tumor biopsies suggested the hypothesis that ER- β expression may be regulated by progestins. This hypothesis was investigated using the PR+ T-47D human breast cancer cell line in culture. The steady-state level of ER- β mRNA was found to decrease after treatment with 10 nM MPA (Fig. 2A). A significant decrease was observed at 6 hours after MPA treatment, and the levels remained decreased for up to 48 hours after treatment. The effect of MPA on the steady-state levels of ER- β mRNA in T-47D cells was first seen with 1 nM MPA and was maximal between 10 and 100 nM MPA (Fig. 2B). The progestin specificity of this response was assessed by treating T-47D cells for 24 hours with MPA, Org 2058, dexamethasone, and the antiprogestin RU 486 (Fig. 3, A and B). Both 10 nM MPA and 10 nM of the synthetic progestin Org 2058 significantly decreased the steady-state levels of ER- β mRNA, whereas little, if any, effect was observed with 100 nM of the synthetic glucocorticoid, dexamethasone. Antiprogestin/anti-glucocorticoid RU 486 (500 nM) had little, if any, effect by itself, but inhibited the down-regulation by 10 nM MPA on the level of ER- β mRNA. It was concluded that progestins can down-regulate the steady-state levels of ER- β mRNA and that an antiprogestin can inhibit this effect in T-47D human breast cancer cells.

Discussion

It was previously documented that the level of ER- β mRNA expression in human breast tumors varied widely (4, 8). This raised the question of whether the expression of ER- β in breast tumors was correlated with known prognostic and treatment-response markers. The measurement of both ERs and PRs in human breast biopsies is routinely used to provide both prognostic and treat-

Fig. 1. A, schematic diagram of the human ER- β cDNA showing the priming sites of the upper and lower primers used for the analysis of ER- β mRNA by RT-PCR. B, expression of ER- β mRNA in human breast tumor biopsy samples, according to ER and PR status determined by ligand binding assay. Top, an autoradiogram of the RT-PCR assays for ER- β mRNA obtained from representative samples of tumors that were classified as ER+/PR+, ER+/PR-, ER-/PR+, and ER-/PR-, as described in "Materials and Methods." Bottom, the ethidium bromide-stained gel of the RT-PCR analysis of GAPDH mRNA run in parallel for the same samples. C, quantification of ER- β mRNA expression within human breast tumors classified according to ER and PR status, as determined by ligand binding assay. Total RNA, extracted from the tumors, was reverse transcribed, PCR-amplified, and PCR products were separated on acrylamide gel as described in "Materials and Methods." Signals have been quantified and normalized, as indicated in "Materials and Methods." ●, ER+/PR+ tumors; ○, ER+/PR- tumors; ■, ER-/PR+ tumors; □, ER-/PR- tumors. Horizontal line, the median value in each group.

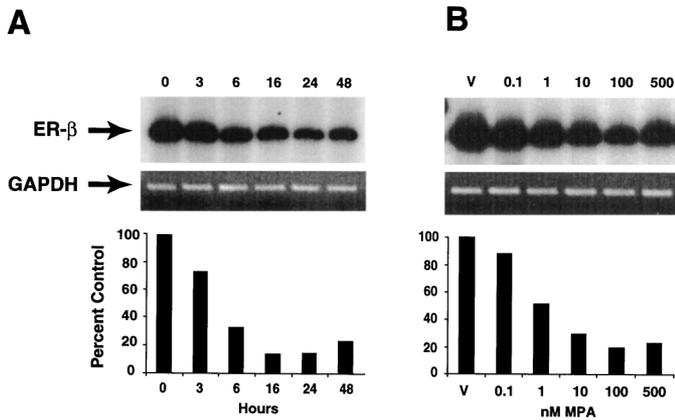


Fig. 2. *A*, time-dependent down-regulation of ER- β mRNA expression in T-47D human breast cancer cells. *Top*, an autoradiogram of ER- β mRNA levels determined by RT-PCR after treatment with 10 nM MPA for the indicated time periods. *Middle*, the ethidium bromide-stained gel of the RT-PCR analysis of GAPDH mRNA run in parallel for the same samples. *Bottom*, the results presented as a histogram after quantification and normalization of the ER- β signal, as described in "Materials and Methods." This experiment was replicated twice. *B*, dose-dependent down-regulation of ER- β mRNA expression in T-47D human breast cancer cells. *Top*, an autoradiogram of ER- β mRNA levels determined by RT-PCR after treatment with vehicle alone (V) and varying concentrations of MPA for 24 h. *Middle*, the ethidium bromide-stained gel of the RT-PCR analysis of GAPDH mRNA run in parallel for the same samples. *Bottom*, the results presented as a histogram after quantification and normalization of the ER- β signal, as described in "Materials and Methods."

ment-response information (11). Because ER- β is structurally and functionally related to ER- α (1–3, 12), it was relevant to determine whether the expression of ER- β was related to the ER and PR status of the tumor, as defined by ligand binding assays. Our analysis established that the expression of ER- β mRNA was inversely correlated with PR status generally. Although there was no significant correlation between ER- β mRNA levels and ER status overall, a significant difference in ER- β mRNA levels in those tumors that were ER+/PR+ (lowest expression) and those tumors that were ER-/PR+ (higher expression) was observed. This could be interpreted to mean that both ER status and PR status could influence ER- β mRNA expression. However, the differences observed could also be explained by the significant difference in the absolute level of PR expression between the two groups (PR levels determined by ligand binding assays expressed as mean \pm SE, 190 ± 24 fmol/mg protein *versus* 97 ± 21 fmol/mg protein, in ER+/PR+ and ER-/PR+ groups, respectively). This would be consistent with the inverse correlation that was seen with ER- β mRNA and the absolute levels of PR determined by ligand binding analysis, considering all groups together.

These data suggested the possibility that the expression of ER- β may be regulated by progestins. In T-47D cells (which express ER- α , ER- β , and PR), the steady-state level of ER- β mRNA was specifically decreased by progestin treatment in a time- and dose-dependent manner. Our data support the hypothesis that the progestin effect is mediated by PR, however, our data do not address whether this occurs via a transcriptional or post-transcriptional mechanism. Interestingly, progestins are known to also decrease the steady-state levels of ER- α mRNA and protein in T-47D cells (13). Therefore, PR is able to regulate the expression of both ER- α and ER- β in human breast cancer cells in a similar fashion. However, the interaction of PR and the two distinct ERs is likely to be different. It has been well documented that there is a general positive correlation between ER and PR levels, as determined by ligand binding assays in human breast tumors (11). ER status, as determined by ligand binding, correlates well with both immunological detection of the ER- α protein (14) and ER- α mRNA detection (15). Such data together with other studies

(6) suggest that the ER level in breast tumors, as determined by ligand binding in most cases, is due to ER- α . Furthermore, ER- β mRNA is the predominant ER mRNA in MDA MB 231 human breast cancer cells (4) and these cells are known to be ER negative by ligand binding assay providing further evidence for the lack of interference of ER- β expression in the determination of ER status by ligand binding assay in the majority of human breast tumors. Interestingly, a significant level of ER- β -like mRNA in human breast cancer cell lines and possibly, therefore, breast tumors may be represented by exon 8 deleted variants (10), which most likely encode nonestrogen binding ER- β variant proteins, which could not contribute to ER ligand binding assays. Therefore, the available data suggest that the previously observed positive correlation of ER and PR in human breast tumors is due to ER- α expression, underscoring the difference in the relationship of ER- α and ER- β with PR in human breast cancer tissue.

Our data are the first to identify a correlation between ER- β mRNA expression and a known prognostic and treatment-response marker in human breast cancer biopsies. The inverse relationship between PR (a good prognostic variable and a marker of response to endocrine therapies) and ER- β suggests that although ER- β is often down-regulated in human breast tumors compared with normal human breast tissue (6), its maintenance and/or increased expression in some breast tumors may correlate with a poorer prognosis and the likelihood of failure of response to endocrine therapies such as antiestrogens. This remains to be tested in samples of breast tumors from patients known to have responded or not to have responded to endocrine therapies, in clinical trials. Furthermore, a functional involvement of ER- β in this phenotype remains to be determined. Interestingly, although no agonist activity of tamoxifen-like antiestrogens can be measured through ER- β in a recombinant expression system using

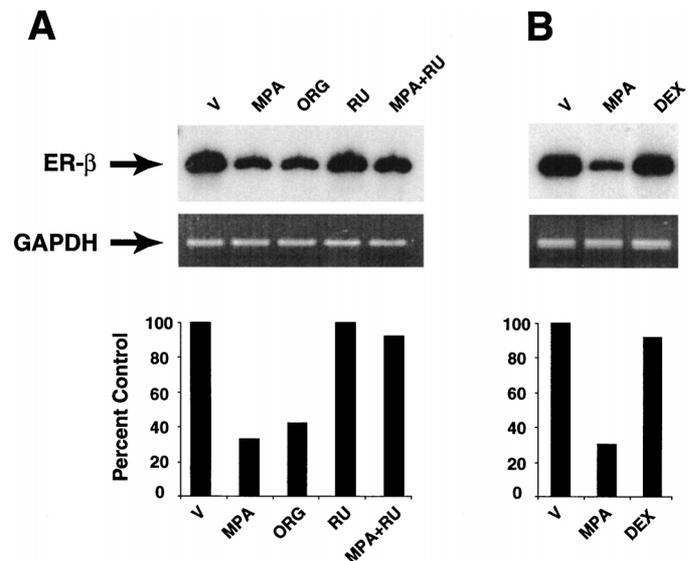


Fig. 3. *A*, steroid specificity of the down-regulation of ER- β mRNA expression in T-47D human breast cancer cells. *Top*, an autoradiogram of ER- β mRNA levels determined by RT-PCR after 24 h of treatment with vehicle alone (V), 10 nM MPA (MPA), 10 nM Org2058 (ORG), 500 nM RU 486 (RU), and 10 nM MPA + 500 nM RU 486 (MPA+RU). *Middle*, the ethidium bromide-stained gel of the RT-PCR analysis of GAPDH mRNA run in parallel for the same samples. *Bottom*, the results presented as a histogram after quantification and normalization of the ER- β signal, as described in "Materials and Methods." This experiment was replicated twice. *B*, steroid specificity of the down-regulation of ER- β mRNA expression in T-47D human breast cancer cells. *Top*, an autoradiogram of ER- β mRNA levels determined by RT-PCR after 24 h of treatment with vehicle alone (V), 10 nM MPA (MPA), and 10 nM dexamethasone (DEX). *Middle*, the ethidium bromide-stained gel of the RT-PCR analysis of GAPDH mRNA run in parallel for the same samples. *Bottom*, the results presented as a histogram after quantification and normalization of the ER- β signal, as described in "Materials and Methods."

transient transfection and a classical ERE-reporter gene (3), all classes of antiestrogens bound to ER- β result in the transcriptional activation of AP-1-driven reporter genes, again in a transient recombinant model system (12). Because AP-1-regulated genes are often associated with growth and proliferation (16–18), it is tempting to speculate that increased expression of ER- β in human breast tumors could play a role in tamoxifen resistance in the small number of tumors that appear to proliferate in response to tamoxifen (19, 20).

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