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13. ABSTRACT (Maximum 200 words)

The overall goal of the proposed research has been to understand the mechanisms by which breast cancers become resistant to hormone treatments. Specifically, the hormones involved are the steroidal agonists, estradiol and progesterone, the antagonists tamoxifen and RU-486, and the receptors to which these hormones bind, namely estrogen (ER) and progesterone receptors (PR). To this end, we initially used as models, the PR of human breast cancer cells and the antiprogestin RU-486. We anticipated that the mechanisms we uncovered for progestins would be relevant to estrogens as well. This has turned out to be the case. We have discovered two proteins, which interact with antagonist-occupied receptors, and alter the direction of receptor-dependent transcription. 1. We isolated a coactivator, which we have dubbed L7/SPA, that interacts with tamoxifen-occupied ER or RU-486 occupied PR and **increases** transcription by 3-10 fold. It does not enhance agonist-dependent transcription, however. 2. We also isolated a corepressor protein that **suppresses** the partial agonist activity of tamoxifen or RU-486. These proteins may enhance (L7/SPA) or inhibit (corepressor) development of hormone resistant breast cancers, we have preliminary data in tumors taken from patients, in which this is being tested.

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Principal Investigator's Signature

(4) TABLE OF CONTENTS

(5) INTRODUCTION

- The research in our laboratory focuses on breast cancer, and how the steroid hormones produced by the ovaries -- estradiol and progesterone -- are involved in the development and growth of these cancers.

- Additionally, because many breast cancers are hormone-dependent, which means that their growth is enhanced by estradiol and progesterone, treatment of the disease often involves the use of drugs that interfere with the actions of these hormones. Such interfering drugs are called steroid hormone antagonists. The best known of these antagonists are the antiestrogen, tamoxifen, and the antiprogestin, RU-486. Research in our laboratory seeks to understand just how steroid antagonists block the stimulatory effects of the steroid hormones in breast cancers.

- A key problem in the use of steroid antagonists to treat breast cancer, is that the tumors regress initially. However, in time, the tumors acquire resistance and resume growing. Research in our laboratory seeks to understand how tumors acquire resistance, with the goal of trying to block this process so that the effectiveness of antagonist treatments can be prolonged. Another outcome of this work is that it may suggest methods to design and screen for better antagonists; perhaps ones against which resistance is less likely to develop.

Therefore, the long-term goals of our research is to improve the strategies and outcomes of hormone therapies in breast cancers: by understanding how steroid hormones control cancer growth, by understanding how tumors become resistant to hormone treatments, and by devising ways through which development of resistance can be avoided.

Estradiol and progesterone are hormonal agonists produced by the ovaries. These hormones then enter the blood and reach their target organs which, in addition to the breast, include the uterus and cervix, bones, blood vessels, skin, brain and other sites. These organs are "targets" for the hormones because their cell nuclei have proteins called "hormone receptors". When the hormone reaches the target cells, it passes through the cell cytoplasm and into the nucleus, where it encounters and binds the appropriate receptors. This binding activates the receptors, which in turn bind to specific DNA sequences located in front of the genes being regulated, and (usually) activates those genes. In other words, steroid hormone receptors are transcription factors whose function is controlled by hormone binding. Breast cancers whose growth is stimulated by estradiol and/or progesterone, do so because the tumor cells have estrogen- (ER) and/or progesterone receptors (PR) which bind these hormones. Like the agonists, the antagonists tamoxifen and RU-486, bind the tumor ER and PR respectively, and block the effects of the hormonal agonists at those sites; hence the term "antagonist" (1,2,3).

The structure of nuclear steroid receptors has been partially characterized. These are large proteins with modular functional domains. At the downstream, or C-terminal end, is the hormone binding domain (HBD). A hinge region separates the HBD from a centrally positioned DNA binding domain (DBD) through which the receptors interact with DNA.

Upstream of this, at the N-terminus, are transcriptional activation functions and other poorly defined domains. Both PR and ER have this same generic structure. There is an additional complexity with respect to PR however, in that there are two receptor isoforms that differ in size: PR B-receptors have a 164 amino-acid extension at the far N-terminus (the B-upstream segment, or BUS), which is missing in PR A-receptors. Because of this, the two PR isoforms have different gene regulatory properties when they are occupied by agonists or antagonists at the HBD (4,5).

We postulate that breast cancers become "resistant" to hormone therapy because antagonists acquire inappropriate, agonist-like, effects. The clinical consequences of such a functional switch are grave. The studies we proposed to address in this grant include the molecular mechanism by which antagonist-occupied progesterone B-receptors become transcriptional agonists.

The aims of the present work were to:

Aim 1. Characterize functional differences between A- and B-receptors in breast cancer cells. Band A-receptors are present together in breast cancers. Transient transfection methods show that antagonist-occupied B-receptors, but not A-receptors, can act like transcriptional agonists in a promoter-specific manner or when cAMP levels are increased. We plan to stably transfect PR-negative breast cancer cell lines with expression vectors encoding B- or A-receptors. This allows each receptor isoform to be studied independently while being expressed at normal levels in a physiological setting. The cells will be used to assess long-term growth effects, and transcriptional regulation, by progesterone agonists and antagonists, with or without increased cAMP levels. For transcriptional studies, different promoter-reporter constructs will be analyzed. These studies will define the effects of each receptor isoform on the functional end-points of transcription and cell proliferation in the physiologically relevant setting of breast cells.

Aim 2. BUS - The B-upstream segment. A third transcriptional activation domain unique to B-receptors? Since only PR B-receptors anomalously induce transcription in the presence of progesterone antagonists, we will focus on their unique 164 amino acid extension -- the B-upstream segment, or BUS. Preliminary data show that BUS contains a novel transcriptional activation function we call AF-3. This is in addition to two other AFs (AF-1 and AF-2) that are common to both receptor isoforms. We postulate that AF-3 functions by binding one or more coactivator proteins. We will construct a series of expression vectors of BUS alone, or of BUS fused to the DNA binding domain (DBD) and nuclear localization signal (NLS) of PR. These constructs will be tested for their ability to constitutively regulate transcription; to "squelch" full-length B-receptor actions; to complement A-receptors; and to be cAMP modulated, all in promoter and cell-specific contexts. Mutants will be constructed of 5 *ser-pro* clusters found on BUS in order to map AF-3, and to analyze the control of B-specific transcription by phosphorylation. The studies in this aim will functionally define and characterize AF-3; a site unique to B-receptors.

Aim 3. Mechanisms of AF-3 action in the BUS segment. Antagonist-occupied B-receptors activate transcription in a promoter-specific fashion: on the mouse mammary tumor virus (MMTV) promoter when cAMP levels are elevated; on the *Herpes simplex* virus thymidine kinase

(*tk*) promoter, through a novel, PRE-independent mechanism. First, we plan to identify cis-acting elements on the MMTV and *tk* promoters through which antagonist-occupied B-receptors stimulate transcription. Site-specific mutants of the MMTV promoter will seek the cis-acting elements that eliminate cAMP effects without loss of PR-regulated transcription. We will test the hypothesis that cAMP acts through novel DNA elements that cooperatively bind the ATF/CREB and HMG family of proteins, and interact with BUS. Linker-scanning mutants of the *tk* promoter will be used to define elements that mediate antagonist-occupied B-receptor stimulation of transcription. Second, protein-protein interactions between antagonist-occupied B-receptors and as yet unknown coactivators will be characterized using bacterially produced BUS fusion proteins, or the yeast two-hybrid system to identify, isolate and clone cDNAs encoding nuclear proteins that interact with BUS and to analyze their tissue-specific distribution. The studies in this aim will define novel coactivator proteins that interact with the B-receptor isoform of PR, and select their direction of transcription.

(6) BODY

In 1997 we showed the following:

(A) We have previously observed that under certain conditions, tamoxifen and RU-486 inappropriately activate gene transcription in a manner that resembles agonists -- that is, they behave as partial agonists. Such a switch in the activity of antagonists could be one mechanism by which resistance develops in a tumor. Thus if the antagonist treatment that is supposed to be inhibitory, becomes stimulatory instead, it is bad news for the patient. We undertook to explain this switching behavior, and speculated that in some tumors, receptors occupied by antagonists are capable of attracting a nuclear protein with "coactivator" properties, to the transcription complex. In the presence of such a coactivator, the transcription apparatus activates, rather than inhibits gene activity. To find such a putative coactivator we went fishing in a library of human proteins, using the PR hinge-HBD as bait. Because we were interested in proteins that bind PR in the presence of antagonists, we treated the hinge-HBD with RU-486. We isolated two interesting proteins that interact with RU-486 occupied PR and tamoxifen-occupied ER:

1. A coactivator that had not previously been described, which we call L7/SPA has precisely the properties we predicted. When we overexpress L7/SPA in breast tumor cells together with tamoxifen-occupied ER or RU-486 occupied PR, the antagonists have 3 to 10 times greater ability to activate a reporter gene than they do in the absence of L7/SPA. Thus L7/SPA strongly enhances the partial agonist transcriptional activity of these antagonists. **We predict that L7/SPA is overexpressed in hormone resistant tumors (ie in patients whose tumors resume growing despite treatment with tamoxifen).**

2. We isolated a second protein, N-CoR, with unanticipated "corepressor" properties. Its overexpression suppresses the partial agonist effects of tamoxifen and RU-486, and reverses the excessive agonist effects produced by L7/SPA. **We predict that high levels of N-CoR expression can be used to identify tumors likely to respond well to treatment with antagonists.**

- *Twila A Jackson, Jennifer K Richer, David L Bain, Glenn S Takimoto, Lin Tung and Kathryn B Horwitz. 1997 The Partial Agonist Activity of Antagonist-Occupied Steroid Receptors is Controlled by a Novel Hinge Domain-Binding Coactivator L7/SPA and the Corepressors N-CoR and SMRT. Molecular Endocrinology 11: 693-705.*

These are key observations that we are pursuing as follows:

1. We have completed the development of a quantitative RT-PCR assay to measure mRNA expression levels for the coactivators L7/SPA and SRC-1, and for the corepressors N-CoR and SMRT.

2. During development of the SMRT assay, we discovered that there are three isoforms of this corepressor. Their levels vary in different breast cancer cell lines.

3. We have cloned the three SMRT isoforms into protein expression vectors and tested their ability to repress the partial agonist effects of tamoxifen and RU486. The three isoforms differ in this effect.

- *A manuscript describing these results is in preparation. Assays in tumors taken from patients are in progress (see Conclusions).*

4. In addition, with respect to L7/SPA, we have tested nine breast cell lines and 10 breast tumors and have interesting preliminary data. First, in six breast cancer cell lines, L7/SPA expression levels are high, while in three cell lines derived from normal breast cells, its levels are low. We require additional data to confirm this relationship. If true, it would suggest that an antiestrogen, like tamoxifen, is more likely to be an agonist in a malignant cell than in a normal cell. Second, in 10 breast tumors taken from patients, L7/SPA levels are high only in three ER+/PR+ tumors. Its levels are low in the remaining seven tumors that are either ER+/PR– or ER–/PR–. This too needs larger numbers for confirmation. These data suggest the hypothesis that L7/SPA levels can be regulated by progesterone. We are now testing this idea.

5. We are developing antibodies to L7/SPA so that levels of the protein can be measured. A putative peptide was synthesized and has been used to generate polyclonal antibodies. We are testing antibodies at the present time.

(B) In my opinion, a key aspect to understanding the differences in function between PR_A and PR_B, is to understand how the B-specific region BUS, modifies the structure of the PR N-terminus. To that end we are undertaking detailed structural analyses of the N-terminal A (NT_A), N-terminal B (NT_B) and of BUS alone or linked to the DBD. The first step is to purify these proteins to homogeneity in anticipation of structural studies involving protease digestion, nuclear magnetic resonance, circular dichroism, high pressure liquid chromatography, and other analytical methods. We have purified NT_A to greater than 98 percent purity by two methods: a) classical chromatographic methods, and b) by a combination of affinity chromatography plus classical methods. We are approaching this purification state for NT_B as well.

6. Other peer-reviewed papers published from 1997-to date include the following:

Petz LN, Nardulli AM, Kim J, **Horwitz KB**, Freedman LP and Shapiro DJ. DNA bending is induced by binding of the glucocorticoid receptor DNA binding domain and progesterone receptors to their response element. J STEROID BIOCHEM MOLEC BIOL 60:31-41, 1997.

*Miller MM, James RA, Richer JK, Gordon DF, Wood WM and **Horwitz KB**. Progesterone regulated expression of flavin-containing monooxygenase 5 is controlled by the B-isoform of progesterone receptors: Implications for tamoxifen carcinogenicity. J CLIN ENDOCRINOL METAB 82:2956-2961, 1997.

Groshong SD, Owen GI, Grimison B, Schauer IE, Daly MC, Langan TA, Sclafani RA, Lange CA and **Horwitz KB**. Biphasic regulation of breast cancer cell growth by progesterone: Role of the cdk inhibitors p21 and $p27^{KIP1}$. MOLEC ENDOCRINOL 11:1593-1607, 1997.

*Richer JK, Lange-Carter C, Wierman AM, Brooks KM, Jackson TA, Tung L, Takimoto GS and **Horwitz KB**. Novel progesterone receptor variants in breast cancers and normal breast cells

repress transcription by wild-type receptors. BREAST CANCER RES TREAT 48:231-241, 1998.

*Hovland AR, Powell RL, Takimoto GS, Tung L and **Horwitz KB**. An N-terminal inhibitory function (IF) suppresses transcription by the A-isoform but not the B-isoform of human progesterone receptors. J BIOL CHEM 273:5455-5460 1998.

Owen GI, Richer JK, Tung L, Takimoto GS and **Horwitz KB**. Progesterone regulates transcription of the $p21^{WAF1}$ cyclin dependent kinase inhibitor gene through Sp1 and CBP/p300. J BIOL CHEM 273:10696-10701, 1998

*Kumar NS, Richer JK, Owen GI, Litman E, **Horwitz KB** and Leslie KK. Selective down-regulation of progesterone receptor isoform B in poorly differentiated human endometrial cancer cells: Implications for unopposed estrogen action. CANCER RES 58:1860-1865, 1998.

Pahl PMB, Hodges-Garcia K, McItesen L, Perryman MB, **Horwitz KB** and Horwitz LD. ZNF207, a novel zinc finger gene on chromosome 6. GENOMICS, In Press, 1998.

*** Related to the goals of this grant and included in the appendix.**

The following papers are under review:

*Sartorius CA, Takimoto GS, Richer JK, Tung L and **Horwitz KB**. Colocalization of poly(ADP-ribose) polymerase and the Ku autoantigen/DNA-dependent protein kinase holoenzyme on the DNA binding domain of progesterone receptors and regulation of phosphorylation in a DNA-independent manner. Submitted, 1998.

Lange CA, Richer JK and **Horwitz KB**. Convergence of progesterone and epidermal growth factor signaling in breast cancer. I. Potentiation of mitogen-activated protein kinase pathways. Submitted, 1998.

Richer JK, Lange CA, Manning NG, Owen GI, Powell RL and **Horwitz KB**. Convergence of progesterone and epidermal growth factor signaling in breast cancer. II. Progesterone receptors regulate Stat5 expression and activity. Submitted, 1998.

(7) CONCLUSIONS

The findings described in (6) address the overall goals of our research, and provide the first practical evidence that hormone resistance can be understood, and perhaps even be prevented.

1. To test the predictions made above, we are now analyzing a series of tumor pairs, taken from patients before tamoxifen treatment was started, and then again after the tumors became resistant to tamoxifen. In these pairs of tumors, we are measuring expression levels of L7/SPA, N-CoR and a related corepressor called SMRT. If our hypotheses are correct, the levels of the two corepressors will be high in the hormone responsive tumors at the start of treatment, and will fall in the resistant tumors coincident with a rise in L7/SPA. If so, we will have gained important insights into the mechanisms of hormone resistance. In future, by measuring the levels of these proteins we could identify tumors likely to respond well to antagonists, and could anticipate development of resistance and possibly block its onset.

2. The purified N-terminal peptides of the progesterone receptors will be analyzed biochemically and biophysically. We hope to understand at a fundamental structural level, why the two receptors have different biological functions.

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2. Spitz IM and Bardin CW. Mifepristone (RU 486) - A modulator of progestin and glucocorticoid action. NEW ENGL J MED 329:404-412, 1993.

3. Yamamoto KR. Steroid receptor regulated transcription of specific genes and gene networks. ANN REV GENET 19:209-252, 1985.

4. Sartorius CA, Tung L, Takimoto GS and **Horwitz KB.** Antagonist-occupied human progesterone receptors bound to DNA are functionally switched to transcriptional agonists by cAMP. J BIOL CBEM, 268:9262-9266, 1993.

5. Tung L, Mohamed KM, Hoeffler JP, Takimoto GS and **Horwitz KB.** Antagonist-occupied human progesterone B-receptors activate transcription without binding to progesterone response elements, and are dominantly inhibited by A-receptors. MOLEC ENDOCRINOL 7:1256-1265, 1993.

(9) APPENDIX

Three copies of the following reprints are submitted:

Miller *et al*, 1997
Richer *et al*, 1997
Hovland *et al*, 1997
Kumar *et al*, 1997

Jackson *et al*, 1997 was submitted last year.

[CANCER RESEARCH 58, 1860–1865, May 1, 1998]

Selective Down-Regulation of Progesterone Receptor Isoform B in Poorly Differentiated Human Endometrial Cancer Cells: Implications for Unopposed Estrogen Action[1]

Nirmala S. Kumar, Jennifer Richer, Gareth Owen, Elizabeth Litman, Kathryn B. Horwitz, and Kimberly K. Leslie[2]

The Reproductive Molecular Biology Laboratory of the Department of Obstetrics and Gynecology [N. S. K., E. L., K. K. L.], the Division of Endocrinology, the Department of Medicine [N. S. K., J. R., G. O., K. B. H.], and the Department of Pathology [K. B. H.], the University of Colorado Health Sciences Center, Denver, Colorado 80262

ABSTRACT

The uterine endometrium responds to unopposed estrogen stimulation with rapid cell proliferation. Progesterone protects the endometrium against the hyperplastic effects of estradiol (E_2) through progesterone receptors (PRs), of which two isoforms are expressed: human (h) PRA and PRB. hPRB has a longer NH_2 terminus and may function differently from hPRA. Thus, the relative expression of hPRA:hPRB is likely to be important for the action of progesterone. We hypothesized that the hPRA:hPRB ratios may be abnormal in endometrial cancer, leading to a lack of normal progesterone protection against the growth-promoting effects of E_2. To test this hypothesis, well-differentiated Ishikawa endometrial cancer cells were compared to poorly differentiated Hec50 and KLE cells. Reverse transcription-PCR was chosen as a sensitive method to detect transcripts for the two forms of PR. The relative expression of PR isoforms under hormonal stimulation was determined by Western blotting. Transient transfections of hPRA and hPRB into endometrial cells allowed the evaluation of the transcriptional activity of each isoform independently on reporter gene transcription under the control of a simple progesterone response element-containing promoter. The effect of coexpressing the estrogen receptor on PR expression was also studied. Ishikawa cells (well-differentiated) express both hPRA and hPRB. Both isoforms, but predominantly hPRB, are up-regulated by E_2 and not by tamoxifen or the pure antiestrogen ICI 182,780. Hec50 and KLE cells (poorly differentiated) express only hPRA. No hPRB is present in the poorly differentiated cells, and it is not induced by estrogen receptor expression and/or estrogen treatment. In all cells, hPRB expression, whether endogenous or produced as a result of transfection, acts as a stronger transcription factor than hPRA on a simple progesterone-dependent promoter. We speculate that down-regulation of hPRB may predict for poorly differentiated endometrial cancers that do not respond to progestin therapy.

INTRODUCTION

Cancer of the uterine endometrium is the most common malignancy of the female genital tract, is the third most common cancer in women, and is responsible for 13% of gynecological cancer deaths (1). The induction of endometrial cancer is related to hyperestrogenism unopposed by progesterone. This conclusion is based on studies linking high rates of endometrial cancer to polycystic ovary disease and other hyperestrogenic states in young women (2–4) and to the use of unopposed estrogen replacement therapy in postmenopausal women (5–8), the tumorigenic effects of which can be completely reversed by the addition of a progestational agent (9).

Progesterone counteracts the growth-stimulatory effects of estrogen by inducing glandular and stromal differentiation (10, 11). Endometrial hyperplasia can be reversed by progestin therapy, and progestin treatment is effective in decreasing the growth of endometrial tumors that express PRs[3] (12). The PR content of endometrial cancers is strongly correlated with successful endocrine treatment and survival (13). Although nearly all endometrial tumors express ER at the high levels observed in the proliferative phase of the menstrual cycle, expression of PR is variable; even in the presence of ER, some tumors do not contain PR (14, 15). Because PRs are normally up-regulated by estrogens via ERs (16–19), this implies that failure to induce PR may be a factor in the genesis and/or progression of endometrial cancer.

hPRs are ligand-activated transcription factors that have a hormone-binding domain at the COOH terminus, a DNA-binding domain through which the receptors contact DNA, and two variable-length NH_2 termini that produce the two major isoforms [truncated A receptors (20) and B receptors] that contain an additional 164 amino acids at the NH_2 terminus (21, 22). Both isoforms have an activation function, AF1, just upstream of the DNA-binding domain and another, AF2, in the hormone-binding domain. Additionally, the unique NH_2-terminal sequence of B receptors contains a strong third transcriptional activating function, AF3 (23).

Based on work in T47D breast cancer cells (21) and in some endometrial tumors (24), it was believed that hPRA and hPRB were expressed in approximately equimolar ratios, were dimerized as homo- and heterodimers with equal frequency, and had similar functions. However, recent studies have uncovered provocative differences between hPRA and hPRB expression and function in normal endometrium (25) and breast cancers (22, 26). When hPRA:hPRB ratios were examined in the normal cycling endometrium by immunoblotting (27, 28), hPRA was found to be expressed more highly than hPRB throughout the cycle. However, hPRB expression increased more sharply than hPRA in response to increasing E_2 levels at midcycle and disappeared completely in the late secretory phase, whereas hPRA expression continued to be present throughout the cycle. In a recently published analysis of PRs in primary breast tumors, a range of isoform ratios was found, with hPRA exceeding hPRB in 76% of the cases (29).

It has also become increasingly apparent that hPRA and hPRB have different functional characteristics. hPRB is a stronger transcriptional activator of many PRE-containing promoters than is hPRA (23, 16, 30), although the differences are cell specific, and at least two examples exist in which the transcriptional activity of hPRA exceeds that of hPRB (31–33). The magnitude of transcriptional activation by hPRB can be significantly greater than that by hPRA. On the mouse mammary tumor virus promoter, for example, transcriptional activation by hPRB exceeds that by hPRA by 10-fold (20).

Whereas hPRB and hPRA are primarily transcriptional activators of progesterone-responsive genes, hPRA also functions as a transcriptional inhibitor of other steroid hormone receptors, including ER (30) and hPRB (32, 34). The term dominant-negative has been applied to hPRA with respect to its inhibitory effects on other receptors (32). These studies have generated the hypothesis that hPRA and hPRB

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[2] To whom requests for reprints should be addressed, at Box B-198, the University of Colorado Health Sciences Center, 4200 East 9th Avenue, Denver, CO 80262.

[3] The abbreviations used are: PR, progesterone receptor; RT-PCR, reverse transcription-PCR; ER, estrogen receptor; h, human; PRE, progesterone response element; E_2, 17β-estradiol; E_1, estrone; CAT, chloramphenicol acetyltransferase.

levels are tightly regulated and comprise a dual system to control the actions of progesterone (16).

The studies reported herein were undertaken to determine the relative abundance of the PR isoforms, their expression in response to estrogens and antiestrogens, and their effects on progesterone-dependent reporter gene transcription in cultured endometrial cancer cells. We hypothesized that functional differences between hPRA and hPRB have significance in the endometrium, especially with respect to the ability of PR to counter estrogen-mediated endometrial glandular proliferation. Low expression of either hPRA or hPRB could lead to unopposed estrogen stimulation by two independent mechanisms: (*a*) loss of hPRB would result in lack of transcription of progesterone-responsive genes, presumably including those responsible for cell differentiation; and (*b*) loss of hPRA would result in lack of dominant-negative inhibition of estrogen-stimulated cell proliferation. In these studies, we demonstrate that differential expression of PR isoforms does occur in cultured endometrial cancer cells with potential consequences for tumor development and progression.

MATERIALS AND METHODS

Cell Lines. Ishikawa and Hec50 cells were a generous gift from Dr. E. Gurpide (Mt. Sinai City University of New York, New York, NY). KLE cells were obtained from the American Type Culture Collection (Bethesda, MD). The cell lines were maintained in Eagle's MEM [MEM-$CaCl_2$ (anhydrous; 200 mg/liter), $Fe[NO_3]\cdot 9H_2O$ (0.10 mg/liter), KCl (400 mg/liter), $MgSO_4$ (anhydrous; 97.67 mg/liter), NaCl (6400 mg/liter), $NaHCO_3$ (3700 mg/liter), $NaH_2PO_4\cdot H_2O$ (125 mg/liter), $C_6H_{12}O_6$ (1 g/liter), and $C_3H_3O_3Na$ (110 mg/liter); Life Technologies, Inc., Gaithersburg, MD) in the presence of 7.5% fetal bovine serum (Gemini Bio Products, Inc. Calabasas, CA), 2 mM L-glutamine (Life Technologies, Inc.), and an antibiotic/antimycotic solution containing penicillin-G (50 units/ml), streptomycin (50 units/μg), and fungizone (0.125 unit/μg; all from Gemini Bio Products, Inc.). Before transfection and after hormone treatment, the cells were plated in phenol red-free MEM containing 7.5% double charcoal-stripped fetal bovine serum and 2 mM L-glutamine to reduce the media concentrations of endogenous contaminating agents such as estrogenic compounds.

RNA Isolation. Cells were washed and lysed using guanidinium isothiocyanate solution. Total RNA was isolated by phenol-chloroform extraction according to Chomczynski and Sacchi (35).

RT-PCR. RT-PCR kits were purchased from Perkin-Elmer Corp. (Branchburg, NJ). cDNAs were synthesized from 1.5 μg of total RNA using random hexamer primers. cDNA synthesis was carried out as suggested by the kit protocol, except that the RNA was incubated at 65°C) for 5 min to denature the RNA before the addition of murine leukemia virus reverse transcriptase. The reverse transcription was carried out for 60 min at 42°C. After an incubation at 99°C for 5 min to inactivate the reverse transcriptase, the entire 20-μl cDNA synthesis reaction was used in subsequent PCR reactions with specific primers to amplify regions of PR common to both isoforms and regions unique to hPRB. β-actin cDNA fragments were also amplified as positive controls (Table 1).

Table 1 *Location and sequence of primers*

Position	Size (bp)	Sequence
B-unique		
Forward-744	429	ACAGAATTCATGACTGAGCTGAAGGCAAAGGGT
Reverse-1173		ACAAGATCTCAAACAGGCACCAAGAGCTGCTGA
Common to A and B		
Forward-1239	243	ACAGAATTCATGAGCCGGTCCGGGTGCAAG
Reverse-1482		ACAAGATCTCCACCCAGAGCCCGAGGTTT
β-Actin[a]		
Forward-1386	229	AAGGCCAACCGCGAGAAGAT
Reverse-2076		TCGGTGAGGATCTTCATGAG
β-Actin[b]		
Forward-1386	771	AAGGCCAACCGCGAGAAGAT
Reverse-2811		GTGGACGATGGAGGGGCCGGACTC

[a] Spans one intron.
[b] Spans two introns.

Primers for β-actin were chosen specifically to cross one or two introns in the β-actin gene. In the presence of contaminating genomic DNA, additional larger bands would be amplified; the lack of amplification of the larger band was used as a control to rule out contamination with genomic DNA. For example, using the first β-actin primer set, which spans a 471-bp intron, only a 229-bp band would be amplified in the absence of genomic DNA; however, a 229-bp fragment and a 700-bp fragment would be amplified if contaminating genomic DNA were present. Negative controls without RNA and without reverse transcriptase were also performed. Ten percent of each reaction was run on a 2% agarose gel, stained with ethidium bromide, and analyzed. Previously, the specific bands had been isolated, cloned, and sequenced to assure that the amplified DNA represented the product expected (data not shown).

Whole Cell Protein Extraction. Cells were plated on 15-cm plates. One day after plating, the cells were treated with either vehicle (ethanol) alone; 0.01 μM E_2, E_1, and *trans*-tamoxifen (all from Sigma, St. Louis, MO); or ICI 182,870 (Imperial Chemical Industries; a gift of Zeneca Pharmaceuticals, Macklesfield, United Kingdom) dissolved in ethanol. Plates were washed with cold buffer (0.2 g/liter KCl, 0.2 g/liter KH_2PO_4, 8 g/liter NaCl, and 2.16 g/liter $Na_2HPO_4 0\cdot 7H_2O$) and scraped with a rubber policeman. The cells were spun down, and the pellets were snap-frozen on dry ice. Cell pellets were then thawed in high-salt buffer [0.4 M KCl, 29 mM HEPES (pH 7.4), 1 mM DTT, and 20% glycerol] plus protease inhibitors for 5–10 min. The extracts were passed through a 28-gauge needle and centrifuged at 50,000 × *g* for 20 min. Bradford assays were performed in duplicate on the supernatant to quantitate the amount of protein (36).

Western Blotting. Enhanced chemiluminescence Western blotting kits were purchased from Amersham (Arlington Heights, IL), and the kit instructions were followed. Briefly, protein extract (800 μg/lane) was loaded on a 7.5% SDS-polyacrylamide gel. This was run at 8 mV overnight in tank buffer [25 mM Tris, 192 mM glycine, and 0.1% SDS (pH 8.3)]. The proteins were transferred to nitrocellulose membranes (Amersham) that were then incubated with a mouse monoclonal antibody whose epitope lies in the NH_2-terminal region common to both hPRA and hPRB (AB-52; Ref. 33). The membranes were subsequently incubated with a goat antimouse secondary antibody (Cappel; Organon Teknika Corp., West Chester, PA) followed by Luminol reagent (Amersham), and chemiluminescence was detected by autoradiography.

Transient Transfections and Reporter Assays. Ishikawa, Hec50, and KLE cells were plated in 10-cm dishes in triplicate and grown to 75–80% confluence. For each plate, 1 μg of the progestin-dependent reporter plasmid PRE_2-$TATA_{tk}$-CAT (23), containing two consensus PREs cloned upstream of the TATA box from the thymidine kinase gene and the CAT reporter gene was transfected. Additionally, 3 μg of the expression vector for β-galactosidase, pCH110, ± 0.1 μg of the expression vector for ER, pSG5-HEGO (a gift from P. Chambon, CRNS/INSERM/ULP, Cedex France), were introduced into cells by lipofection using 20 μl of LipofectAMINE (Life Technologies, Inc.). In experiments designed to test the expression and transcriptional efficiency of ER introduced into KLE and Hec50 cells, the reporter plasmid vit-tk-CAT was used (a gift from P. Chambon). This vector encodes the consensus estrogen response element from the vitellogenin A2 gene upstream from the thymidine kinase promoter (37). Twenty-four h posttransfection, the cells were treated with vehicle alone or with hormone. At 48–72 h posttransfection, CAT activity was measured in cellular extracts by TLC according to previously published methods (38). Each reaction was normalized to β-galactosidase activity to correct for the efficiency of transfection between plates. Experiments were performed in triplicate and repeated at least three times. The results are shown as representative experiments analyzed by the Student's *t* test, and presented as the mean ± SE.

RESULTS

PR Isoform Expression and Regulation by E_2

RT-PCR and Western blotting were used to determine the differential expression of PR isoform transcripts and proteins and whether estrogens induce PR in Ishikawa, KLE, and Hec50 endometrial cancer cells.

mRNAs for hPRA Are Present in Ishikawa, Hec50, and KLE Cells; However, Only Ishikawa Cells Express hPRB mRNAs. Fig. 1*a* shows data from well-differentiated Ishikawa cells. Fig. 1*b* shows data from poorly differentiated KLE cells. Results identical to those for KLE were obtained for the Hec50 cells (data not shown). In Fig. 1, *a* and *b*, *Lane 1* represents amplification of PR mRNAs encoding sequences common to both A and B receptors. A band is seen in both cell types. However, *Lane 2* represents B receptors only. Clearly, whereas B-receptors are present in Ishikawa cells, they are missing in KLE cells. When both sets of primers are combined in the same reaction (primers common to both A and B receptors plus primers specific to B receptors) as shown in *Lane 3*, amplification of transcripts unique to B receptors occurs only in Ishikawa cells. β-Actin products of two different sizes (771 and 229 bp) were used as positive controls. Negative controls, one without RNA (*Lane 5*) and the other without reverse transcriptase (*Lane 6*), were included.

hPRA Proteins Are Detectable in Ishikawa, Hec50, and KLE Cells, While hPRB Proteins Are Only Expressed in Ishikawa Cells. Fig. 2 is a representative Western blot showing hPRA and hPRB expression in Ishikawa, KLE, and Hec50 cells in the presence or absence of E_2. The 116–120-kDa triplet band characteristic of

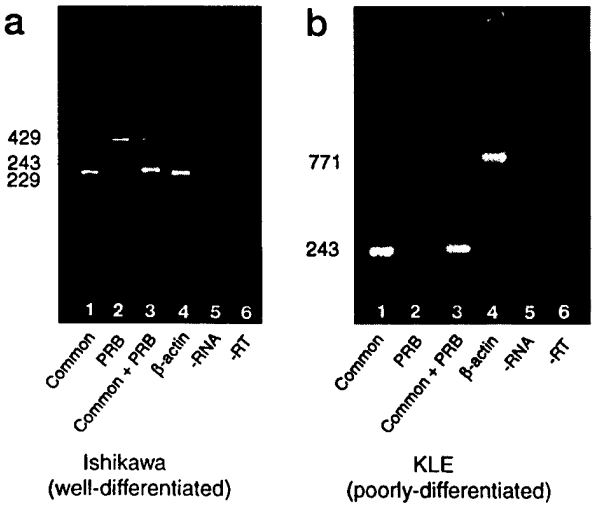

Fig. 1. Ishikawa cells express transcripts encoding hPRB, but KLE cells do not. RT-PCR of total RNA extracted from Ishikawa cells (*a*) and KLE cells (*b*). *Lane 1* shows amplification of cDNAs using primers common to both hPRA and hPRB. *Lane 2* shows data when primers unique to the NH_2 terminus of hPRB are used. Data are also shown when both sets of primers are combined (*Lane 3*). Two different β-actin products of 771 and 229 bp were amplified as positive controls (*Lane 4*). Negative controls without RNA (*Lane 5*) or reverse-transcriptase (*Lane 6*) were included.

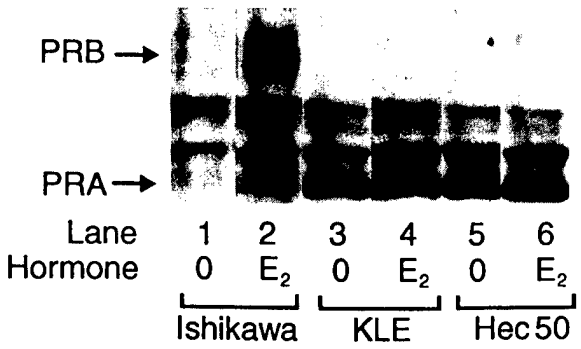

Fig. 2. E_2 induces hPRB only in Ishikawa cells, and hPRA is constitutive in KLE and Hec50 cells. Protein was extracted as detailed in "Materials and Methods," and 800 μg of total protein/lane were resolved on a 7.5% SDS-PAGE gel. Proteins were transferred to nitrocellulose, probed with the anti-PR monoclonal antibody AB52, and visualized by chemiluminescence. E_2, 0.01 μM E_2 dissolved in ethanol. *0*, ethanol alone.

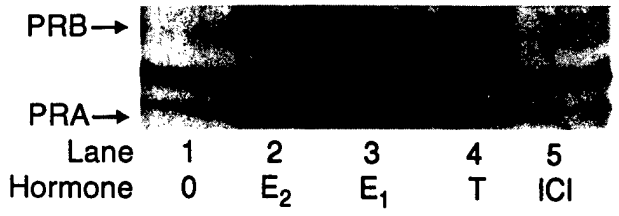

Fig. 3. Estrogens, but not antiestrogens, induce hPRB in Ishikawa cells. A horm survey of estrogens (E_2 and E_1, *Lanes 2* and *3*, respectively) *versus* antiestro (tamoxifen and ICI 182,780, *Lanes 4* and *5*, respectively) is shown. *0*, ethanol alon *trans*-tamoxifen; *ICI*, ICI 182,780.

hPRB is only present in well-differentiated Ishikawa cells (*Lane* and is significantly up-regulated after E_2 treatment (*Lane 2*), wher the 96-kDa hPRA band is present in all cell lines. A receptors up-regulated by E_2 in Ishikawa cells (*Lane 2*) but seem to be con tutively expressed in KLE and Hec50 cells (*Lanes 3–6*). Whereas further enhances hPRA expression in Ishikawa cells, it does induce hPRA expression in KLE or Hec50 cells. These data confi at the protein level those obtained for mRNA by RT-PCR and indic that only well-differentiated Ishikawa cell express B recept whereas A receptors are present in all three cell lines.

The Up-Regulation of hPRA and hPRB Proteins Requires Estrogen and Is Not Induced in the Presence of the Antiestrog Tamoxifen or ICI 182,780. Fig. 3 is a representative Western b showing the presence of hPR proteins in Ishikawa cells after E_2, tamoxifen (*T*), or ICI 182,780 treatment. Note the up-regulat primarily of hPRB and, to a lesser extent, hPRA in response to E_2. is a weak estrogen in Ishikawa cells (39).

Reporter Gene Transcription by hPRA and hPRB

Transfection experiments were used to answer three questions: What is the transcriptional activity of endogenous hPR on a sim PRE-containing promoter in Ishikawa, KLE, and Hec50 cells? What is the effect of enhancing hPRA *versus* hPRB expression transfection on reporter gene transcription under the control o PRE-containing promoter? (*c*) In KLE and Hec50 cells that do express ER, is it possible to reinstate PR-mediated gene transcript by introducing ER and thereby up-regulating endogenous PR expr sion? The data addressing each question are presented below.

Endogenous Expression of hPRB in Ishikawa Cells Allo Progestin-mediated Transcription of a Reporter Plasmid C trolled by a Simple PRE-containing Promoter; No Transcripti Occurs in hPRB-negative Hec50 and KLE Cells. Fig. 4 is a r resentative experiment showing CAT activity in Ishikawa and K cells transfected with PRE_2-$TATA_{tk}$-CAT in response to E_2 and/or progesterone agonist R5020. Ishikawa cells demonstrate low basel CAT activity in the absence of hormone. R5020 alone doubles transcriptional activity, suggesting that the low constitutive levels PR are functional. However, the effect of R5020 is significan enhanced by pretreatment with E_2 for 24 h to further induce PR. Wh this is followed by R5020 treatment, an additional 2–3-fold incre in CAT activity occurs. No significant induction of CAT activ occurs when the cells are pretreated with ligands that act as anti trogens, such as tamoxifen or ICI 182,780. Interestingly, the w estrogen E_1 can mimic the effects of the potent estrogen E_2 w respect to PR induction in Ishikawa cells. We have shown that E_1 converted to E_2 in these cells;[4] therefore, we speculate that response to E_1 is actually due to its conversion to E_2.

Based on Western blotting data (Figs. 2 and 3), we hypothesize t

[4] K. K. Leslie, unpublished data.

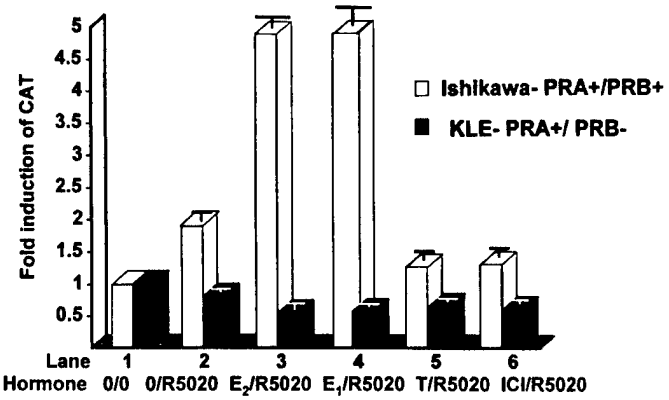

Fig. 4. E_2 enhances the transcription resulting from endogenous PR in Ishikawa cells. Cells were transfected with a reporter vector, PRE_2-$TATA_{tk}$-CAT, and then treated with ethanol alone (*Lanes 1* and *2*), estrogens (E_2 and E_1, *Lanes 3* and *4*, respectively), or antiestrogens (tamoxifen and ICI 182,780, *Lanes 5* and *6*, respectively) 24 h posttransfection. Forty-eight h posttransfection, the cells were treated with ethanol alone (*Lanes 1* and *2*) or R5020, a synthetic progestin (*Lanes 3–6*). Lysates were normalized to β-galactosidase activity, CAT activity was measured by TLC, and the average of triplicate determinations is shown. *0*, ethanol alone; *T*, *trans*-tamoxifen; *ICI*, ICI 182,780.

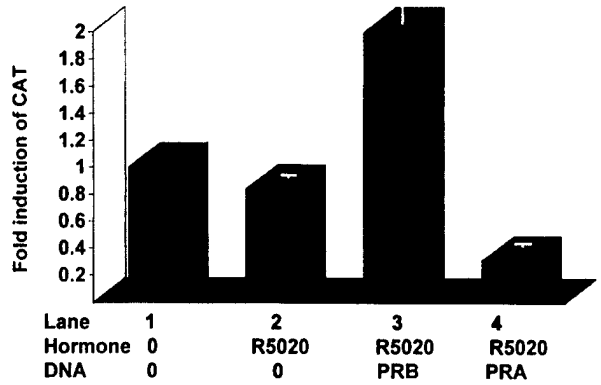

Fig. 5. Exogenously expressed B receptors activate transcription in KLE cells, whereas A receptors inhibit basal transcription. KLE cells were transfected with PRE_2-$TATA_{tk}$-CAT and expression vectors for hPRB (*Lane 3*) or hPRA (*Lane 4*). Transfections resulted in an increase in PR expression of approximately 40 fm/mg protein. Twenty-four h posttransfection, the cells were treated with ethanol alone (*Lane 1*) or R5020, a synthetic progestin (*Lanes 2–4*). Lysates were normalized to β-galactosidase activity, CAT activity was measured by TLC, and the average of triplicate determinations is shown.

the increased CAT activity in Ishikawa cells in response to E_2 is associated with the specific up-regulation of endogenous hPRB, which, in contradistinction to hPRA, is known to strongly activate many PRE-containing promoters (23, 16, 30). Fig. 4 shows that KLE cells exhibit very little baseline CAT activity, and that this is not increased with E_2 treatment, consistent with the Western blot data showing that E_2 cannot up-regulate hPRB expression in these cells. However, KLE cells do express hPRA (Fig. 2). Therefore, the data in Fig. 4 also indicate that hPRA is not a strong activator of PRE_2-$TATA_{tk}$-CAT, as has been demonstrated previously for other PRE-containing promoters (32). The response of Hec50 cells, which also express only hPRA, is similar to that of KLE cells (data not shown).

Introduction of hPRB, but not hPRA, Restores Progestin-mediated Induction of PRE_2-$TATA_{tk}$-CAT in KLE Cells. As has been demonstrated, KLE cells express only endogenous hPRA, not hPRB. The experiment in Fig. 5 was designed to test the effect of introducing hPRB into KLE cells. CAT activity controlled by the PRE_2-$TATA_{tk}$ promoter was used as an indicator of PR action. Fig. 5 is a representative experiment demonstrating CAT activity in KLE cells transfected with expression vectors for hPRB and hPRA. Transfection of the hPRB expression vector led to a 2-fold induction of CAT activity above control levels and a 5-fold induction of CAT over cells transfected with the hPRA expression vector. Therefore, these data confirm the increased *trans*-activating capability of hPRB compared to hPRA on the PRE_2-$TATA_{tk}$-CAT vector.

Another finding of interest relates to the magnitude of hPRB induction of CAT in these experiments. KLE cells express relatively high endogenous levels of hPRA. When hPRB expression is introduced, CAT induction is increased, but only modestly, resulting in a 2-fold induction. This is low compared to the 3–4-fold levels observed in Ishikawa cells (Fig. 4), which express relatively higher levels of endogenous hPRB and lower levels of hPRA. We hypothesize that the induction of CAT in KLE cells by hPRB is dampened by the endogenous hPRA. In addition, comparing *Lane 1* with *Lane 4* in Fig. 5, CAT expression is consistently below baseline when hPRA is overexpressed in KLE cells. These data may be examples of the dominant-negative effects (32) of hPRA on the basal transcription apparatus of the PRE_2-$TATA_{tk}$ promoter.

Estrogen Responsiveness Can Be Induced in Hec50 and KLE Cells by Transfection of an Expression Vector for ER. Before evaluating whether ER could induce hPRB expression in ER-negative KLE and Hec50 cells, we first determined whether the cells could be successfully transfected with the ER expression vector, pSG5-HEGO, and whether estrogen responsiveness could be restored. The representative experiment shown in Fig. 6 demonstrates that E_2 treatment results in CAT induction when KLE and Hec50 cells are transfected with an estrogen-responsive reporter gene, vit-tk-CAT, and pSG5-HEGO. Estrogen-mediated gene transcription is reinstated by this manipulation in both cell lines. We next asked whether endogenous genes normally regulated by E_2, including PR, could be induced in KLE and Hec50 if ER expression was restored.

ER Expression in KLE Cells Fails to Reinstate PR Expression. We showed in Fig. 2 that no induction of either PR isoform occurs in wild-type KLE and Hec50 cells in response to E_2. This is an expected result because, unlike Ishikawa cells, KLE and Hec50 cells do not express endogenous ER. In the experiment shown in Fig. 7, we wished to test the premise that reintroduction of ER would result in hPRB up-regulation with subsequent induction of the PRE_2-$TATA_{tk}$ promoter in response to E_2 plus R5020. A representative experiment is shown in which KLE cells were transfected with the ER expression vector and with the promoter/reporter PRE_2-$TATA_{tk}$-CAT to measure the transcriptional activity of any induced endogenous PR. The cells were treated with E_2 for 24 h to induce PR, followed by R5020 to

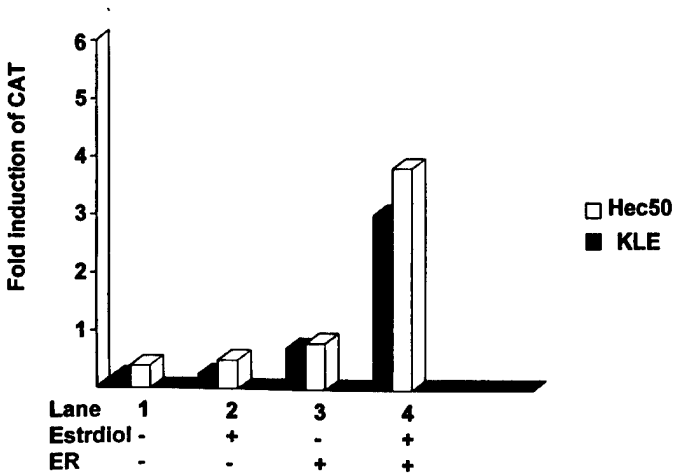

Fig. 6. Introduction of exogenous ER into KLE or Hec50 cells activates a synthetic estrogen-responsive reporter. KLE and Hec50 cells were transfected with the ER reporter vector vit-tk-CAT and with (*Lanes 3* and *4*) or without (*Lanes 1* and *2*) the expression vector for ER, pSG5-HEGO. Twenty-four h posttransfection, cells were treated with vehicle alone or with vehicle + 0.01 μM E_2, and CAT activity was measured.

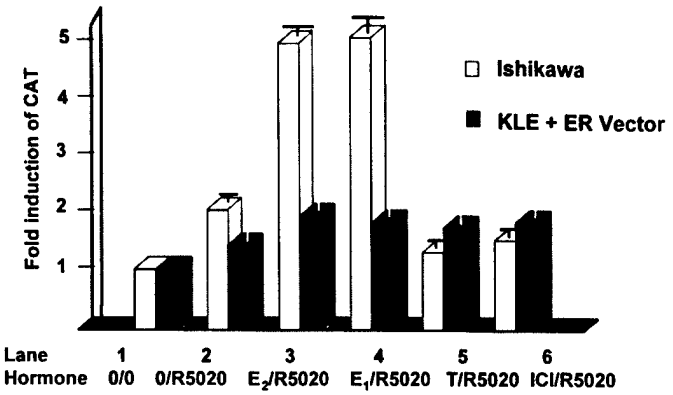

Fig. 7. Introduction of ER into KLE cells fails to activate endogenous PR. KLE cells were transfected with the progesterone-responsive reporter PRE_2-$TATA_{tk}$-CAT and an expression vector for ER, pSG5-HEGO (*Lanes 1–6*). Cells were treated with ethanol alone (*Lanes 1* and *2*), E_2 or E_1 (*Lanes 3* and *4*, respectively), or antiestrogens (tamoxifen or ICI 182,780, *Lanes 5* and *6*, respectively) 24 h posttransfection. Forty-eight h posttransfection, the cells were treated with ethanol alone (*Lane 1*) or with the progestin R5020 dissolved in ethanol (*Lanes 2–6*). CAT activity driven by PR was measured. *0*, ethanol alone; *T*, *trans*-tamoxifen; *ICI*, ICI 182,780.

activate PR. Interestingly, little significant induction of R5020-driven CAT activity occurs in the E_2-treated cells (*Lane 3*) compared to the untreated cells (*Lane 2*), suggesting that hPRB expression is not reinstated simply by reintroducing ER into the KLE cells. Similar results were obtained for Hec50 cells (data not shown). In contrast, Ishikawa cells show a strong R5020 response after E_2 priming. These data suggest that compared to estrogen-responsive Ishikawa cells, KLE and Hec50 cells have at least two alterations: (*a*) the ER is not synthesized; and (*b*) at least some ER-responsive pathways including PR expression have become desensitized.

DISCUSSION

We report an analysis of PR expression and function in well-differentiated endometrial cancer cells, Ishikawa cells, and poorly differentiated Hec50 and KLE cells that represents one of the first attempts to determine the consequences of differential PR isoform expression in endometrial cancer. Ishikawa cells are estrogen responsive and express endogenous ER (30–50 fm/mg protein; data not shown). Like most endometrial cells grown *in vitro*, neither Hec50 nor KLE cells express endogenous ER; however, they can be rendered responsive to estrogen (as measured by activation of an estrogen response element-containing promoter) by cotransfecting an expression vector encoding ER. This is shown in Fig. 6.

In general, the vast majority of endometrial cancers, and these cell lines in particular, arise from the glandular epithelium. Such cells are excellent models to study PR action, because in the endometrial glandular epithelium, the role of progesterone through its receptor is primarily antagonistic to estrogen-mediated cell proliferation (10). This is in contrast to the breast, where progesterone mediates both proliferative and antiproliferative effects (40, 41). Therefore, the study of the expression and function of hPR isoforms has particular importance for the endometrium, which relies on progesterone to induce cellular differentiation.

Differential expression of hPR isoforms was determined by RT-PCR and by Western blotting. The functional significance of the expression of hPRA *versus* hPRB in Ishikawa, Hec50, and KLE cells was investigated by transfection experiments using the CAT reporter gene under the control of a progesterone-responsive promoter (PRE_2-$TATA_{tk}$).

All cells tested express hPRA (Figs. 1, *a* and *b*, and 2). In addition, well-differentiated Ishikawa cells express hPRB; however, poorly differentiated Hec50 and KLE cells do not (Figs. 1 and 2). hPRB is up-regulated by estrogen in Ishikawa cells (Figs. 2 and 3) but is not induced by estrogen in Hec50 and KLE cells (Fig. 2). This seems to be true even in the presence of ER, because transfection of an expression vector encoding ER and estrogen treatment do not reinstate hPRB-mediated gene expression in KLE cells (Fig. 7).

In experiments designed to test the strength of transcriptional activation of hPRA *versus* hPRB on the simple PRE_2-$TATA_{tk}$ promoter, we find that hPRB is a more powerful activator of reporter gene transcription than is hPRA. This is true for cells that are selectively transfected with equal amounts of expression vectors for hPRA and hPRB (Fig. 5). As has been reported in other systems (32), we note that hPRA may be an inhibitor of basal transcription in KLE cells (Fig. 5). It should be noted that unlike PRE_2-$TATA_{tk}$, other promoters are more strongly induced by hPRA than hPRB (31, 32, 33), so we anticipate that there are subsets of genes activated primarily by hPRA as well. The study of which progesterone-responsive promoters are induced by each PR isoform is currently underway in our laboratory.

Unopposed estrogen stimulation is the common thread that underlies the development of many endometrial cancers. How does unopposed estrogen stimulation occur? We hypothesize that down-regulation of hPRA or hPRB may contribute to unchecked cell proliferation. It is likely that both isoforms are important to protect the endometrium (hPRA for its dominant-negative effect on ER, and hPRB for its ability to strongly up-regulate the transcription of genes required for differentiation). Poorly differentiated KLE and Hec50 cells demonstrate abnormal expression of PR isoforms compared to Ishikawa cells, which are well-differentiated and responsive to progestins (42). KLE and Hec50 cells no longer express hPRB but retain expression of hPRA. If the dominant-negative effects of hPRA are indeed important in countering ER-induced transcription (30), this function is likely to be preserved in cells such as KLE and Hec50. However, it is predicted that such cells do not express the hPRB-dependent genes required for progesterone-mediated cell differentiation. These studies indicate that low expression of hPRB portends a poorly differentiated endometrial cancer phenotype that may not respond to progestin therapy.

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An N-terminal Inhibitory Function, IF, Suppresses Transcription by the A-isoform but Not the B-isoform of Human Progesterone Receptors*

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The B-isoform of human progesterone receptors (PR) contains three activation functions (AF3, AF1, and AF2), two of which (AF1 and AF2) are shared with the A-isoform. AF3 is in the B-upstream segment (BUS), the far N-terminal 164 amino acids of B-receptors; AF1 is in the 392-amino acid N-terminal region common to both receptors; and AF2 is in the C-terminal hormone binding domain. B-receptors are usually stronger transactivators than A-receptors due to transcriptional synergism between AF3 and one of the two downstream AFs. We now show that the N terminus of PR common to both isoforms contains an inhibitory function (IF) located in a 292-amino acid segment lying upstream of AF1. IF represses the activity of A-receptors but is not inhibitory in the context of B-receptors due to constraints imparted by BUS. As a result, IF inhibits AF1 or AF2 but not AF3, regardless of the position of IF relative to BUS. IF is functionally independent and strongly represses transcription when it is fused upstream of estrogen receptors. These data demonstrate the existence of a novel, transferable inhibitory function, mapping to the PR N terminus, which begins to assign specific roles to this large undefined region.

Transcriptional control in response to extracellular signals involves the binding of regulatory proteins to specific enhancer elements of target genes. These proteins contain activation functions (AFs)[1] through which contact is made with the basal transcription machinery either directly or indirectly by means of intermediary coregulatory proteins (1). Progesterone receptors (PR) are members of the nuclear receptor family of ligand-inducible transcription factors. These are structurally complex proteins containing multiple functional domains, including a highly conserved central DNA-binding domain (DBD), a moderately well conserved C-terminal hormone-binding domain (HBD), and a poorly conserved, N-terminal region whose function is largely unknown (1).

There are two naturally occurring isoforms of PR. The 933-amino acid B-receptors contain an N-terminal 164-amino acid upstream segment (BUS) that is missing in the truncated 769-amino acid A-receptors (2–5). The two PR isoforms have AF1 and AF2 in common (5, 6). AF1 maps to a 91-amino acid "proline-rich" segment located just upstream of the DBD and AF2 is located in the HBD (6). BUS, restricted to B-receptors, contains AF3 (5). In general, B-receptors are stronger transactivators than A-receptors (5, 7–9), and only B-receptors can activate transcription in the presence of antiprogestins (9–11). On the other hand, A-receptors can dominantly inhibit B-receptors (9, 12, 13) as well as other members of the steroid receptor family (14).

In addition to AFs, some transcription factors also contain inhibitory domains (IDs) that modulate the activity of the AFs. Such IDs have been identified by deletion mutagenesis that generate proteins with enhanced transcriptional activities. Examples include members of the AP1 family c-Jun (15), c-Fos, and the related protein, FosB (16); ATF-2, a member of the ATF/cAMP regulatory element-binding protein subfamily of basic region leucine zipper (bZIP)-containing transcription factors (17); and the lymphoid-specific transcription factor, Oct-2a (18). An ID has also been found in the proto-oncogene c-Myb, which plays a key role in hematopoesis (19). Finally, IDs have been characterized in two yeast transcription factors: PHO4, which is regulated by phosphate levels (20), and ADR1, which regulates glycerol metabolism genes (21). To date, no ID has been described in the nuclear receptor family of transcription factors.

The IDs are structurally distinct from the AFs that they regulate (15–21). In some cases, inhibition is transferable to heterologous AFs, suggesting that the IDs are functionally independent. For example, when fused to the *Escherichia coli* polypeptide B42, the inhibitory regions of ADR1 repress transcription (21). Similarly, the IDs of c-Myb and c-Jun can inhibit the activity of VP16, a potent transactivator (15, 19). Inhibition by other IDs, however, is restricted to either the cognate AFs or a certain subset of AFs. For example, the bZIP domain of ATF-2 inhibits the related AFs of ATF-2 and E1a but not the acidic AF of VP16 or the glutamine-rich AF of Sp1 (17). Similarly, the N-terminal ID of c-Fos specifically silences the HOB1 subset of AFs found in c-Fos and c-Jun but not other phosphorylation-dependent AFs such as that found in cAMP regulatory element-binding protein (16).

Because A-receptors are weak transactivators compared

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[1] The abbreviations used are: AF, activation function; PR, progesterone receptor; DBD, DNA-binding domain; HBD, hormone-binding domain; ID, inhibitory domain; bZIP, basic region leucine zipper; IF, inhibitory function; ER, estrogen receptor; NT_A, N-terminal A; NT_B, N-terminal B; NLS, nuclear localization signal; PCR, polymerase chain reaction; nt, nucleotide(s); MMTV, mouse mammary tumor virus; CAT, chloramphenicol acetyltransferase; PRE, progesterone response element; h, human; BUS, B-upstream segment.

with B-receptors and are *trans*-dominant inhibitors of other nuclear receptors, we postulated that A-receptors contain inhibitory sequences distinct from the three defined AFs and that these sequences are inoperative in B-receptors. We sought this inhibitory function (IF) in a previously uncharacterized 292-amino acid region of the A-receptor N terminus. In this paper we have compared the activity of several PR constructs that either contain or lack IF. We show that IF expresses a novel inhibitory function, distinct from the AFs, that inhibits AF1 and AF2 but not AF3. Therefore, IF removal converts A-receptors from weak into strong transactivators. Additionally, IF is transferable and suppresses estrogen receptor (ER) activity when it is cloned upstream of ER.

MATERIALS AND METHODS

Recombinant Plasmids—Complementary DNAs, hPR2 and hPR1, encoding human A- and B-receptors, respectively, and HEGO, encoding human estrogen receptors, cloned into pSG5 (4), were gifts from P. Chambon (Strasbourg, France). Construction of BUS-DBD, N-terminal B (NT_B)-DBD, N-terminal A (NT_A)-DBD, DBD-HBD, and BUS-DBD-HBD expression vectors, all containing a nuclear localization signal (NLS), was described in Sartorius *et al.* (5).

NT_A-ΔIF was made by polymerase chain reaction (PCR) amplification of PR nucleotide (nt) sequences 2109–2678 encoding AF1, DBD, and the NLS (4). The 5′-sense primer contained an *Eco*RI site, a Kozak consensus sequence (22), and ATG initiation codon. The 3′-antisense primer contained a STOP codon and *Bgl*II site (5). The resulting PCR fragment was cloned into pSG5 digested with *Eco*RI/*Bgl*II. A-ΔIF was made by digesting NT_A-ΔIF with *Bbs*I/*Bgl*II and isolating the larger fragment, which contains pSG5 sequences, AF1, and the DBD N terminus upstream of the *Bbs*I site. This was used as a recipient for ligation of the *Bbs*I/*Bgl*II fragment from BUS-DBD-HBD, which contains the DBD C terminus, NLS, and HBD.

For B-ΔAF1 a fragment encoding sequences upstream of AF1 (fragment 1) was made by amplification of hPR1 sequences using a 5′-sense primer (nt 1855–1878) containing an *Mlu*I site and a 3′-antisense primer (nt 2096–2116) containing a *Sal*I site. Fragment 2, which spans sequences from the 3′ border of AF1 to the end of the HBD, was made with a 5′-sense primer (nt 2385–2404) containing a *Sal*I site and a 3′-antisense primer (nt 3525–3545) containing a *Bgl*II site (5). The two fragments were combined with an *Mlu*I/*Bgl*II vector-containing fragment (fragment 3) derived from hPR1. All three fragments were ligated to produce B-ΔAF1. A-ΔAF1 was made from B-ΔAF1 by removing an *Eco*RI/*Bam*HI fragment, filling in overhanging ends, and ligating the blunt ends. For NT_B-ΔAF1, fragment 4, which spans sequences from the 3′ end of AF1 through the NLS, was amplified from NT_B-DBD using the 5′-sense primer for fragment 2 containing a *Sal*I site and a 3′-antisense primer (nt 2646–2678) containing a *Bgl*II site, coding sequences homologous to the 3′ end of NT_B-DBD and a STOP codon (5). PCR fragments 1 and 4 were combined with the *Mlu*I/*Bgl*II vector-containing fragment 3 and ligated.

IF-BUS-DBD was made by amplification of IF (spanning nt 1239–2108) from hPR1 using primer pairs containing *Eco*RI sites. BUS-DBD-NLS was amplified from BUS-DBD with a 5′-sense primer containing an *Eco*RI site and a 3′-antisense primer containing a *Bgl*II site and STOP codon (5). The two fragments were ligated and digested with *Mlu*I/*Bgl*II. The larger fragment was cloned into the vector-containing fragment derived from NT_A-DBD digested with *Mlu*I/*Bgl*II. IF-NLS was made by amplification of IF using a 5′-sense primer containing a *Bam*HI site and a 3′-antisense primer containing an *Eco*RI site. This fragment was cloned into pSG5–2TK digested with *Eco*RI and *Bam*HI. pSG5–2TK was made by digesting pSG5 with *Eco*RI and *Bgl*II and inserting an oligo linker containing a Kozak consensus sequence (22), *Bgl*II site, the PR NLS, multi-cloning sites for *Bam*HI, *Sma*I, and *Eco*RI, and three STOP codons. IF-ER was made by amplification of ER coding sequences (nt 233–2020) (23) using primer pairs containing *Eco*RI sites. The resulting fragment was ligated into the IF-NLS expression vector at the *Eco*RI site.

cDNA sequences were verified by dideoxy (U. S. Biochemical Corp.) or automated fluorescent sequencing (Cancer Center Sequencing Core Lab, University of Colorado Health Sciences Center, Denver, CO), and protein expression was verified by immunoblotting.

Mouse mammary tumor virus (MMTV)-chloramphenicol acetyltransferase (CAT), thymidine kinase (*tk*-CAT) and ERE_2-$TATA_{tk}$-CAT reporters were gifts from P. Chambon. For PRE_2-$TATA_{tk}$-CAT, two copies of the tyrosine aminotransferase progesterone response element (PRE) were cloned upstream of a truncated fragment (−60/+51) of the *tk* gene as described previously (9).

Immunoblotting—Whole-cell 0.5 M KCl extracts were prepared from COS cells transiently transfected with the expression vectors described. The expressed PR fragments were resolved by electrophoresis on 7.5% or 10% SDS-containing denaturing polyacrylamide gels and transferred to nitrocellulose. Protein blots were probed with our anti-PR monoclonal antibodies, AB-52 and B-30 (24), and the anti-DBD polyclonal antibody α266 (25) provided by D. Toft (Rochester, MN). For detection of ER or IF-ER, the anti-ER antibody SRA 1,000 was used (StressGen, Victoria, BC). Bands were detected by enhanced chemiluminescence (Amersham Corp.) as described previously (11).

Transfection and Transcription Assays—HeLa cells were plated in 100-mm tissue culture dishes in 10 ml of minimum essential medium supplemented with 5% twice charcoal-stripped, heat-inactivated fetal calf serum (DCC-MEM). Duplicate plates were transfected by calcium phosphate coprecipitation with 2 μg of the reporter plasmid, variable amounts (indicated in the figures) of the receptor expression vectors, 3 μg of the β-galactosidase expression plasmid pCH110 (Pharmacia Biotechnology Inc.) to correct for transfection efficiency, and Bluescribe (Stratagene, La Jolla, CA) carrier plasmid for a total of 20 μg/plate (11). 24 h later, the medium was changed to 7.5% DCC-MEM, and cells were either left untreated or were incubated with 10 nM of the synthetic progestin R5020 (Roussel UCLAF, France) or 17β-estradiol, for an additional 24 h. Cells were harvested, and lysates were normalized to β-galactosidase activity and analyzed for CAT activity by TLC as described previously (9, 10). Standard deviations of phosphorimaging (Image Quant, Molecular Dynamics, Sunnyvale, CA) data were determined using Microsoft Excel, version 5.0 (Microsoft Corporation, Seattle, WA) for the number of sets indicated in the figure legends.

RESULTS

A series of expression vectors was constructed in which each region of PR that contains an AF was fused, either alone or in combination with another AF, to the PR DBD-NLS (5). Additionally, the constructs contained or lacked IF, the 291 amino acids lying upstream of AF1. IF was also cloned upstream of full-length ER. The detailed structure of all the constructs is shown in Fig. 1.

Expression of the receptor proteins was verified by SDS-polyacrylamide gel electrophoresis and immunoblotting of whole-cell extracts derived from COS cells (Fig. 2). They range in size from 191 to 933 amino acids and are all well expressed. The presence of multiple bands for some constructs, particularly ones that contain BUS (Fig. 2*A*, *lane 8*, for example), is due to phosphorylation (5, 26). Interestingly, this multiple banding pattern is amplified by removal of the HBD (Fig. 2*A*, *lanes 4* and *8*) and is reduced by juxtaposition of IF upstream of BUS (Fig. 2, compare *lane 6* in *A* and *lane 1* in *B*). Each of the receptors shown in Fig. 2 binds to a perfect palindromic PRE in an electrophoretic mobility shift assay (data not shown).

An Inhibitory Function—To search for an inhibitory function, an A-receptor variant was constructed (A-ΔIF) that lacks the N-terminal 292 amino acids located upstream of AF1 (Fig. 1). This previously uncharacterized domain, designated IF (amino acids 165–455), although common to both receptor isoforms, has the potential to function differently when free at the N terminus of A-receptors but constrained by BUS in B-receptors. Strikingly, on the PRE_2-$TATA_{tk}$ promoter (Fig. 3*A*) or on the MMTV promoter (Fig. 3*B*), deletion of IF converts A-receptors from weak into strong transactivators equivalent to B-receptors. However, unlike B-receptors, but like A-receptors, A-ΔIF displays strong "self-squelching" behavior. Therefore, as the concentration of A-ΔIF is increased, the high levels of CAT activity fall.

IF Inhibits AF1 and AF2 but Not AF3—Fig. 3 shows that IF contains a potent inhibitory function that suppresses the activity of A-receptors. However, because B-receptors, which also contain IF sequences, are strong transactivators, we postulated that IF does not influence AF3 but that its inhibitory effects are

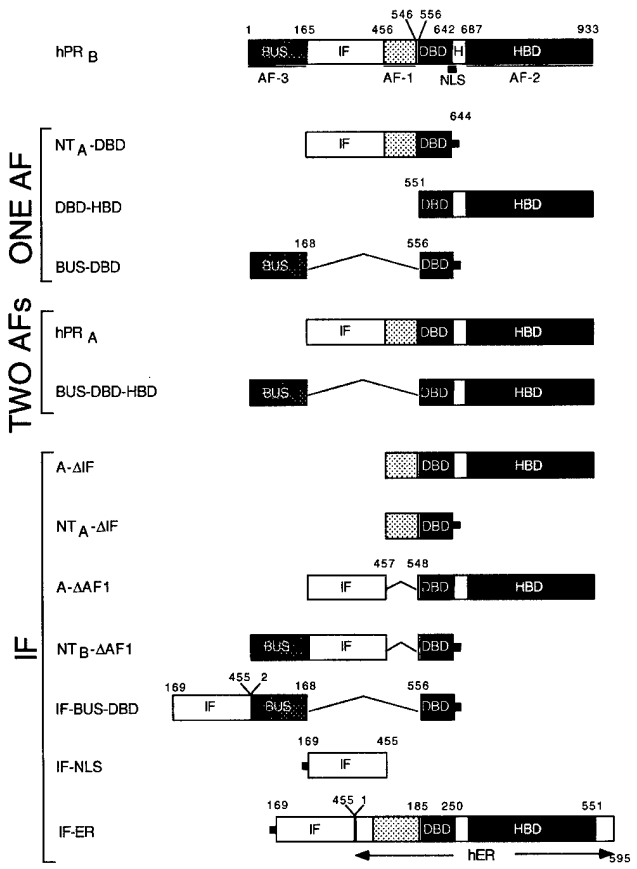

FIG. 1. **hPR and hER test constructs.** PR and ER constructs used to test the effects of deletion (Δ) or presence of IF on the activity of individual AFs or combinations of AFs. Key amino acids are numbered.

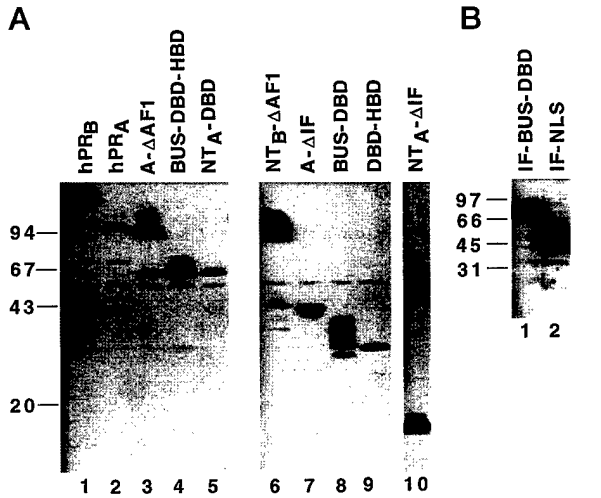

FIG. 2. **Protein expression levels of PR constructs.** Expression vectors (1.25 or 1.0 μg/100-mm plate in *A* and *B*, respectively) for PR isoforms and variant constructs were transiently expressed in COS cells, and proteins in whole-cell extracts were resolved by either 7.5 (*A*) or 10% (*B*) SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose. Immunoblot A was probed with a mixture of the A- plus B-specific monoclonal antibody AB-52, the B-specific monoclonal antibody B-30, and the anti-DBD polyclonal antibody α266. The latter was generously provided by David Toft (25). Immunoblot B was probed with AB-52. The *numbers* represent the molecular mass of protein standards that were run simultaneously and detected by Coomassie Brilliant Blue staining.

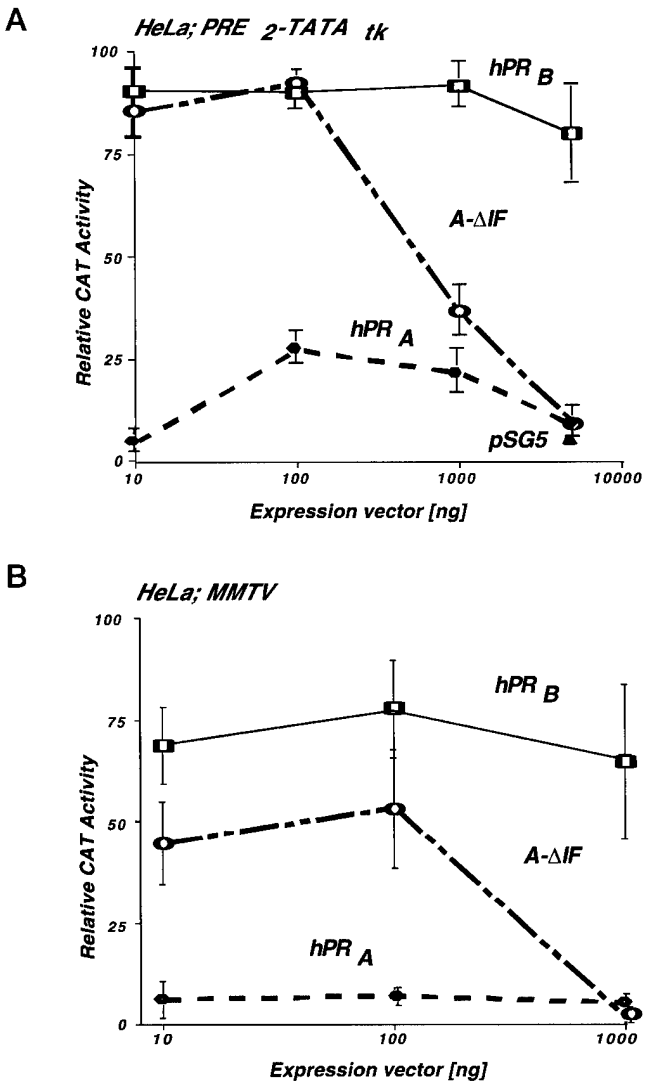

FIG. 3. **Removal of IF from A-receptors converts weak transactivators into strong transactivators.** HeLa cells were transfected with increasing concentrations of expression vectors for wild-type A-receptors (hPR_A), A-receptors lacking IF (*A-ΔIF*), B-receptors (hPR_B), or the empty expression vector (*pSG5*). Cells were treated with 10 nM R5020, and CAT activity from 2 μg of PRE_2-$TATA_{tk}$-CAT (*A*) or MMTV-CAT (*B*) was measured by TLC using β-galactosidase-normalized cell extracts. CAT levels were quantified by phosphorimaging. Data points represent averages of duplicate samples, and the range of those values is indicated by the *error bars* (*A*), or averages of four to six data points and the *error bars* represent the standard deviations (*B*).

restricted to AF1 and/or AF2 (Fig. 4). To test this hypothesis, constructs were made that contained each AF alone, with or without IF (Fig. 1). Dose-response data using PRE_2-$TATA_{tk}$-CAT are shown in Fig. 4 for AF1 (NT_A-DBD) with (+) and without (−) IF (Fig. 4*A*); AF2 (DBD-HBD) with (+) and without (−) IF (Fig. 4*B*); and AF3 (BUS-DBD) with (+) and without (−) IF (Fig. 4*C*) compared with full-length B-receptors. Analogous to its role in full-length A-receptors, we find that IF has its strongest effect on AF1 and AF2 at low receptor concentrations.

Deletion of IF strongly increases AF1-dependent transcription (Fig. 4*A*, 100 ng) but its inhibitory effects on AF1 can be overcome at higher concentrations because of the failure of NT_A-DBD to self-squelch. Note that NT_A-DBD is the only A-receptor construct that does not self-squelch (not shown). These and other data (Fig. 4*B* and not shown) suggest that the HBD is required for this property.

Deletion of IF from an AF2-containing HBD construct also converts a weak transactivator into a stronger one at low concentrations (Fig. 4*B*). At higher concentrations, however, the influence of self-squelching abolishes AF2 activity. Thus,

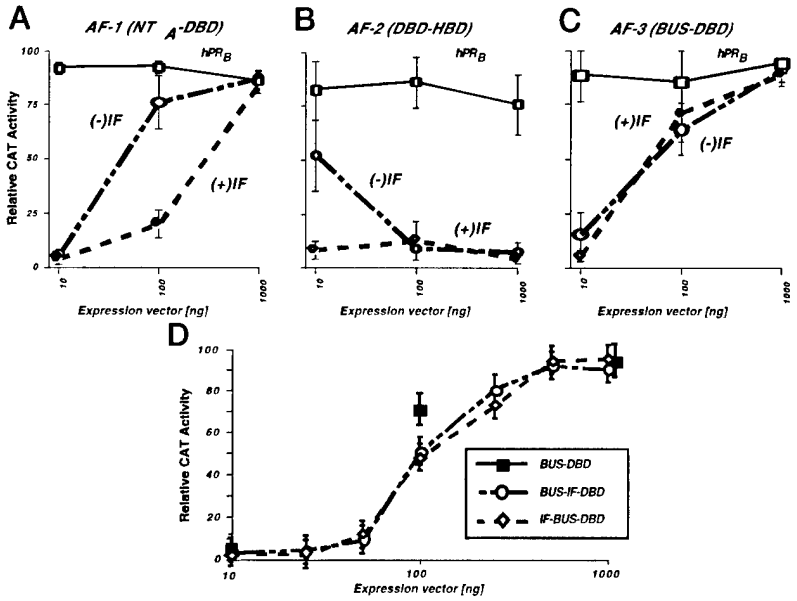

FIG. 4. **IF represses AF1 and AF2 but not AF3 regardless of orientation.** HeLa cells were cotransfected with 2 μg of PRE_2-$TATA_{tk}$-CAT and increasing concentrations (10–1000 ng) of expression vectors for hPR_B (*A–C*), NT_A-DBD plus or minus IF (*A*), DBD-HBD plus or minus IF (*B*), or BUS-DBD plus or minus IF (*C*). *D*, HeLa cells were cotransfected with 2 μg of PRE_2-$TATA_{tk}$-CAT and 10–1000 ng of expression vectors for BUS-IF-DBD (*NT_B-DBD*), IF-BUS-DBD, or BUS-DBD. CAT assays were quantified by phosphorimaging. *Error bars* represent standard deviations from three to eight data points (*A–C*) or the range of values for duplicates (*D*).

the HBD mediates two opposing activities: a stimulatory AF2 function and an inhibitory self-squelching function. IF can suppress the former but has no effect on the latter. We speculate that IF inhibition of AF1 and AF2 is additive in full-length A-receptors, accounting for their weak activity at all concentrations.

Neither the inhibitory function of IF nor the self-squelching activity of the HBD operates in B-receptors. Removal of IF does not enhance AF3 activity at low concentrations (Fig. 4*C*) nor does it effect AF3 at higher concentrations, and, like full-length B-receptors (Fig. 3), the B-receptor derivatives BUS-DBD-HBD or B-ΔAF1 do not self-squelch despite presence of the HBD (data not shown).

Mechanisms of PR Auto-inhibition by IF—Several possible mechanisms can be invoked for auto-inhibition of A-receptors by IF. One is binding of a repressor at IF. However, we find that overexpression of an IF-NLS construct does not squelch the putative repressor (not shown). Another possible mechanism is steric hindrance of AF1 and AF2 by IF due to the latter's upstream position. To test this hypothesis, IF was cloned upstream of AF3. We reasoned that if IF acts by steric hindrance in A-receptors, then perhaps juxtaposition of IF upstream of BUS would inhibit AF3 activity. To that end, IF-BUS-DBD was constructed and compared with NT_B-ΔAF1 (BUS-IF-DBD) on PRE_2-$TATA_{tk}$-CAT (Fig. 4*D*). The only difference between these two constructs is the position of IF relative to AF3. BUS-DBD was used as a control. At all concentrations tested, the two IF-containing constructs had equivalent transcriptional activity. Therefore, BUS appears to be insensitive to the inhibitory effects of IF, regardless of the position of IF. In addition, we find that IF has no effect in other B-receptor derivatives. Specifically, constructs containing AF2 plus AF3 (B-ΔAF1 and BUS-DBD-HBD) had identical transcriptional profiles with and without IF (not shown). Taken together, these data suggest that IF does not act simply by steric hindrance of any AF to which it is linked; rather IF inhibition is specific for AF1 and AF2. We therefore asked whether IF could suppress AF1 and AF2 of another member of the steroid receptor family.

IF Is Transferable to the Heterologous AFs of ER—Inhibitory domains, like activation domains, can be discrete and modular. To determine whether IF effects were transferable, we tested the ability of IF to inhibit the heterologous AFs of ER. ERs contain AF1 and AF2 and, in this respect, structurally resemble A-receptors (14, 27, 28). However, ER have no sequences homologous to IF. To test the effects of IF on ER, an IF-ER chimera was constructed (Fig. 1) in which IF was cloned upstream of ER. Fig. 5*A* shows transcription by wild-type ER or IF-ER of the ERE_2-$TATA_{tk}$-CAT reporter in the absence (*open symbols*) or presence of 10 nM 17β-estradiol (*solid symbols*). CAT activity induced by ER is maximal at 0.1 μg of the expression vector and then decreases at higher concentrations due to self-squelching. This has previously been described (7, 14). At the same cDNA concentrations, IF cloned upstream of ER markedly reduces transcription. Fig. 5 (*B* and *C*) compares the transcriptional efficacy of ER and IF-ER when the two are expressed at similar protein levels. We find that IF-ER is expressed at lower efficiency than ER. Thus, 1 μg of the IF-ER expression vector and 0.1 μg of HEGO produce equivalent amounts of immunoreactive protein (Fig. 5*B*). Note that the expected molecular mass of ER is 65 kDa and that of IF-ER is 97 kDA. Fig. 5*C* shows that at these equivalent protein concentrations, wild-type ER strongly activate transcription, whereas little or no transcription is produced by IF-ER. We conclude that when IF is transferred upstream of ER, it silences ER-dependent transcription.

DISCUSSION

This paper describes a novel, transferable inhibitory function, designated IF, which lies in the 292-amino acid N-terminal region upstream of the PR AF1 but operates only in the context of A-receptors.

A- versus B-receptors—Why progesterone target tissues contain two receptor isoforms remains an intriguing physiological puzzle. They were first described in chick oviducts (29) and then in human cells (2). In humans, the two proteins are the products of a single gene that has two promoters, from which at least nine messages, two of which are A-receptor specific, are transcribed (30). An internal AUG present in some messages may also encode A-receptors (30). Thus, there is complex regulatory control over protein levels of the two isoforms, the details of which are still unclear. In initial studies using breast cancer cell lines, the two isoforms were found in approximately equimolar amounts (3). However, it is now clear that their relative levels are under tight developmental and hormonal control in chicken oviducts (31–33) and the female rat brain (34), and preliminary data in the human uterus also show a discordance, with A:B ratios ranging between 50:1 and 2:1 during the menstrual cycle due to large excursions in the levels of B-receptors (35). In breast cancers 25% of tumors have a

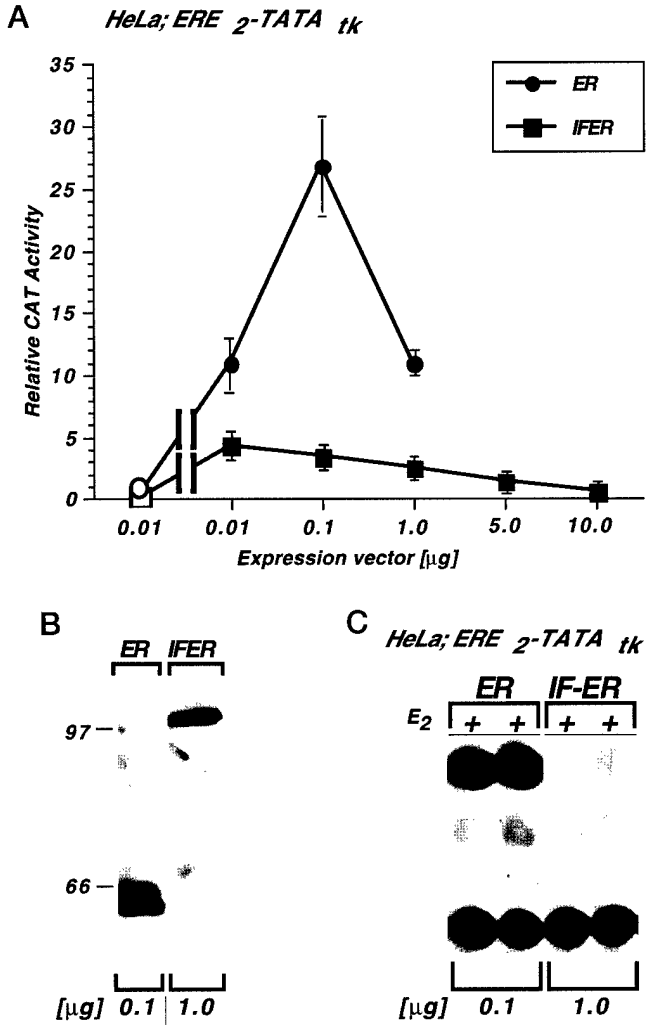

FIG. 5. **IF is transferable and inhibits the heterologous AFs of hER.** *A*, HeLa cells were transiently cotransfected with 2 μg of ERE_2-$TATA_{tk}$-CAT and varying amounts of the wild-type hER expression vector, HEGO (0.01–1 μg), or IF-ER (0.1–10 μg) and either left untreated (*open symbols*) or treated with 10 nM 17β-estradiol (*solid symbols*). CAT levels were analyzed by TLC and autoradiography from β-galactosidase-normalized cell extracts; the average of duplicates is shown. The range of values is shown by the *error bars*. *B*, COS cells were transiently transfected with 0.1 μg of HEGO or 1.0 μg of IF-ER. Extracts were resolved by SDS-polyacrylamide gel electrophoresis and immunoblotted with the anti-ER hinge region monoclonal antibody SRA 1000. Prestained molecular weight markers were used to estimate size. *C*, duplicate sets of HeLa cells were transiently transfected with 0.1 μg of HEGO or 1.0 μg of IF-ER and the ERE_2-$TATA_{tk}$-CAT reporter. Normalized cell extracts were measured for CAT activity by TLC.

significant excess of A-receptors (36). Given the functional transcriptional differences between the two isoforms, their unequal distribution in tissues and tumors could be biologically important. For example, an excess of B-receptors in the uterus may mark those patients at greatest risk of developing tamoxifen-induced endometrial cancers (37).

Transferable Inhibition of AF1 and AF2—Much of the work devoted to understanding regulation of transcription by steroid receptors has focused on AFs and their stimulatory actions. However, transcriptional inhibition may be equally important as a way of preventing or terminating activation. Studies that deal with inhibition have focused on composite DNA elements and invoke mechanisms in which receptor occupancy at one DNA site interferes with transcription by an activator at an adjoining site (38, 39). Heterodimerization of an activator by a repressor and recruitment of corepressors are other silencing mechanisms (40). We now demonstrate that negative signaling elements can exist in the receptor molecule itself.

We show that IF markedly suppresses the transcriptional activity of AF1 and AF2 of A-receptors (Fig. 4). The ability of IF to also strongly suppress AF1 plus AF2 of ER (Fig. 5) suggests that its inhibitory mechanisms involve general steroid receptor-related processes. It is tempting to speculate that IF prevents the binding of key AF1 or AF2 transcriptional coregulators that are shared by all steroid receptors (40). However the inability of soluble IF (*i.e.* IF-NLS) to squelch such activity suggests that IF acts structurally, perhaps through intramolecular contacts. Our data show that the inhibitory activity can be transferred to the cognate AFs of ER. In that respect, IF resembles the bZIP domain of ATF-2 and the N-terminal ID of c-Fos (16, 17). Whether IF can also suppress heterologous AFs remains to be determined.

IF Cannot Inhibit AF3—B-receptors also contain the IF element, but its repressor activity appears to be constrained by BUS, which is located further upstream. Therefore, IF specifically inhibits AF1 and AF2 of PR but not AF3 (Fig. 4). Furthermore, IF cannot inhibit AF3 regardless of its position relative to BUS (Fig. 4, *C* and *D*). We have previously demonstrated that AF3 transcriptional activity is unusual in that it is critically dependent on the presence of the PR DBD. In gel mobility shift studies, BUS-DBD binds to a PRE only if a bivalent monoclonal antibody is added, which appears to supply a dimerization function. The possibility exists that BUS and the DBD of PR are linked through intramolecular contacts so that the mechanisms of AF3 action may be quite different from those of AF1 and AF2.

There is now compelling evidence that alterations in the three-dimensional structure of steroid receptors modifies their transcriptional behavior. Most of that work comes from analyses of the HBD. For example, using protease accessibility as a probe for receptor structure, it has been shown that PR (41) and ER (42, 43) assume altered conformational states when the HBD is occupied by agonists or antagonists. More recently, crystallographic analyses of the HBDs of unliganded RXRα (44) and liganded retinoic acid receptor γ (45) and thyroid receptor α1 (46) have yielded information about the position of residues critical to the function of AF2. These studies also show that binding of ligand alters the conformation of the HBD, which, depending on the ligand, may be interpreted as a positive or negative signal by the transcriptional machinery (41).

The present studies focus on the PR N terminus to explain functional differences between the two isoforms. As yet, nothing is known about the three-dimensional structure of the N termini of any nuclear receptor. This region is, however, structurally the most divergent among members of this family of proteins, suggesting that each receptor will take on unique N-terminal conformations that determine its specificity. We postulate that structural differences between the N termini of the two PR isoforms, due to the presence or absence of BUS, influence the functional role of IF.

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Progesterone Regulated Expression of Flavin-Containing Monooxygenase 5 by the B-Isoform of Progesterone Receptors: Implications for Tamoxifen Carcinogenicity

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ABSTRACT

Progesterone is a key developmental, proliferative, and differentiative hormone in the breast and endometrium, and it can accelerate carcinogenesis in the mammary gland epithelium. In the breast and uterus, progesterone acts through two coexpressed isoforms of progesterone receptors, the B- and A-receptors. To study the function of each isoform in isolation, we previously constructed two breast cancer cell lines that stably and independently express either B-receptors (YB cells) or A-receptors (YA cells). In the present study, YA or YB cells were left untreated, or were treated with the synthetic progestin R5020, and the messages present in each cell line under the two conditions were analyzed by differential display. Two message species are described that are regulated only by B-receptors. One of these is regulated in a ligand-independent manner. A third set of messages, encoding flavin-containing monooxygenase 5 (FMO5), was induced by R5020 only in YB cells. A-receptors appear to be inhibitory. FMOs are involved in the metabolic activation of drugs and xenobiotic compounds, including the antiestrogen tamoxifen, to carcinogenic intermediates. It is possible, therefore, that by upregulating the levels of FMO5, progesterone enhances the carcinogenicity of tamoxifen in target tissues that overexpress progesterone B-receptors. (*J Clin Endocrinol Metab* **82:** 2956–2961, 1997)

PROGESTERONE is a key developmental, growth, and differentiative hormone in the breast and uterus (1, 2), and under appropriate conditions, progesterone can accelerate carcinogenesis in mammary gland epithelium (Ref. 3 and references therein). However, only a few progesterone-regulated genes have been defined in any tissue (4–6). To understand the role of progesterone in normal and malignant cell processes, the present study sought to define the subset of proteins regulated by progesterone, using human breast cancer cells as models.

Analysis of progesterone action is complicated by the fact that two progesterone receptor (PR) isoforms are coexpressed in human target cells (7): 120-kilodalton B-receptors, and N-terminally truncated 94-kilodalton A-receptors. Thus, when equimolar levels of the two isoforms are expressed, A:A, A:B, or B:B homo- and heterodimers, form at 1:2:1 molar ratios. This is important, as the two receptor isoforms regulate transcription unequally when occupied by progesterone agonists (8). with A:A homodimers usually weaker than B:B homodimers. Additionally, in the A:B heterodimer, the inhibitory transcriptional phenotype of A-receptors is dominant. On the other hand, only B-receptors can paradoxically activate transcription when bound by antagonists (9–11). All known progesterone-dependent target tissues and cells express both PR isoforms, but recent data in breast cancers (12) and uteri (13) suggest that the ratio of B- to A-receptors can flucuate widely. This structural variability would influence the responsiveness of tissues to progestational signals. To study the function of the two PR isoforms independently, we isolated a PR-negative subline (Y cells) of the A- plus B-receptor-positive T47D breast cancer cell line, and then stably reintroduced expression vectors encoding either A- or B-receptors into Y cells to create, respectively, YA and YB cells (14). The protein levels of each PR isoform expressed in these new cell lines is equivalent to the levels of that isoform present in wild-type T47D cells (14). We have used these new cells to study isoform-specific regulation of PR target genes by the method of differential display (15).

The present study demonstrates that the overall pattern of expressed messenger RNAs (mRNAs) in YA and YB cells is remarkably similar, that some messages may be regulated in a ligand-independent manner, and that the progestin agonist R5020 modulates transcription of a small but unique subset of messages. Among the messages regulated by R5020, but only in cells expressing the B-isoform, are those encoding flavin-containing monooxygenase 5 (FMO5). In the liver, studies suggest that FMOs can metabolize drugs and xenobiotics to reactive intermediates that bind covalently with microsomal proteins and DNA (16, 17). Tamoxifen, for example, can be metabolized to a reactive intermediate by these

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enzymes (16), and such tamoxifen adducts have been described in the uterine DNA of breast cancer patients (18). Our data suggest the interesting hypothesis, that in target cells that overexpress PR B-receptors, progesterone can induce FMO5 and enhance the carcinogenicity of tamoxifen, and therefore, that an excess of B-receptors may serve as a risk marker.

Materials and Methods

Cell culture

Construction and characterization of YA and YB breast cancer cell lines that stably express either PR A-receptors or B-receptors, respectively, has been previously described (14). Cells were plated at 1×10^7 cells/75 cm^2 tissue culture flask and allowed to grow for 6 days in MEM supplemented with 5% FCS until they were approximately 75% confluent. YA and YB cells were then treated with 20 nM of the progestin R5020 (New England Nuclear, Dupont, Boston, MA) or vehicle alone for 18 h, before harvesting as previously described (14).

Isolation and purification of total RNA and poly A+ mRNA

Total cellular RNA was isolated with guanidinium isothiocyanate followed by centrifugation through a CsCl cushion. DNA was eliminated from total RNA using RNase-free DNase I (Message Clean, GenHunter, Brookline, MA). Poly(A)$^+$ RNA was separated from total RNA by affinity chromatography on oligo(dT)-cellulose (type 7; Pharmacia; Piscataway, NJ) according to our previously published procedures (19).

Differential display of mRNA

We evaluated approximately half (~5000 mRNAs) of the mRNA species estimated to be present in eukaryotic cells (15). Ten arbitrary primers were examined with each of four anchored primers (RNAmap kit II, GenHunter, Brookline, MA). RT-PCR was performed on purified total RNA as described, using Moloney Murine leukemia virus reverse transcriptase. Complementary DNAs (cDNAs) were then selectively amplified by PCR with the appropriate primer pairs in the presence of [^{35}S]deoxycytidine ATP. Aliquots of separate duplicate PCR reactions containing amplified cDNA fragments were then electrophoretically resolved on adjacent lanes of a 6% polyacrylamide/urea denaturing gel. Following transfer, drying, and autoradiography, bands were evaluated for differentially displayed candidate messages.

Recovery, reamplification, and cloning of cDNAs

The autoradiographed film was accurately aligned with the dried gel and differentially regulated PCR fragments were excised, eluted by boiling, ethanol precipitated, and reamplified by PCR with the original primer pairs. The PCR product of the expected size was excised from a 1% agarose gel, and the DNA was purified by silica gel adsorption (QIAEX II kit, Qiagen, Chatsworth, CA). PCR products were ligated into pCRII and transformed into competent INF F′ *E. coli* (One Shot Invitrogen TA Cloning, Invitrogen, San Diego, CA). Following blue/white selection, appropriate clones were grown and plasmid DNA was isolated. Sequencing was performed by the Cancer Center Core Laboratory at the University of Colorado Health Sciences Center, Denver, CO. Sequences were searched against the NIH GenBANK database using a BLAST algorithm.

Northern blotting

Total RNA (20 μg) was separated on a 1% agarose/6% formaldehyde gel according to our published protocol (19). The RNA was transferred overnight to a nylon membrane (TurboBlotter; Schleicher and Schuell, Keene, NH) and fixed by UV cross-linking (Stratalinker; Stratagene, La Jolla, CA). Blots were probed with 2.0×10^7 cpm [^{32}P]cDNA labeled by nick translation from the cloned candidate PCR fragments. Northern blots were subsequently probed with ^{32}P-labeled β-actin to assess loading uniformity.

Rapid amplication of cDNA ends (RACE) was used to obtain further 5′ sequence information from the 360-bp cDNA fragment regulated by R5020 in YB cells (Fig. 3). A 26-bp primer complementary to a region of the 360-bp sequence was used for first-strand synthesis using Moloney Murine leukemia virus reverse transcriptase (Clontech Marathon cDNA Amplification Kit; Clontech Laboratories, Palo Alto, CA) using poly(A)$^+$ RNA from YB cells treated with R5020. Following second-stand synthesis, double-stranded cDNA was blunt ended with T4 DNA polymerase I and ligated to an adapter primer supplied with the Marathon Kit. Adapter ligated cDNAs were amplified by long-distance PCR (Boehringer Mannheim, Indianapolis, IN). The PCR conditions consisted of 30 sec at 94 C for denaturation, 30 cycles at 68 C, and 30 sec for annealing and extension. Amplifed PCR products were size separated on a 1% agarose gel, transferred to a Nytran membrane, and hybridized to a 36-bp oligonucleotide just 5′ of the 26 nucleotide RT primer to verify the specificity of the amplification. Positively hybridizing PCR products were subcloned into PCR 2.1 (Invitrogen), and miniprep DNAs were screened for internal sequences by Southern blot analysis (20) using the radiolabeled 36 mer. An 800-bp positively hybridizing insert was sequenced from both ends using M13 forward and reverse primers, and searched against the GenBANK database. To reconfirm its differential expression pattern, an approximately 300-bp *Eco*RI/*Bam*HI fragment from the 5′ end of the 800-bp clone was labeled and used for Northern blotting.

Results

Four sets of cells were analyzed for constitutive and progesterone-regulated mRNA expression. Untreated YA and YB cells were compared with each other (YA− *vs.* YB−) and to messages in YA and YB cells following 18 h of treatment (YA+ *vs.* YB+) with the progestin R5020. As expected, the vast majority of the message fragments were neither modulated by the PR isoform present in the cell nor by the presence of hormone. Figure 1 shows an example of the differential display patterns obtained. Two bands, one marked by a star, the other labeled FMO5, are discussed below.

Ligand-free regulation by B-receptors

The Northern blot in Fig. 2A shows a 3200-bp RNA transcript labeled by a 380-bp band excised from a differential display gel. The same Northern blot hybridized with a labeled β-actin cDNA demonstrates the relative amounts of RNA loaded (Fig. 2B). A BLAST search with the sequencing data indicates that this message encodes a novel protein. This Northern blot confirms an unusual ligand-independent upregulation restricted to B-receptor-containing cells. Gene regulation by ligand-free steroid receptors has recently been described (21). The authors speculate that the receptors are activated by means of cross-talk with other signaling pathways. A second example of this type of regulation by ligand-free B-receptors is demonstrated in Fig. 1 (*starred product*) in which the product is down-regulated (lanes 5 and 6). It has not been characterized further.

Figure 3A shows a 4000-kilobase (kb) transcript that hybridized to a radiolabeled 450-bp band excised from a differential display gel that was seen in YB cells whether or not they received hormone, but was absent in YA cells irrespective of hormone treatment. Equal loading of RNA was demonstrated by hybridization to a labeled β-actin cDNA (Fig. 3B). This message is either constitutively regulated and represents an extremely rare message that is unique to YB cells, or it is a message that is regulated by B-receptors through both ligand-dependent and -independent mechanisms (21).

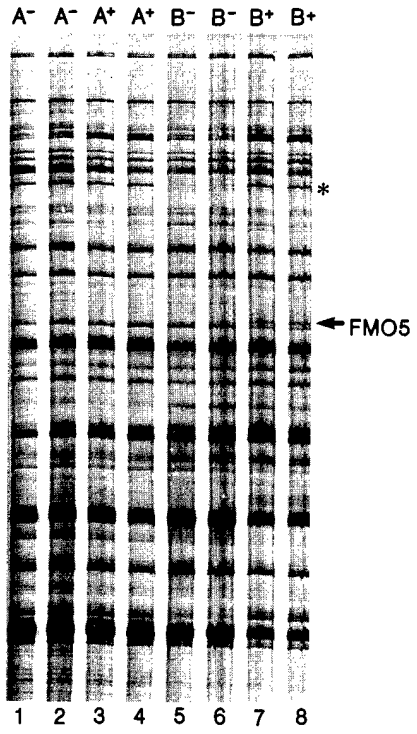

FIG. 1. An example of differentially displayed mRNA fragments derived from breast cancer cell lines expressing either B- or A-receptors in the presence or absence of R5020. Total RNA from YA (A) and YB (B) cells, either untreated (−) or treated (+) with R5020, was reverse transcribed with T12 MA. Product cDNAs were PCR amplified with a T12MA primer/anchor primer 13 combination in the presence of [^{35}S]deoxycytidine ATP. Products were resolved on a 6% urea denaturing gel. *Arrow*, Indicates a ~360-bp band present only in hormone-treated YB cells (YB+). An (~520-bp) band (*star*) was present in all cell types except hormone-untreated YB cells. The vast majority of bands are identical in two cell types. Duplicate sets are shown.

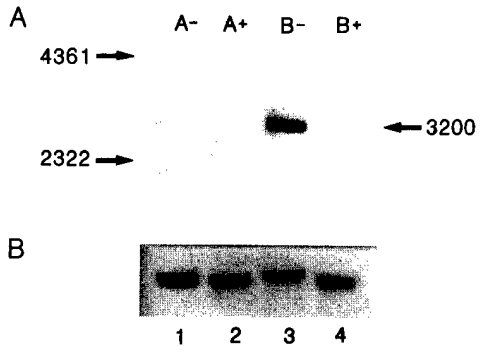

FIG. 2. B-receptor specific message regulated by ligand-independent mechanisms. A, mRNAs from YA (A) or (YB) (B) cells either untreated (−) or treated with (+) R5020 were resolved on agarose gels, transferred to a nylon membrane, and probed with a 380-bp ^{32}P-labeled fragment isolated by differential display. *Arrow*, Indicates an ~3200-bp message. B, Same Northern blot probed with ^{32}P-labeled β-actin demonstrating relative amounts of RNA loaded from YA or YB cells either untreated (−) or treated (+) with R5020.

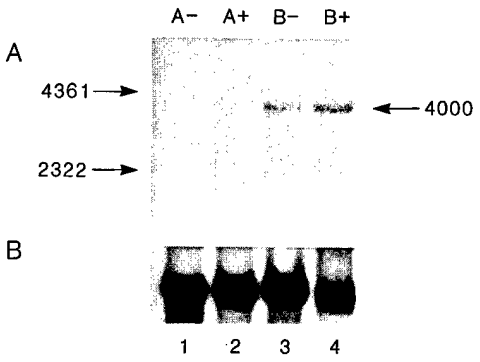

FIG. 3. Constitutively regulated B-receptor-specific message. A, Similar mRNAs were probed with a 450-bp ^{32}P-labeled fragment isolated by differential display. *Arrow*, Indicates an ~4000-bp message. B, Same Northern blot hybridized with a labeled β-actin probe to demonstrate equal RNA loading.

Sequence analysis shows that the protein encoded by this message is also novel.

B-receptor-specific and progestin-dependent regulation of FMO5

An approximately 360-bp mRNA fragment was amplified by the combination of arbitrary primer 13 and anchor primer T12 MA, from R5020-treated YB cells (Fig. 1, *arrow*, lanes 7 and 8) and represents a classically hormone-regulated message. Interestingly, it is only regulated by PR B-receptors. This cDNA fragment was excised from the duplicate lanes of the differential display gel, reamplified, and cloned. The insert was sequenced, radiolabeled, and used as a probe on a Northern blot (Fig. 4A) using total RNA from the basal and R5020-treated YA and YB cell lines. The labeled probe hybridized to a major transcript of approximately 3.8 kb and a minor one of approximately 2.6 kb that were expressed only in R5020-treated YB cells (Fig. 4A, lane 4). The 360-bp fragment was extended in the 5′ direction by RACE using RNA from YB+ cells, to yield an 800-bp product. After the probe was removed, the Northern blot shown in Fig. 3A was reprobed with an approximate 300-bp *Eco*RI/*Bam*HI fragment corresponding to the 5′ end of the 800-bp RACE product. This Northern blot yielded the same two 3.8-kb and 2.6-kb transcripts, and an additional 1.8-kb species (Fig. 4B). The blot was then probed with a [^{32}P]cDNA encoding β-actin (Fig. 4C). This demonstrates relatively uniform RNA loading in lanes 1–3, with lane 4 somewhat underloaded. Thus, the extent of mRNA induction in lane 4 is probably underestimated.

The 800-bp RACE product was sequenced, and a search of the DNA database showed that the first 197 nucleotides were 100% identical to the 3′ noncoding region of an mRNA that encodes FMO5 (17). The remainder of the 800-bp fragment probably represents sequences further 3′ in the untranslated region of the FMO5 message that have not been previously reported, but matched the sequence of the original 360-bp cDNA isolated from the differential display gel.

Figure 5 shows a study with YA and YB cells, similar to that in Fig. 4, which also includes a Northern blot of wild-type T47D cells. These cells express approximately equimolar amounts of A- and B-receptors (7). As shown, FMO5 mRNAs are up-regulated 9- to 10-fold in R5020-treated YB cells, but not in YA cells. Interestingly, in wild-type T47D cells, this up-regulation is greatly attenuated. This is to be expected if only 25% of the receptors bind the FMO5 pro-

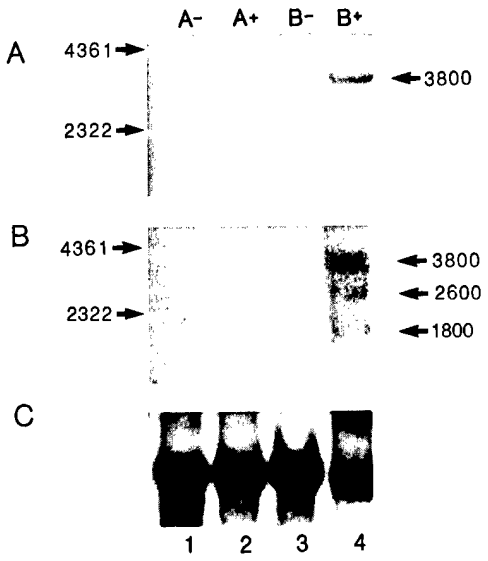

FIG. 4. FMO5 transcripts are strongly up-regulated by R5020 in B-receptor-containing T47D breast cancer cells. A, mRNAs from YA (A) or (YB) (B) cells either untreated (−) or treated with (+) R5020 were resolved on agarose gels, transferred to a nylon membrane, and probed with a 360-bp ^{32}P-labeled fragment isolated by differential display. B, Blot from *A* was stripped and reprobed with a ^{32}P-labeled 300-bp fragment from the 5′ end of the 800-bp RACE product derived from the original 360-bp fragment. C, Blot from *B* was stripped and reprobed with a ^{32}P-labeled β-actin probe. Mol wt of messages are shown on the *right*; mol wt of standards are shown on the left.

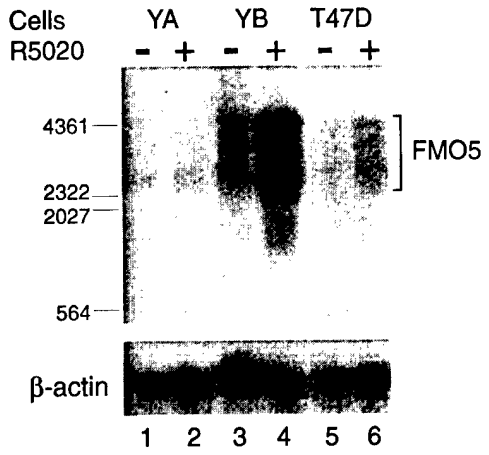

FIG. 5. Suppression of FMO5 up-regulation in T47D cells expressing equimolar levels of B- and A-receptors. YA, YB or wild-type T47D cells (that express equimolar levels of B- and A-receptors) were untreated (−) or treated (+) with R5020. mRNAs were resolved on agarose gels, then transferred to a nylon membrane and probed with the 360 bp ^{32}P-labeled FMO5 fragment isolated by differential display. Numbers to the left indicate the position of λ HindIII DNA size standards. The blot was stripped and reprobed with a ^{32}P-labeled β-actin probe.

moter as B:B homodimers. The remaining 75% bind either as A:A homodimers or A:B heterodimers, both of which would fail to activate transcription of this promoter (10, 11).

Discussion

YA/YB cells and differential display

Because PR are important in breast cancers (22), and the A- and B-isoforms of PR are functionally different (8, 9), we have constructed model T47D breast cancer cell lines in which the physiological role of each isoform can be independently evaluated (14). These cells stably express each isoform separately, but at the same levels seen in wild-type T47D cells (14). Differential display of a subset of the messages expressed in these T47D cells suggests that, for the most part, the two stable cell lines, A-receptor containing YA cells and B-receptor containing YB cells, contain the same mRNA populations, confirming their value for analysis of isoform-specific effects. We report here data for only 50% of possible primer pairs and for only one progesterone treatment time. Thus it is clear that we have analyzed only a subset of the messages present in these cells that are capable of being regulated by progesterone and that can be displayed by the method used. This is supported by the fact that several messages known to be regulated by progesterone in breast cancer cells, including ones encoding lactate dehydrogenase (4), alkaline phosphatase (5), and fatty acid synthetase (6) were not identified in our analysis. Of course some of the differentially displayed bands among those that have not yet been sequenced could, in theory, encode these proteins. Also of interest is the fact that all three of the progestin-regulated messages we detected were under B-receptor control. This PR isoform is usually, but not always (8), the stronger transcriptional activator in experimental model systems. Nevertheless, there are conditions under which A-receptor effects predominate (9), and at least one gene, the multidrug resistance gene encoding P-glycoprotein, is under progesterone control by the A-isoform (23). However, our observation that only a low frequency of transcripts are progesterone-regulated is supported by earlier data (24).

Progesterone regulation of FMO5 in breast cancer cells

Our data unequivocally show that three mRNAs containing FMO5 sequences are progesterone regulated in breast cancer cells specifically under the control of the PR B-isoform. To our knowledge no other B-receptor-specific regulated gene has been previously described. Additionally, we found no previous reports showing that FMO5 messages are expressed in normal or malignant breast cells. However, evidence is accumulating for both developmental and progesterone regulation of the five known FMOs in several tissues. Early studies showed that the enzyme is induced in rabbit lung during pregnancy (25) and in hepatic microsomes of CD1 mice during late gestation (26). More recently, modulation of FMO isoform B levels has been demonstrated in rabbit lung and kidney during pregnancy and after progesterone administration (27). Similarly, elevated levels of plasma progesterone, but not of cortisol, correlate with elevated FMO2 enzyme levels in the maternal and fetal rabbit lung, both during gestation and postpartum (28). On the other hand, FMO2 is induced by both progesterone and cortisol in the rabbit kidney (28). Interestingly, the FMO2 isoform is also induced by 17β-estradiol in the lung and by glucocorticoids in the liver (27), demonstrating tissue specific modulation of these enzymes by other steroid hormones. Regulation by progesterone and/or cortisol may be a direct

consequence of the binding of PR B-receptors or glucocorticoid receptors to the FMO5 promoter, which contains a progesterone/glucocorticoid response element (R.M. Philpot, personal communication). Why PR A-receptors do not activate this promoter will be the subject of future studies.

We observed three transcripts (3.8 kb, 2.6 kb, and 1.8 kb) in poly(A)$^+$ RNA isolated from human breast cancer cells that were labeled with the FMO5-specific probe. A recent study using human, rabbit, and guinea pig liver RNA demonstrated only two FMO5 products (3.8 kb and 2.6 kb) (17). The third, a 1.8-kb band that we observe may be caused by tissue-specific differences among FMO5 mRNAs produced in the breast compared with the liver, to the detection of a third FMO5 message that has not previously been reported, or to the presence of an unrelated mRNA that has a sequence similar to that of the FMO5 message. At present we cannot distinguish among these possibilities.

Our finding that FMO5 is progesterone regulated in breast cancer cells has interesting implications regarding drug metabolism in the breast and other progesterone target tissues. FMOs are flavin-containing microsomal monooxygenases that use NADPH as a cofactor. They catalyze the oxidation of a diverse array of substrates including hydrophobic foreign molecules and drugs (29, 30), and have a metabolic role akin to that of the cytochrome P450s (27). Indeed, FMOs as a group appear to be important in the detoxification of drugs, pesticides, and a variety of industrial, chemical, and other xenobiotics.

However, in addition to detoxification, under the influence of FMOs many xenobiotic compounds undergo metabolic activation and produce highly reactive and toxic intermediates that bind covalently to proteins or DNA (16, 31). For example, it has been suggested that the antiestrogen tamoxifen is a potential substrate for FMOs (16). The isoform of liver FMO that is responsible for the formation of the reactive tamoxifen intermediate has not yet been reported, and the FMO5 isoform of the enzyme was only recently cloned and sequenced from a human hepatic library (17). Although the antiestrogenic activity of tamoxifen is the major indicator for its current therapeutic and prophylactic use in breast cancer (32), concerns have been raised about site-specific second cancers arising after long-term therapy with this drug (33, 34). Specifically, tamoxifen-induced DNA adducts were recently described in five of seven endometrial samples of breast cancer patients (18). Our data offer the intriguing possibility that metabolic activation of a variety of drugs mediated by FMOs, including tamoxifen (16, 17), can be controlled by progesterone in target tissues like the breast and the uterus, that overexpress PR B-receptors. If so, in conditions of B-receptor excess, progesterone might accelerate tamoxifen-induced carcinogenesis. This also implies that heightened uterine B- to A-ratios might identify those women at greatest risk of developing tamoxifen-induced malignancies.

Acknowledgments

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Report

Progesterone receptor variants found in breast cells repress transcription by wild-type receptors

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Key words: progesterone receptors, splice variants, dominant negative variants, hormone dependence, markers

Summary

Progesterone, through its nuclear receptors (PR), regulates the development and growth of breast cancers. PR also serve as markers of hormone dependence and prognosis in patients with this disease, and functional PR are required to mediate the antiproliferative effects of progestin therapies. We find that normal and malignant breast cells and tissues can express anomalous forms of PR transcripts. We have isolated four variant PR mRNAs that contain precise deletions of exons encoding sections of the DNA- and hormone-binding domains. The transcripts lack exon 2 (PRΔ2), exon 4 (PRΔ4), exon 6 (PRΔ6), or exons 5 and 6 (PRΔ5,6). On immunoblots, PRΔ4, Δ6, and Δ5, 6 cloned into the background of the PR A-isoform comigrate with similar proteins present in breast tumor extracts; Δ6 and Δ5, 6 are dominant-negative transcriptional inhibitors of wild-type A- and B-receptors. We propose that expression of variant PR can compromise the accuracy of receptor measurements as markers of hormone-dependent cancers, and can modify the responses of tumors to progestin therapies.

Introduction

Progesterone is a key developmental and growth regulatory hormone of the breast. It also accelerates the development and growth of mammary tumors while antiprogestins inhibit both processes [1, 2]. The role of progestins in human breast disease is still poorly understood, but expression of progesterone receptors (PR) in breast cancers is a key marker of hormone dependence [3] and of disease-free survival [4]. PR are also direct targets of endocrine therapies. Synthetic progestins are routinely used for second-line treatment of tamoxifen-resistant tumors, and antiprogestins are undergoing clinical trials. Both the agonists and the antagonists depend on the presence of functional PR for their anti-tumor effects [2].

Steroid receptors, including PR, are modular transcription factors composed of a C-terminal nuclear localization signal (NLS) and hormone binding domain (HBD), a central DNA binding domain (DBD) and several activation functions (AFs) located in both the C- and N-termini. These and other functional domains are encoded by transcripts assembled from eight exons: exon 1 encodes the N-terminus, exons 2 and 3 the DBD, exon 4 the NLS

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and hinge region, and exons 5 to 8 the HBD [2, 5]. Cloning of many members of the steroid receptor family a decade ago defined the structure of the most common receptor messenger RNA that was present in the tissue from which the cDNA library was derived. Since then, these 'wild-type' structures have served as the bases for extensive characterization of receptor functional domains. More recently, as interest has focused on tissue-specific differences in the actions of steroid hormones and on mechanisms by which hormone resistance syndromes develop, it has become evident that the original transcripts are often the most common among other naturally occurring isoforms.

Since most steroid receptor transcripts are transcribed from a single gene, isoforms usually arise from alternate promoter usage, or by post-transcriptional mechanisms involving exonic deletions and alternate splicing. However, authentic genomic mutations have also been described. For example, variant, possibly tissue-specific estrogen receptor (ER) transcripts have been identified in normal and malignant tissues [5–10], while the only known example of somatic estrogen resistance in man is caused by a point mutation in the ER gene that generates a premature stop codon [11]. Two natural isoforms of androgen receptors (AR) exist [12], and a variety of AR mutations are associated with prostatic malignancies and somatic androgen resistance syndromes [13, 14]. Similarly, it now appears that there are two glucocorticoid receptors (GR) [15], while mutant GR have long been associated with glucocorticoid resistance syndromes [16]. A variety of natural receptor isoforms exist among members of the retinoic acid/thyroid receptor subfamily of nuclear receptors [17]. This receptor subfamily is encoded by multiple genes, explaining the origin of some of the heterogeneity. Recently, it has been found that heterogeneity among steroid receptors may arise by similar mechanisms. For example, a novel ER designated ER-β with unique tissue distribution is encoded by a gene that differs from that of classical ER [9]. Mutations associated with resistance syndromes have also been described in the retinoic acid/thyroid receptor subfamily [18, 19].

Knowledge that PR exist as two isoforms predates their cloning. The two human receptors – 120 kDa B-receptors and N-terminally truncated, 94 kDa A-receptors [20] – are the products of at least nine mRNAs [21] transcribed from two promoters in the PR gene [22]. Neither structural details about the transcripts nor reasons for their extensive heterogeneity are known. The relative levels of the two isoforms are developmentally and hormonally regulated in normal target tissues [23–25]. This complex regulation may be important, since the two isoforms have significant functional differences resulting from structural differences at their N-termini [26–29]. Additionally, a third isoform – the $\sim$ 60 kDa C-receptors – may represent exon 1 deletion variants, although their precise structure is unknown [21, 30]. Additional heterogeneity in PR protein expression has recently been demonstrated in breast cancers, in which, besides the B- and/or A-isoforms, a prominent 78 kDa PR is present in 25% of tumors, and other, lower Mr proteins, are sporadically detected [31]. The molecular structure of these tumor variants is also unknown.

We now describe unusual PR transcripts present in normal and malignant breast cells that encode receptors with DBD and HBD anomalies. Specifically, we find transcripts in which exons 2, 4, 5, and 6 are deleted. These mRNAs produce frame shifts resulting in C-terminally truncated proteins, or proteins in which key functional domains are wholly or partly deleted. The expressed recombinant exon 4, exon 6, and exon 5, 6 deletion variants comigrate with the $\sim$ 78 kDa PR variants commonly found in breast cancers [31]. The exon 6 and exon 5,6 variants are dominant-negative inhibitors of transcription by agonist-occupied wild-type PR A- and B-receptors. Our data suggest that if the exon deletion variants are expressed as proteins, they could alter the responsiveness of normal and malignant breast cells to progestins, and alter the interpretation of PR assays as markers of hormone-dependent tumors.

Methods

Cells and tissues

MCF-7 and $T47D_{CO}$ human breast cancer cell lines were grown as previously described [26]. Surgical

tumor specimens were cooled to 4° C. After removal of samples for pathological review, the remaining tissues were quick-frozen and stored at −70° C. Their use for research purposes was approved by the UCHSC Institutional Review Board.

Reverse transcription-polymerase chain reaction (RT-PCR)

Cells and tissues were lysed in TRI-REAGENT (Molecular Research Center, Inc., Cincinnati, OH) or by a modified guanidinium thiocyanate method with proteinase K digestion (Applied Biosystems, Foster City, CA). Contaminating DNA was removed with MessageClean (GenHunter Corp, Brookline, MA). RT-PCR was performed with GeneAmp (Roche Molecular Systems, Branchburg, NJ) using primers homologous to sequences in the human PR [32], ER [5], and β-actin [33] genes. For PR they were: set 1 (nt 744–768 and 1152–1173), which amplifies a 429 bp far N-terminal fragment in the B-upstream segment (BUS or B) unique to B-receptors in exon 1; set 2 (nt 1239–1260 and 1461–1482) which amplifies a 243 bp N-terminal fragment (A+B) common to both A- and B-receptors in exon 1; set 3 (nt 2355–2375 just upstream of the exon 1, 2 boundary, and nt 2675–2697 just downstream of the exon 3, 4 boundary), which amplifies a 342 nt fragment derived from exons 2 and 3 that encodes the two zinc fingers of the DBD; set 4 (nt 2619–2642 in exon 3 and nt 3393–3416 in exon 8) which amplifies a 797 nt fragment including exon 4 to the 5′ border of exon 8, encoding the hinge region NLS and HBD; and set 5 consisting of the 5′ sense primer of set 3 and the 3′ antisense primer of set 4, which amplifies a 1061 nt fragment that encodes the DBD plus HBD. Similar primer pairs designed for ER include set 1 (nt 617–637 and 1082–1103) to amplify a 486 nt exon 2 plus 3 DBD fragment, and set 2 (nt 971–994 and 1787–1811) to amplify an 840 nt exon 4 through 8 HBD fragment. Primers were designed to span large introns, work at high annealing temperatures and contain restriction enzyme sites for cloning. Controls included β-actin primers spanning one or three introns, and reaction mixtures lacking reverse transcriptase or RNA. RT-PCR products were resolved on agarose gels and stained with ethidium bromide, or probed by Southern blotting [34]. In some studies [^{32}P]dCTP was incorporated into the DNA strands during the amplification reactions. RT-PCR products were cloned into the EcoRI and BglII sites of pGEM7Zf+ (Promega, Madison, WI) and sequenced (Sequenase, Amersham, Arlington Heights, IL).

Construction of exon deletion mutants

A cDNA encoding the Δ6 deletion mutant was constructed in the background of B-receptors from the original PR Δ6 pGEM clone, by subcloning a mutant 207 bp fragment into wild-type pSG5-hPR1. cDNAs encoding the other mutants were recreated by PCR using pSG5-hPR1 [35] as a template, and oligonucleotides designed to selectively eliminate the desired exon. To create cDNAs encoding the identical mutants in A-receptors, fragments containing the mutations were subcloned into pSG5-hPR2. The constructs were verified by sequencing.

Immunoblotting and gel mobility shift assays

Whole cell extracts (WCE) were prepared from COS-1 cells transfected with the variant PR expression vectors, and tumors were homogenized in RIPA buffer [36]. PR were detected in protein blots using mAb B-30 or AB-52, as previously described [26, 27, 29].

Gell mobility shift assays were performed as described [27] using [^{32}P]labeled 27 bp oligonucleotides containing a palindromic progesterone response element (PRE), incubated with WCE prepared from COS-1 cells transiently transfected with 1 μg cDNA encoding each variant or wild-type PR and treated 1 hr or not with 10 nM R5020 prior to harvest.

Transfection and transcription assays

HeLa cells were cotransfected by calcium phosphate precipitation with 2 μg of the PRE_2-$TATA_{tk}$-

chloramphenicol acetyltransferase (CAT) reporter [27], the PR expression vectors, 1 μg of a β-galactosidase expression plasmid (Pharmacia-LKB Biotechnology, Piscataway, NJ), and carrier plasmid (Bluescribe, Stratagene, La Jolla, CA) for a total of 15 μg/plate). Twenty-four hours later, cells were incubated without or with 50 nM R5020 for an additional 24 hr. Cells were harvested, and lysates were analyzed for CAT activity by thin layer chromatography (TLC) as previously described [26, 27, 29].

Results

RT-PCR products from the MCF-7 human breast cancer cell line exhibit extensive heterogeneity in the PR DBD and HBD (not shown). The wild-type and variant bands from these amplification reactions were excised, subcloned, and sequenced (Figure 1, top). We have identified four splice variants carrying deletions of exon 2 (panel A, PRΔ2), exon 4 (panel B, PRΔ4), exon 6 (panel C, PRΔ6), and exons 5 and 6 (panel D, PRΔ5,6). All the deletions mapped precisely to the exon/intron boundaries, suggesting that the variant transcripts were generated by alternative splicing. We have isolated similar mRNA splice variants from a subline of $T47D_{CO}$ [20] human breast cancer cells (not shown).

These variant PR transcripts would encode proteins with the structures shown in Figure 1, bottom. Wild-type B-receptors are 933 amino acids in length, and the functional domains are assembled from 8 exons. In PRΔ2, AG of the AG/G triplet encoding Arg516 at the exon 1/2 boundary is spliced to the GG of G/GG at the exon 2/3 boundary, which shifts the reading frame of exon 3. As a result Arg516 is followed by 16 unique amino acids ending in a termination codon, which would produce a truncated protein consisting of the PR N-terminus lacking both DBD and HBD. In PRΔ4 Gly636 is encoded by G/GT which derives its first nucleotide from exon 3, and the other two from exon 4. The exon 4/5 boundary is also a G/GT triplet encoding Gly738 so that the exon 3/5 splice junction remains in the frame to the end of the mRNA. The resulting protein would contain a complete N-terminus and DBD but is missing amino acids 637 to 737 encoding

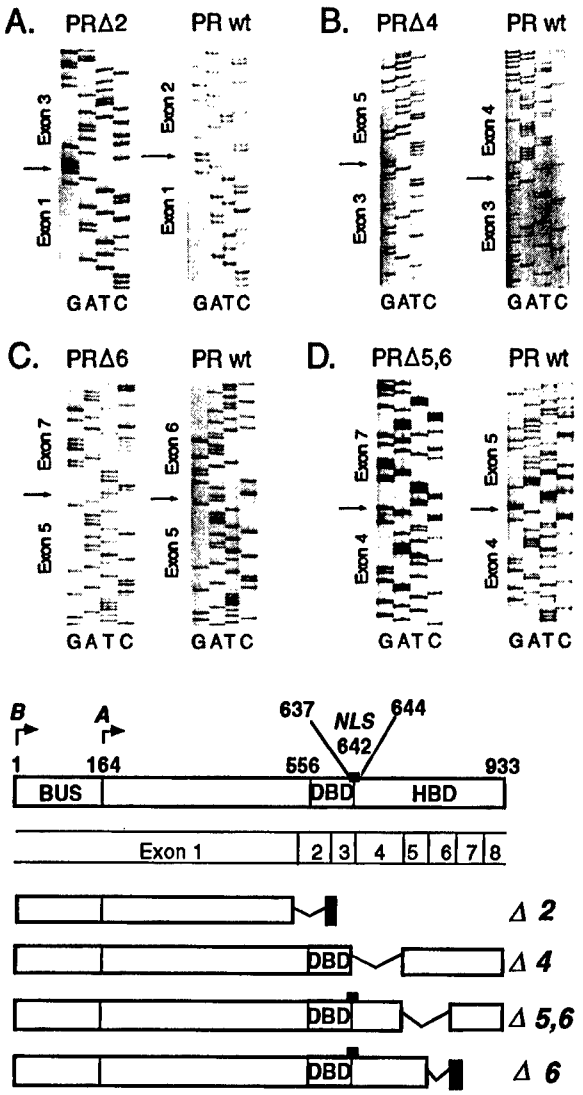

Figure 1. RT-PCR products and deduced protein structure of four PR variants isolated from MCF-7 breast cancer cells, compared to wild-type PR.

Top: Sequences surrounding the splice junction (arrows) of variant transcripts amplified by RT-PCR from MCF-7 cell total RNA, are shown in comparison to wild-type (wt) sequences at the same positions, demonstrating the missing exons. Four PR variants missing exon 2 (panel A), exon 4 (panel B), or exon 6 (panel C) and exons 5 and 6 (panel D) were isolated.

Bottom: Deduced protein structure of the four PR variants isolated from MCF-7 cells. B and A refer to the N-termini of the B- and A-isoforms, respectively; BUS, B-upstream segment; DBD, DNA binding domain; NLS, nuclear localization signal; HBD, hormone binding domain. Numbers indicate the amino acid borders of key domains. The black boxes at the C-terminus in Δ2 and Δ5, 6 indicate missense amino acids following a frame shift in the variant transcript, which terminate in a premature stop codon. Note that the Δ2 and Δ4 variants lack an NLS.

the NLS and a proximal segment of the HBD. In PRΔ5,6 the exon 4/5 border G/GT is spliced to the exon 6/7 border A/TT. In the resulting protein, Gly738 and Ile830 are replaced by a Val and the intervening amino acids are spliced out. The protein stays in frame and contains an intact N-terminus, DBD, NLS, and the far C-terminus, but it is missing the central core of the HBD. In PRΔ6 the GA/A exon 5/6 border is spliced to the A/TT exon 6/7 border leading to a frame shift and a premature termination codon. The resulting protein is truncated in the center of the HBD at Glu786 and contains 12 unique amino acids at the C-terminus.

Figure 2 shows the PR and ER RT-PCR products of RNA isolated from an intraductal carcinoma (panel B), and the surrounding normal breast tissue from the same patient (panel A). The tumor contained both invasive and *in situ* components, and was strongly ER-positive but PR-negative by immunohistochemistry (not shown). Receptor data for the normal tissue were unavailable. All four wild-type PR bands were amplified from the normal breast tissue including the BUS fragment labeled B (Figure 2A, lane 2), the A+B fragment (lane 3), the DBD fragment (lane 4), and a weak wild-type HBD fragment (lane 5). A weakly staining heterogeneous region of lower molecular weight was also discernible in the HBD, lane 5. To

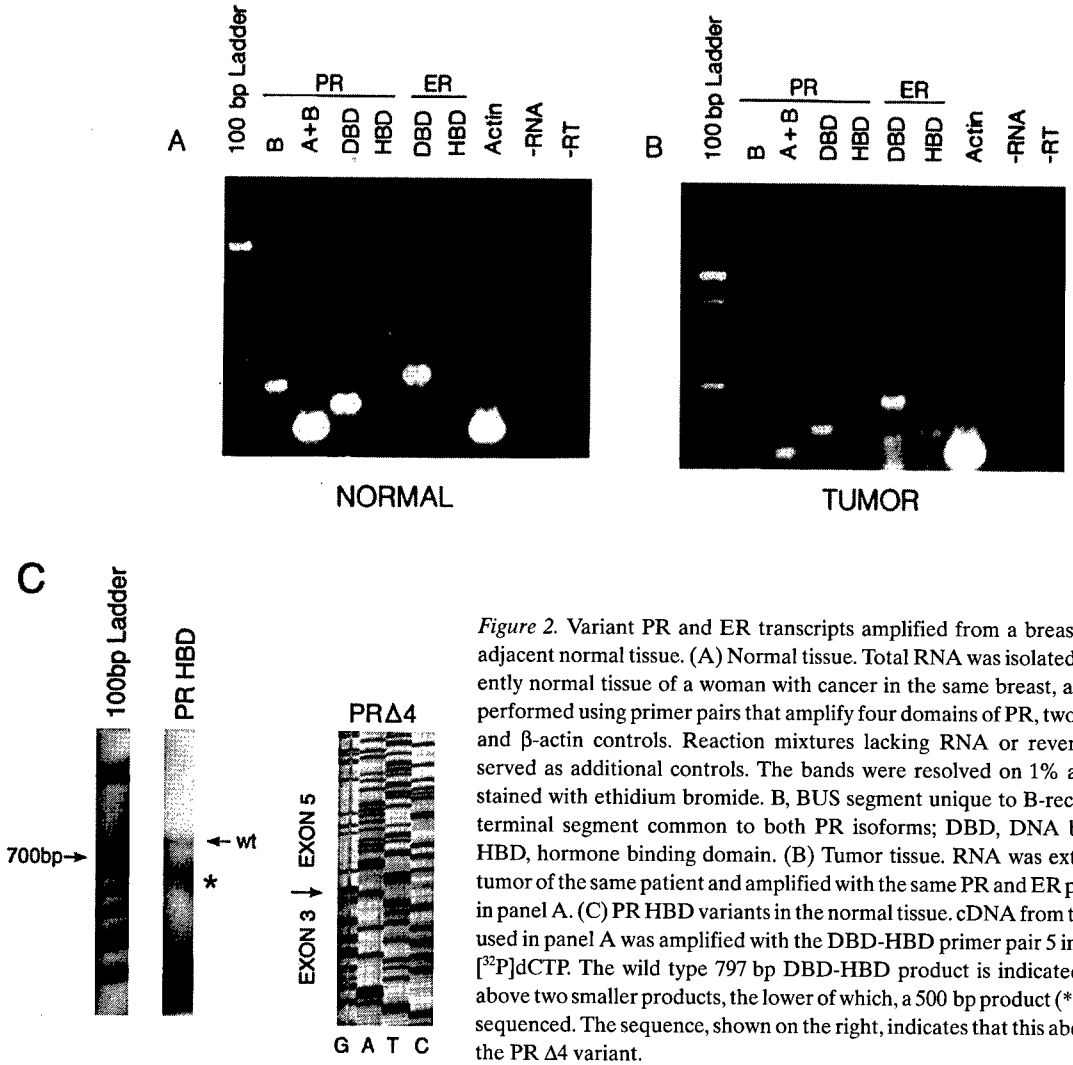

Figure 2. Variant PR and ER transcripts amplified from a breast tumor and the adjacent normal tissue. (A) Normal tissue. Total RNA was isolated from the apparently normal tissue of a woman with cancer in the same breast, and RT-PCR was performed using primer pairs that amplify four domains of PR, two domains of ER, and β-actin controls. Reaction mixtures lacking RNA or reverse transcriptase served as additional controls. The bands were resolved on 1% agarose gels and stained with ethidium bromide. B, BUS segment unique to B-receptors; A+B, N-terminal segment common to both PR isoforms; DBD, DNA binding domain; HBD, hormone binding domain. (B) Tumor tissue. RNA was extracted from the tumor of the same patient and amplified with the same PR and ER primer pairs used in panel A. (C) PR HBD variants in the normal tissue. cDNA from the normal tissue used in panel A was amplified with the DBD-HBD primer pair 5 in the presence of [^{32}P]dCTP. The wild type 797 bp DBD-HBD product is indicated with an arrow above two smaller products, the lower of which, a 500 bp product (*) was cloned and sequenced. The sequence, shown on the right, indicates that this aberrant product is the PR Δ4 variant.

visualize this region [^{32}P]dCTP was incorporated into the DNA strands during the amplification reactions using PR primer set 4 (panel C, left). This clearly shows, in addition to the wild-type 797 bp HBD band (arrow), two smaller bands of ~ 600 and 500 bp. The lowermost of these (star) was subcloned and sequenced (panel C, right), and found to lack exon 4 analogous to the PRΔ4 deletion variant present in MCF-7 cells (Figure 1B).

The ER from the normal breast tissue (Figure 2A) is also of interest. There is a strong wild-type band in the DBD lane, plus a minor lower molecular weight variant in the same lane that has not been characterized. However, from the same RT cDNA product, no HBD band was amplifiable. Thus the ER transcripts in this presumably normal tissue are anomalous.

The PR and ER RT-PCR products amplified from the tumor of this patient are shown in Figure 2B. Despite a strong β-actin signal from the cDNA, no PR BUS (lane 2) or HBD (lane 5) signals were amplified. However, the A+B products (lane 3) and the DBD products (lane 4) were present. Thus, unlike the normal tissue, which appears to express mRNAs encoding all four functional domains for both PR isoforms, the tumor has few if any B-receptor specific transcripts, and lacks mRNAs encoding an intact HBD. Note that failure to amplify HBD sequences with primer pair 4 (which anneal to nucleotides in exons 3 and 8) probably results from a deletion or mutations in one of the primer binding sites. We know that exon 3 sequences are preserved based on amplification of a DBD product of predicted size from the same cDNA synthesis reaction. Thus failure of HBD amplification may result from anomalies in exon 8. We have noted similar discordance in the structure of PR transcripts when comparing tumors and the surrounding normal tissue, in several paired sets taken from breast and endometrial cancers (not shown). The tumor ER DBD (Figure 2B) shows the same variant seen in the normal tissue (Figure 2A), in addition to the wild-type DBD band. Like the normal tissue, no 840 bp wild-type ER HBD band was amplified from the tumor, but two aberrant smaller HBD bands were present that were not observed in the normal specimen.

To study functions of the PR variants, they were

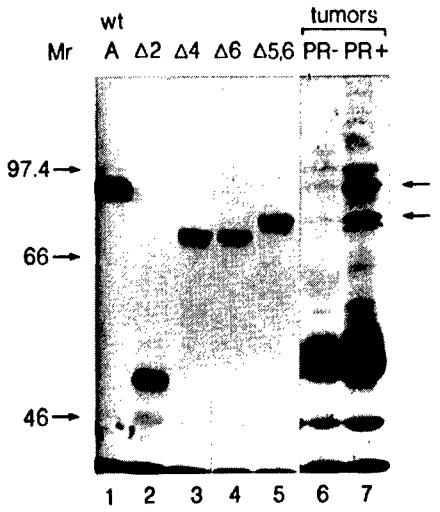

Figure 3. PR immunoblot of recombinant wild-type and variant A-receptors, and extracts from a PR-negative and PR-positive breast cancer. Whole cell extracts of COS-1 cells transiently transfected with expression vectors encoding recombinant wild-type and variant A-receptors (lanes 1–5), or natural tissue extracts from a PR-negative (lane 6) and a PR-positive (lane 7) breast tumor, were electrophoretically resolved, transferred to nitrocellulose, and probed with the anti-PR mAb AB-52. The upper arrow indicates the wild-type A-receptors. The lower arrow indicates smaller Mr, A-receptor variants in the PR-positive tumor. B-receptors (Mr 120 kDa) are not expressed in this PR-positive tumor.

reconstructed in the background of both B- and A-receptors. The cDNA constructs were transfected into COS-1 cells, and protein expression levels of wild-type A-receptors (lane 1) and the four exon deletion variants cloned into the background of A-receptors (lanes 2–5) were measured by immunoblotting, as shown in Figure 3. Also shown in Figure 3 are immunoblots of extracts from a PR-negative (lane 6) and a PR-positive (lane 7) breast tumor. Wild-type A-receptors have an Mr of ~ 94 kDa. The AΔ2 variant is truncated upstream of the DBD (Figure 1) and resolves as an ~ 50 kDa fragment, while the Mr of the other three variants is 70–80 kDa. The PR-positive tumor (lane 7) lacks wild-type B-receptors, but it expresses wild-type 94 kDa A-receptors (upper arrow) as well as two lower molecular weight A-receptor variants (lower arrow) of 75–80 kDa that are undetectable with a B-receptor specific antibody (not shown). These smaller bands resemble the 78 kDa PR proteins found in 25% of breast cancers [31]. The PR varia-

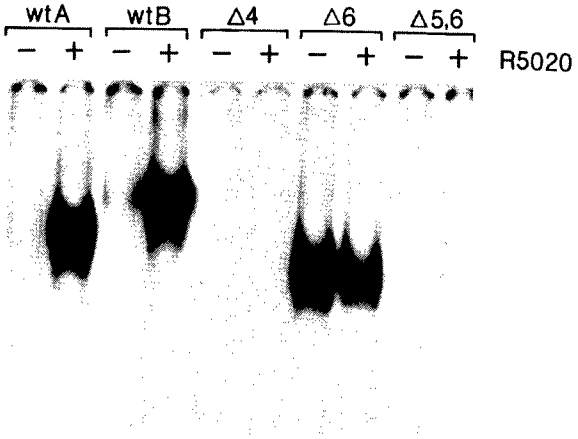

Figure 4. Gel mobility shift assay showing the DNA binding properties of wild-type and variant PR. Whole cell extracts were prepared from COS-1 cells transiently transfected with expression vectors encoding wild-type PR A- and B-isoforms and three variants cloned into the background of A-receptors. Cells were treated with (+) or without (−) 10 nM of the progestin R5020 for 1 hr. Extracts were incubated with a 27 bp [^{32}P]labeled oligomer containing one copy of the palindromic PRE from the tyrosine amino transferase promoter, then resolved on a nondenaturing electrophoretic gel, dried, and autoradiographed.

nts in the tumor (lane 7) resolve at a similar position as the AΔ4, AΔ6, and AΔ5,6 recombinant variants in lanes 3–5, suggesting a possible molecular structure for the naturally occurring proteins. However, only protein microsequencing can unequivocally define the structure of the natural variants.

Because A-receptor variants may be more abundant in tumors than their B-receptor counterparts (Figure 3 and reference [31]) their functional properties are described below. The gel mobility shift assay shown in Figure 4 compares the ability of liganded or unliganded wild-type A- and B-receptors and three A-receptor variants to bind DNA at a PRE *in vitro*, after they are extracted from transfected COS-1 cells. As shown, the two wild-type PR isoforms do not bind DNA unless they are occupied by R5020. The AΔ4 and AΔ5,6 variants and the AΔ2 variant (not shown) fail to bind DNA whether or not ligand is present, while AΔ6 binds DNA constitutively.

The variant B- and A-receptors were also tested for their ability to regulate transcription. Data for the A-receptor variants are shown in Figure 5. When each is expressed alone, AΔ4, AΔ6 and AΔ5, 6 have little or no intrinsic transcriptional activity in the absence of ligand, or when occupied by R5020

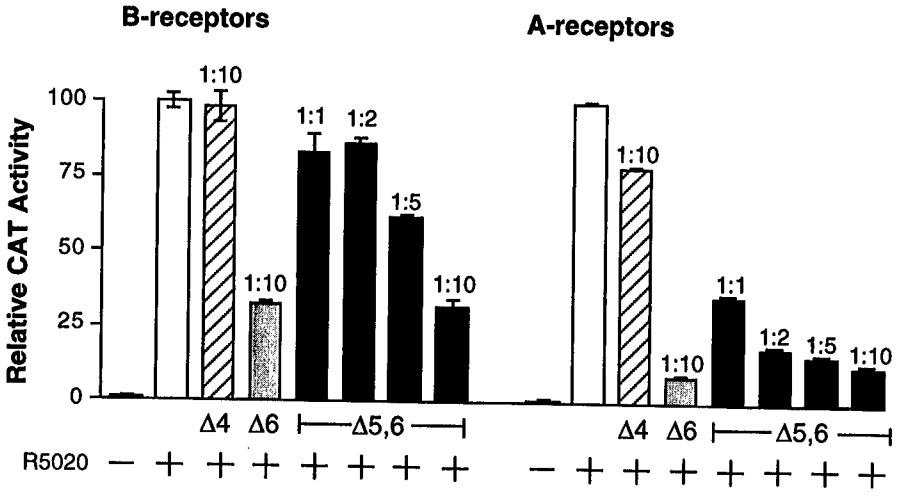

Figure 5. The PR variants Δ6 and Δ5, 6 are dominant-negative inhibitors of transcription by wild-type B- and A-receptors. HeLa cells were cotransfected with expression vectors encoding wild-type B-receptors (50 ng, left) or wild-type A-receptors (150 ng, right) in the absence (open bar) or presence (filled bars) of equimolar (1:1) to 10-fold (1:10) molar excess of expression vectors for the variants indicated, cloned into the background of A-receptors. Cells were also cotransfected with a β-galactosidase expression vector to control for transfection efficiency and with the PRE_2-$TATA_{tk}$-CAT reporter, then left untreated (−) or treated (+) with R5020 for 24 hrs. Cells were harvested and β-galactosidase-normalized extracts were analyzed for CAT activity by TLC. The ligand-dependent transcriptional activity of wild-type B-receptors (left) or A-receptors (right) was set at 100%. The error bars show the variation within assays for duplicate plates. The results shown here are representative of three similar experiments.

or the antiprogestin RU486 (not shown). However, Figure 5 shows that AΔ6 and AΔ5,6 are dominant-negative inhibitors of transcription when they are co-expressed with wild-type PR B-receptors (left) or A-receptors (right). In this study, HeLa cells were cotransfected with the PRE_2-$TATA_{tk}$-CAT reporter, the expression vectors for wild-type B- or A-receptors, and equimolar to 10-fold higher concentrations of cDNAs for the variants, cloned into the background of A-receptors. Transcription by the wild-type PR B- or A-receptors was set at 100%. Note, however, that wild-type, R5020-occupied B-receptors are 5–10 fold stronger transactivators than A-receptors. Co-expression of 10-fold higher concentrations of the AΔ4 cDNA has no effect on transcription of either wild-type receptor, due perhaps to the fact that this variant lacks an NLS (Figure 1) and is therefore located in a different cellular compartment than the wild-type receptors. On the other hand, at 10-fold molar excess, AΔ6 or AΔ5,6 reduce the transcriptional activity of B-receptors by 60–70%. Interestingly, wild-type A-receptors appear to be more sensitive than B-receptors to the presence of the co-expressed variants (right panel). Even at equimolar concentrations (as confirmed by immunoblotting), AΔ5,6 reduces the activity of wild-type A-receptors by 65%, and higher concentrations of AΔ5,6 or AΔ6 suppress A-receptor activity by $\sim$ 90%. A similar dominant-negative effect of the PR BΔ5,6 and BΔ6 variants on transcription by wild-type B- and A-receptors was observed (not shown) indicating that the inhibition is not A-isoform specific. Overall, these data suggest that expression of variant receptors could profoundly alter the progesterone responsiveness of tumors or tissues in which they are expressed.

Discussion

Steroid receptors are complex transcription factors that interact with an array of coregulatory proteins, form a variety of heterodimeric partnerships, are subject to cell- and promoter-specific controls, can regulate transcription without binding to DNA, and are influenced by cross-talk with other signaling pathways [39, 40]. This complexity extends to PR, in which the two isoforms assemble into three dimeric species, each having a different biological activity [3]. In general, A-receptors are weaker transactivators than B-receptors. Additionally, in the presence of antiprogestins, A-receptors can inhibit the activity of B-receptors. In our hands, agonist-occupied A-receptors are not inhibitory. The expression of novel PR variants has the potential to broadly expand the diversity of PR actions. During the preparation of this paper, the PR variants Δ4 and Δ6 were reported in T47D-5 cells and breast tissue [41]. The PR Δ6 variant was seen more frequently in the breast tumors examined than in reduction mammoplasties [41]. However, no functional analyses were done with these variants.

In the present study, we characterize PR Δ6 and Δ5,6 as dominant-negative inhibitors of agonist-dependent transcription by wild-type PR. In contrast, the PR Δ4 variant has no effect on the activity of wild-type receptors, probably because it lacks an NLS. The PR Δ2 variant, which is truncated upstream of the DBD, also lacks an NLS. Deletion of this domain segregates these two variants to the cytoplasm, away from the transcriptional machinery. We tested PR Δ4, however, because of the theoretical possibility that through its HBD it can dimerize with wild-type PR and be indirectly translocated to the nucleus. The detailed mechanisms involved in the inhibitory effects of the other two variants remain to be determined. A variant like Δ6, which binds DNA constitutively but is transcriptionally inactive, could theoretically block access of wild-type receptors to DNA. A variant like Δ5,6 which – at least *in vitro* – does not bind to DNA, could theoretically 'squelch' transcriptional coregulatory factors that are required for function by the wild-type receptors. The possibility that some of these variant transcripts are expressed as proteins in breast cancers is suggested by immunoblotting studies which show anomalous receptor bands in many tumors [31].

Our demonstration that unusual forms of PR transcripts are expressed in normal and malignant breast cells, and that some of the resultant variant proteins can inhibit the agonist-dependent transcriptional activity of wild-type PR, could have interesting clinical implications. First, while PR are

routinely measured in breast cancers as a guide to endocrine therapy, approximately 30% of PR-positive tumors fail to respond to hormone treatments [42]. The clinical assay methods currently in use to measure PR levels in breast cancers cannot distinguish between the two PR isoforms, and would not detect PR variants. Variant PR could account for some of these false-positive tumors. Thus, PR immunoassays that use an antibody whose epitope lies in the N-terminus, will score a tumor as PR-positive even if the receptors lack key functional domains lying downstream of that epitope. Similarly, an assay that is based on agonist binding properties of the HBD will score a tumor as PR-negative even if key activation domains, antagonist binding sites, or DNA binding functions of the receptors are retained. Second, tumors that express variant PR can theoretically respond anomalously to progestins. At present, Megace or medroxyprogesterone acetate (MPA) are commonly used for second-line endocrine therapy of antiestrogen-resistant breast cancers [43]. We know little about the mechanism by which these progestins inhibit the growth of some, but not all, PR-positive tumors, or about the differential biological activity that they may have on the two PR isoforms or the variants. Third, progestins are widely used by healthy women for oral contraception and hormone replacement therapy [44]. The existence in normal tissues, not only of two functionally different PR isoforms, but of additional PR variants, may lead to the tissue-specific differences in progestin actions that are commonly observed. For example, progestins are antiproliferative in the uterus, and are therefore added to estrogens for hormone replacement therapy at menopause because they block the proliferative and tumorigenic effects of unopposed estrogens. On the other hand, in the breast, the same concentrations of progestins are not antiproliferative, and therefore are not protective [1, 2, 44]. Perhaps a detailed analysis of the endometrium and breast, for the varieties of PR forms that they express, would explain the heterogeneity of tissue responses to progestins. Finally, advanced hormone-responsive breast cancers are fatal because the tumors acquire resistance to endocrine therapies [45]. Overexpression of dominant-negative receptor variants during tumor progression could provide one explanation for progestin resistance.

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