[CANCER RESEARCH 54, 3868–3877, July 15, 1994]

New T47D Breast Cancer Cell Lines for the Independent Study of Progesterone B- and A-Receptors: Only Antiprogestin-occupied B-Receptors Are Switched to Transcriptional Agonists by cAMP[1]

Carol A. Sartorius, Steve D. Groshong, Louise A. Miller, Roger L. Powell, Lin Tung, Glenn S. Takimoto, and Kathryn B. Horwitz[2]

Departments of Medicine [R. L. P., L. T., G. S. T., K. B. H] and Pathology [L. A. M., K. B. H.] and the Molecular Biology Program [C. A. S., S. D. G., K. B. H], University of Colorado Health Sciences Center, Denver, Colorado 80262

ABSTRACT

Because progesterone antagonists are growth inhibitors, they are in Phase III clinical trials for the treatment of breast cancer. However, when cellular cAMP levels are elevated, some antiprogestins inappropriately activate transcription. We have proposed that hormone "resistance" may result from such unintended stimulation of breast cancer by antagonists. In transient expression systems, the two natural isoforms of human progesterone receptors (PR), B-receptors and truncated A-receptors, have dissimilar effects on agonist-mediated transcription. We show here that in the presence of 8-Br-cAMP, antiprogestin-occupied B-receptors but not A-receptors become transcriptional activators. Therefore, we developed new model systems to study each PR isoform independently in a breast cancer setting: (*a*) a stable PR-negative monoclonal subline (T47D-Y) of PR-positive T47D breast cancer cells was selected by flow cytometric PR screening. T47D-Y cells are PR-negative by immunoassays, by ligand binding assay, by growth resistance to progestins, by failure to bind a progesterone response element (PRE) *in vitro*, and by failure to transactivate PRE-regulated promoters; and (*b*) T47D-Y cells were stably transfected with expression vectors encoding one or the other PR isoform, and two monoclonal cell lines were selected that express either B-receptors (T47D-YB) or A-receptors (T47D-YA) at levels equal to those seen in natural T47D cells. The ectopically expressed receptors are properly phosphorylated, and like endogenously expressed receptors, they undergo ligand-dependent down-regulation. The expected B:B or A:A homodimers are present in cell extracts from each cell line, but A:B heterodimers are missing in both. In the presence of agonists, cAMP-dependent, transcriptional synergism of PRE-regulated promoters is seen in both cell lines. By contrast, in the presence of the antiprogestins RU486 or ZK112993, inappropriate transactivation occurs in YB cells but not in YA cells. The class of antiprogestins represented by ZK98299, which blocks PR binding to DNA, does not activate transcription in either cell line. We propose that these new cell lines are physiological models for the study of PR isoform-specific antiprogestin resistance in breast cancer.

INTRODUCTION

Because T47D breast cancer cells express constitutively high levels of PR,[3] they have become the major model to study the actions of progesterone and synthetic progestins in human cells. For example, using T47D cells, we (1) and others (2, 3) recently showed that in the presence of elevated intracellular levels of cAMP, PR occupied by progesterone antagonists can, unexpectedly, become strong transcriptional activators. Since progesterone antagonists are now in Phase III clinical trials for the treatment of breast cancer, the ominous consequences of such inappropriate transcriptional activation are clear. Therefore, it is important to understand, in breast cancer cells, the basic molecular mechanisms responsible for both the inhibitory as well as the stimulatory activity of these hormones.

One difficulty in unraveling the mechanisms of antiprogestin action is that the PR in T47D cells, like those in other human tissues, exist as two natural isoforms, hPR_B that are 933 amino acids in length and truncated hPR_A that lack 164 NH_2-terminal amino acids (4–8). In the few human tissues in which the receptor ratio has been analyzed, B- and A-receptors have been found in approximately equimolar amounts (5, 9, 10). The two isoforms are believed to be generated by translation initiation from two in-frame AUGs present on nine different, isoform-specific mRNAs, transcribed from two different promoters in the single PR gene (7, 11–13). Hormone binding leads to dimerization of the two isoforms so that three dimeric species are produced: A:A and B:B homodimers and A:B heterodimers. The receptor dimers then bind to DNA at specific PRE on the promoters of progesterone responsive genes and regulate transcription (Ref. 14 and references therein; Ref. 15). Until recently, the mixture of two receptor proteins in one cell has not been an issue in studies of progestin actions because the hetero- and homodimers have been presumed to be functionally equivalent. Considerable data are, however, now accumulating, suggesting that this presumption is incorrect (16). Tora *et al.* (16) have shown by transient transfection methods that the two chicken PR isoforms activate transcription unequally from some promoter-reporter constructs, and Meyer *et al.* (17) showed that the antiprogestin RU486 can have weak agonist-like activities when bound to B-receptors but not A-receptors. We recently showed that the transcriptional activation by antagonist-occupied B-receptor homodimers can occur through an unusual mechanism that does not require binding of the receptors to DNA at PRE (18). Furthermore, antagonist-occupied A-receptor homodimers are not only transcriptionally silent in this setting, but when present in the A:B heterodimer, A-receptors dominantly inhibit the inappropriate activation by B-receptors. Thus, it is now becoming evident that B- and A-receptors are functionally different and that the ratio of B- to A-receptors present in normal or malignant cells may dictate the direction of response to an antiprogestin.

Most of the studies described above were done by transient transfection of PR expression vectors and promoter-reporter constructs into HeLa human cervicocarcinoma cells or CV-1 monkey kidney cells and their derivatives. While these methods have been extremely valuable for molecular analyses of steroid-regulated transcription, they have some drawbacks: (*a*) only 8–10% of cells express the transfected receptors; (*b*) in the PR-positive subpopulation, receptor levels are overexpressed to supraphysiological if not pharmacological levels, raising the possibility that "squelching" artifacts account for some of the observed effects; (*c*) consistent levels of expressed

Received 2/25/94; accepted 5/17/94.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] These studies were supported by NIH Grants CA26869 and DK48238. C. A. S. and S. D. G. were supported by a stipend from the Lucille P. Markey Charitable Trust.

[2] To whom requests for reprints should be addressed, at Departments of Medicine and Pathology, University of Colorado Health Sciences Center, Campus Box B151, 4200 East Ninth Avenue, Denver, CO 80262.

[3] The abbreviations used are: PR, progesterone receptor(s); hPR_B, progesterone B-receptors; hPR_A, progesterone A-receptors; PRE, progesterone response element(s); TAT, tyrosine aminotransferase; tk, thymidine kinase; CAT, chloramphenicol acetyltransferase; MMTV, mouse mammary tumor virus; ER, estrogen receptor(s); MEM, minimal essential medium; FCS, fetal calf serum; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; cDNA, complementary DNA; GR, glucocorticoid receptor(s); AR, androgen receptor(s).

receptors are difficult to achieve from one experiment to the next; (*d*) the recipient cells lack appropriate tissue-specific factors and may not even arise from progesterone target cells; and (*e*) transient expression of the receptors precludes performing long-term experiments. Our interest is to study the independent effects of B- and A-receptors under physiological conditions in the setting of human breast cancer cells. This is impossible with existing PR-positive breast cancer cell lines, all of which contain mixtures of B- and A-receptors. To circumvent both the weaknesses of transient transcription methods and the lack of suitable models, we describe here the development of new cell lines. First, a PR-negative monoclonal T47D cell line (called T47D-Y cells) was selected by flow cytometric PR screening after cloning by limiting dilution. Then, T47D-Y cells were stably transfected with either B-receptor (pSG5-hPR1) or A-receptor (pSG5-hPR2) expression vectors (11), and two monoclonal cell lines, independently expressing approximately equal levels of either hPR_B (called T47D-YB cells) or hPR_A (called T47D-YA cells), were selected by immunoblotting. These cells were used to demonstrate that elevated levels of cAMP switch the transcriptional phenotype of some progesterone antagonists to render them strong agonists, but this occurs only when these hormones are bound to B-receptors. In additional studies,[4] we demonstrate that these new cell lines are indeed suitable for long-term growth experiments. We find that the inappropriate transcriptional stimulation produced by antiprogestin-occupied B-receptors is reflected physiologically by enhanced growth of 8-Br-cAMP plus antagonist-treated T47D-YB cells but not T47D-YA cells.

MATERIALS AND METHODS

Recombinant Plasmids. pSG5-hPR2 and pSG5-hPR1, the hPR_A and hPR_B expression vectors cloned into pSG5 (11), were a gift of P. Chambon (Strasbourg, France). PRE-*tk*-CAT, a reporter plasmid, contains one copy of the TAT gene promoter PRE (19), cloned upstream of the *tk* promoter (−105/+51) and linked to the CAT reporter as described (18). MMTV-CAT contains the *Hae*III fragment (−631/+125) of the MMTV long terminal repeat linked to CAT (20) and was obtained from P. Chambon. PRE_2-$TATA_{tk}$-CAT contains the TATA box and flanking sequences of the *tk* promoter (−60/+51) cloned downstream of two copies of the TAT promoter PRE.

Cell Lines. The ER-positive, PR-positive parental T47D cell line was cultured by Keydar *et al.* (21) from a patient with breast cancer metastatic to the lung. Several phenotypically different, clonally derived sublines have been described including estrogen-sensitive T47D-clone 11 (21), and estrogen-resistant $T47D_{co}$ (22) and its clonal derivatives T47D-V22 and T47D-D (23). T47D-D have been progressively losing PR. At their current passage (~p570), they are functionally PR negative. $T47D_{co}$ stably transfected with MMTV-CAT were renamed T47D-B11 (24). $T47D_{co}$, V22, and clone 11 are used interchangeably herein as "wild-type" T47D cells since they have similar PR levels. MDA-231 are also of breast cancer origin but unrelated to the T47D cell line and were cultured by Cailleau *et al.* (25). They have always been ER and PR negative.

T47D-Y and T47D-X Cloning by Limiting Dilution. The PR-negative T47D-Y and T47D-X lines were obtained by cloning the T47D-D cell line by limiting dilution. Two confluent T175 flasks, containing approximately 64 million *Mycoplasma*-free T47D-D, were harvested at passage 485 after flow cytometry (26, 27) indicated that these cells contained a large (~55%) PR-negative subpopulation. Cells were diluted in antibiotic-free MEM-based T47D-D cell-conditioned growth media containing 5% FCS to obtain suspensions of 50, 10, or 5 cells/ml. Each well of six 96-well plates was plated with 0.1 ml of each cell dilution together with 0.1 ml of cell-free conditioned growth media. After 7 and 14 days, wells were inspected, and those containing a single colony were scored. Of 576 wells, 91 were scored twice as a single colony and were transferred to 24-well plates (passage 1) at confluence. Cells were again grown to confluence and were transferred to progressively larger flasks. Doubling times varied considerably among the clones. Sixty-eight of the 91 subclones survived and were analyzed by flow cytometry to determine their PR content. Fifteen subclones containing approximately 10 PR fluorescence units or less were then analyzed by immunoblotting using the enhanced chemiluminescence detection system (Amersham). Two cell lines (T47D-X and T47D-Y) were identified as being PR-negative at passage 6 (Fig. 1). T47D-Y cells were placed in continuous culture and remained PR-negative without selective medium at passage 65.

Construction of YA and YB Cells. T47D-Y cells (5–7.5 × 10^5) were plated in each of eight 100-mm culture dishes using MEM supplemented with 5% FCS. Four plates were transfected with 10 μg of pSG5-hPR2, the hPR_A expression vector and four others with pSG5-hPR1, the hPR_B expression vector. Cells were cotransfected with 1 μg of a plasmid encoding the neomycin resistance gene pSV2neo. Calcium phosphate precipitation was used to introduce the DNA into cells (28). Cells were cultured for 4 weeks in media supplemented with 500 μg/ml of the neomycin analogue G418 (GIBCO), and surviving colonies were selected. Individual neomycin-resistant colonies were transferred to multiwell plates and expanded in the presence of 200 μg/ml G418. Twenty cell lines transfected with pSG5-hPR2 and 34 cell lines transfected with pSG5-hPR1 were analyzed for PR expression by immunoblot analysis using the monoclonal anti-PR antibodies AB-52 or B-30 (29). Three of 20 cell lines expressed hPR_A, and 1 of 34 expressed hPR_B. One hPR_A expressor (T47D-YA) and the hPR_B expressor (T47D-YB) have been maintained in continuous culture in the presence of G418 through at least 30 passage generations. The A-receptor containing cell line YA was selected based on comparison of its receptor levels with those of YB.

Immunoblotting. hPRs were extracted from whole cells by freeze-thawing in 0.4 M KCl-containing buffer, separated by SDS-PAGE, and transferred to nitrocellulose as described (30). After incubation with the anti-PR antibody AB-52, the receptor bands were detected by enhanced chemiluminescence (Amersham) and quantified by densitometry.

Gel Mobility Shift Assay. Whole-cell extracts containing receptors, obtained by freeze-thawing in 300 μl 0.4 M KCl, were prepared from T47D cells growing in T25 flasks in MEM supplemented with 5% twice charcoal-stripped, heat-inactivated FCS. Binding reactions contained 25 mM 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid, 2.5% Ficoll 400, 1.0 mM $MgCl_2$, 2.0 mM dithiothreitol, 0.15 mM EDTA, 10% glycerol, and 2 μg (poly)dIC in a total volume of 20 μl. Four μl of cell extract were preincubated 10 min at room temperature in binding buffer and then incubated 15 min at room temperature with 40,000 cpm of a 27-base pair perfect palindromic PRE (5′-AAA GTC AGA ACA CAG TGT TCT GAT CAA-3′). The reaction mixture was analyzed by nondenaturing gel electrophoresis using a 5% acrylamide-0.125% bis-acrylamide gel that was pre-run for 1 h at 10 mA and then run for 3 h at 20 mA/gel. The dried gel was autoradiographed as described previously (30).

Transfection and Transcriptional Analyses. T47D cells were plated and grown in 100-mm^2 cell culture plates in MEM supplemented with 5% twice charcoal-stripped, heat-inactivated FCS. After 24 (T47D-Y) or 72 h (YA or YB), transfection of plasmid DNA into cells was performed by calcium phosphate coprecipitation (28) using 1 μg of the hPR expression plasmids (for T47D-Y cells), 1 μg of the appropriate reporter, 3 μg of the β-galactosidase expression plasmid pCH110 (Pharmacia) to correct for transfection efficiency, and 15 or 16 μg of Bluescribe carrier plasmid (Stratagene) for a total of 20 μg DNA. To improve the transfection efficiency of T47D-Y cells, 24 h after transfection, the medium was aspirated, and the cells were shocked at room temperature for 4 min with 5 ml of Hank's balanced salt solution containing 20% glycerol. After washing the cells twice with 10 ml of serum-free MEM to remove the glycerol, 10 ml of MEM containing 7.5% of stripped FCS with or without 0.5% penicillin-streptomycin were added per dish, together with the hormone additions indicated. Cells were harvested after an additional 48 (YA or YB) or 72 (T47D-Y) h. The steroids tested were R5020 (Roussel-Uclaf) at 10–50 nM and RU486 (Roussel-Uclaf), ZK98299 and ZK112993 (Schering, AG) usually at 100 nM, present alone or with 1 mM 8-Br-cAMP (Sigma). Duplicate plates of cells were lysed by freeze-thawing in 200 μl of 0.25 M Tris, pH 7.8. Lysates (50–100 μl) were assayed for β-galactosidase activity; normalized lysate aliquots were assayed for CAT activity by TLC as described (1, 18), and the plates were autoradiographed and also quantified by phosphorimaging and computer densitometry (Molecular Dynamics).

PR Immuno-Flow Cytometry. The simultaneous flow cytometry assay for PR and DNA was described in detail elsewhere (26, 27). Briefly, 1 to 2 million cells were fixed with 0.5% paraformaldehyde and permeabilized with

[4] S. D. Groshong and K. B. Horwitz, manuscript in preparation.

0.1% Triton X-100. Cells were washed and then incubated with the anti-PR monoclonal antibody AB-52. Nonspecific binding was determined in parallel sets of cells incubated with a mouse monoclonal IgG1 (Coulter Immunology, Hialeah, FL). The cells were washed and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse $F(ab')_2$ (Boehringer-Mannheim, Indianapolis, IN). DNA was then labeled at 37°C by simultaneous treatment with RNase A and propidium iodide. Cells were passed through a nylon mesh, and 10,000 cells were analyzed on a Coulter Epics 752 flow cytometer. DNA content and cell cycle stages are not shown; PR levels were quantified with the 1-Par data analysis program.

Dextran-coated Charcoal PR Assay. Progestin binding to PR was measured by modification of the charcoal-dextran method as described previously (22). Briefly, cytosols were incubated in triplicate with 20 nM (final concentration) of the progestin [^{3}H]R5020 in the presence or absence of 2 μM unlabeled R5020. After 16 h at 4°C, a charcoal suspension was added and incubated for 15 min. The charcoal was sedimented, and an aliquot of the charcoal-resistant supernatant was counted. The data were normalized to extract protein content.

RESULTS

PR-Negative T47D-Y Cells. T47D cells are the major model for studies of hPR and progesterone action in breast cancer, but they are phenotypically different from other breast cancer cell lines. Therefore, it seemed important that the T47D cellular milieu be preserved without the selective pressures imparted by hormone treatments when developing any new hPR and breast cancer models. A PR immunoassay based on flow cytometry (26, 27) showed that our T47D-D subline (23) contained a majority of PR-negative cells. Cells from this line were cloned by limiting dilution. The initial PR analysis was by immuno-flow cytometry, which requires less than 1 million cells, and is particularly suitable for the rapid screening of multiple clonal lines without extensive cell number expansion. Surprisingly, of 68 clonally derived lines initially scored as PR-negative, only two remained PR negative (not shown), suggesting that there is considerable instability in PR expression in the T47D-D cell line. Fig. 1 shows an immunoblot of eight clonal sublines at passage 6 that were selected based on their low immunoreactivity by flow cytometry at passages 3 or 4. Six of the cell lines had moderate PR levels by immunoblotting, but two clones named T47D-X and T47D-Y were PR negative. The PR-negative phenotype of T47D-Y cells appeared to be especially stable, and they were chosen for further study. Karyotype analyses and marker chromosomes (not shown) confirm their monoclonal origin and their genetic homology to T47D-D cells and to the parental T47D line established by Keydar *et al.* (21). They are aneuploid with a modal chromosome number of 59. There are four copies of chromosome 11, which carries the PR gene (31).

Table 1 *Total PR levels in breast cancer cell lines measured by ligand binding*

PR in cytosols prepared from each cell line were labeled with [^{3}H]R5020 (20 nM) in the presence or absence of unlabeled R5020 (2 μM), and the charcoal resistant radioactivity was counted. Receptor levels are expressed in terms of total protein present in the cytosols.

Cell line	PR fmoles/mg protein
MDA-231	7.8
T47D-Y	4.7
T47D-YA	873.0
T47D-YB	1329.0
T47D-D[a]	27.0
T47D-V22	1963.0

[a] T47D-D at passage 492.

T47D-Y cells were maintained in continuous culture without selective medium and remained PR negative at passage 65. They are PR negative by the following tests: immunologically by Western blotting (Figs. 1 and 5) and flow cytometry; ligand binding assay (Table 1); lack of growth response to progestin agonists or antagonists[4]; failure of cell extracts to bind a PRE on gel mobility shift assays (Fig. 6); and failure of progestin agonists and antagonists to induce transcription of transiently transfected, PRE-regulated promoters (Fig. 2). A study showing transcriptional regulation by the agonist R5020, of the PRE-*tk*-CAT and MMTV-CAT reporters in T47D-Y cells with or without transient expression of B-receptors, is shown in Fig. 2. T47D-Y cells were cotransfected with each reporter and with the hPR expression vector pSG5 either lacking (−PR) or containing hPR1 (+PR_B), the cDNA that encodes B-receptors. Cells were then left untreated or treated with the agonist R5020. As shown, background levels of CAT transcription are low with PRE-*tk*-CAT, and there is no remarkable ligand-induced transcription unless hPR_B are present and hormone activated. This is demonstrated again using the MMTV-CAT reporter, from which there is no basal CAT transcription and from which high levels of CAT expression are seen only when hPR_B are transiently

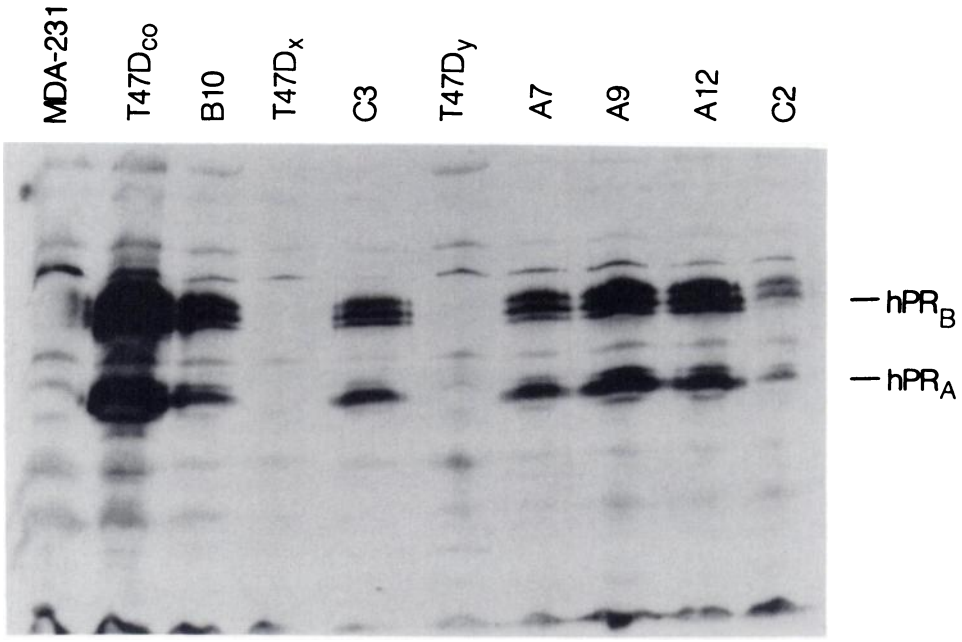

Fig. 1. Isolation of two PR-negative cell lines from the PR-positive T47D-D cell line. Single cells from T47D-D were cloned by limiting dilution, and PR-negative or low PR-positive sublines were selected by flow cytometry. After continued culture, eight cell lines were assayed for PR content by immunoblotting with MAb AB-52 at passage 6. Two clonal sublines called T47D-X and T47D-Y were PR negative as compared to PR-positive ($T47D_{co}$) and PR-negative (MDA-231) controls. Six other clonal sublines of T47D-D were PR positive. This immunoblot was purposely overexposed to reveal the presence, if any, of even minor receptor bands.

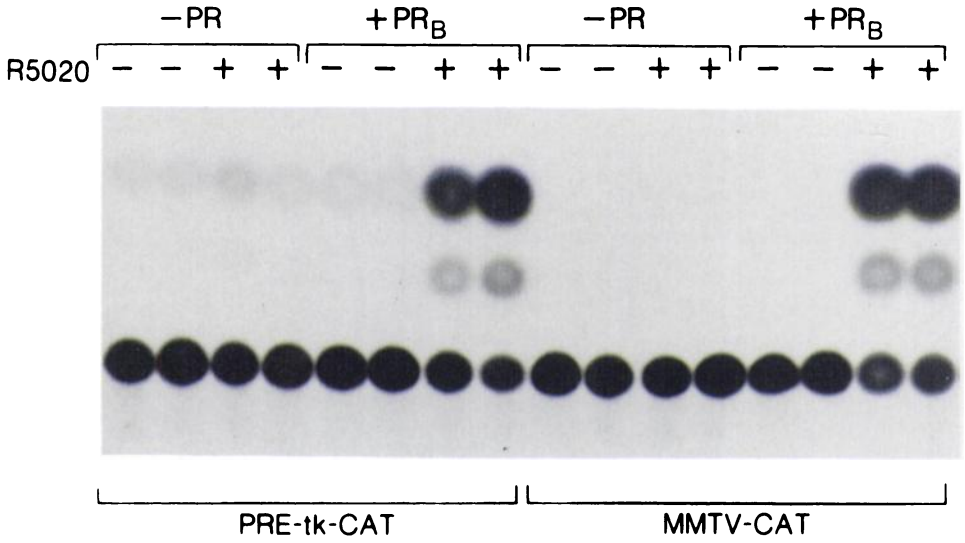

Fig. 2. T47D-Y cells lack transcriptionally active PR. Duplicate sets of T47D-Y cells were transiently transfected with pSG5-hPR1, the B-receptor expression vector (+PR$_B$), or with the empty pSG5 plasmid (−PR), and cotransfected with either PRE-*tk*-CAT or MMTV-CAT promoter-reporters. Cells were treated or not treated with 10 nM of the agonist R5020 as shown, and levels of CAT activity, normalized for β-galactosidase activity, were measured by TLC.

expressed and R5020 is added to the cells. Thus, T47D-Y cells appear to lack sufficient levels of endogenous PR to activate transcription from a PRE-regulated promoter but retain appropriate cofactors to support such transcription if PR are restored.

An experiment that underscores the need for stable new models is shown in Fig. 3. PR-negative T47D-Y cells were transiently transfected with either pSG5-hPR1 or pSG5-hPR2 to express B- or A-receptors and cotransfected with the MMTV-CAT reporter. Cells were untreated or treated with R5020 or the antiprogestin RU486 in the presence or absence of 8-Br-cAMP. Elevated levels of CAT expression are seen following agonist treatment in the presence of B-receptors (Fig. 3, *Lanes 3* and *4*) or A-receptors (Fig. 3, *Lane 14*). Note that in T47D-Y cells, A-receptors have 10–20% the transcription stimulatory activity of B-receptors on the MMTV promoter, as previously reported by Meyer *et al.* (17) using HeLa cells. In T47D-Y cells, the antiprogestin RU486 has little or no agonist-like activity on B-receptors (Fig. 3, *Lanes 9* and *10*) or A-receptors (Fig. 3, *Lanes 19* and *20*). 8-Br-cAMP alone is also without transcriptional activity on transiently expressed promoters (Fig. 3, *Lanes 5* and *6* and *Lanes 15* and *16*), but in the presence of elevated levels of cAMP, RU486 becomes a strong transcriptional activator on B-receptors (Fig. 3, *Lanes 11* and *12*) but not on A-receptors (Fig. 3, *Lanes 21* and *22*). Thus, the antagonist-to-agonist functional switch produced by antiprogestins in the presence of 8-Br-cAMP that we previously described (1) in native T47D cells which contain endogenous mixtures of both receptor isoforms, appears to be due solely to the activity of B-receptors. Under these conditions, RU486-occupied B-receptors (Fig. 3, *Lanes 11* and *12*) are better agonists than R5020-occupied A-receptors (Fig. 3, *Lane 14*).

Construction and Analysis of T47D-YB and T47D-YA Cells. At present, B-receptors or A-receptors can be analyzed independently only within the circumscribed limits of the transient expression systems discussed above. To develop more physiological models, we stably reintroduced either B-receptors or A-receptors into T47D-Y cells to create the T47D-YB and T47D-YA cell lines. In Fig. 4, flow cytometry was used to quantitate PR in the new cell lines in comparison to T47D-Y cells and PR-positive wild-type T47D-V22 cells. Compared to PR-negative T47D-Y cells, the two curves for PR fluorescence intensity units in YB and YA cells are shifted to the right into the PR-positive region. The extensive overlap between the two curves suggests that the PR levels in each cell line are similar. They are, however, lower than the PR levels present in wild-type T47D-V22 cells, which contain mixtures of B- plus A-receptors at high levels. Based on the overlap between the YB and YA curves with that

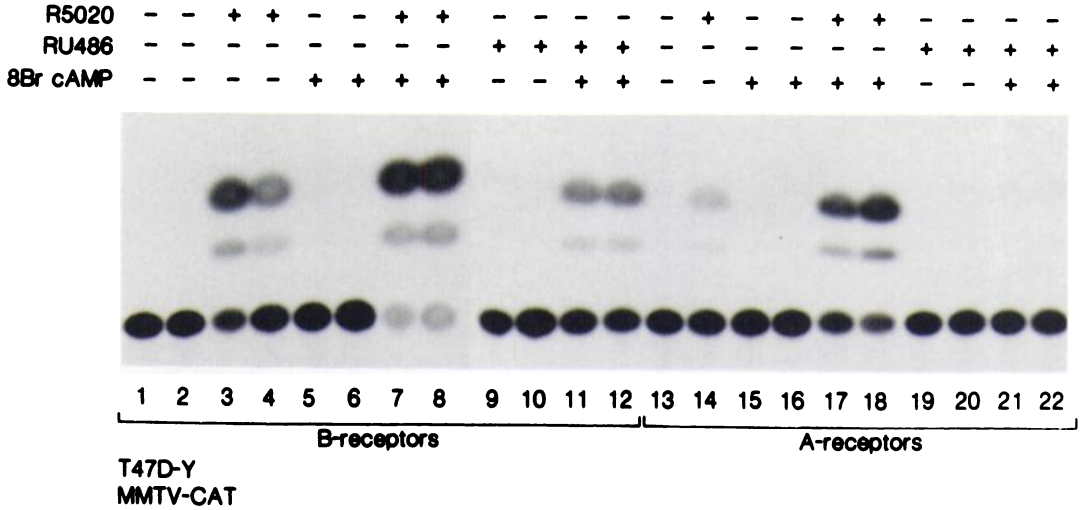

Fig. 3. Transcriptional activation of RU486-occupied PR in the presence of 8-Br-cAMP is restricted to B-receptors. PR-negative T47D-Y cells were transfected with the B-receptor or A-receptor expression vectors and cotransfected with the MMTV-CAT promoter-reporter. Cells were treated with 10 nM of the agonist R5020, 100 nM of the antagonist RU486, and/or 1 mM 8-Br-cAMP as indicated and then harvested. β-galactosidase-normalized cell extracts were assayed for CAT activity by TLC.

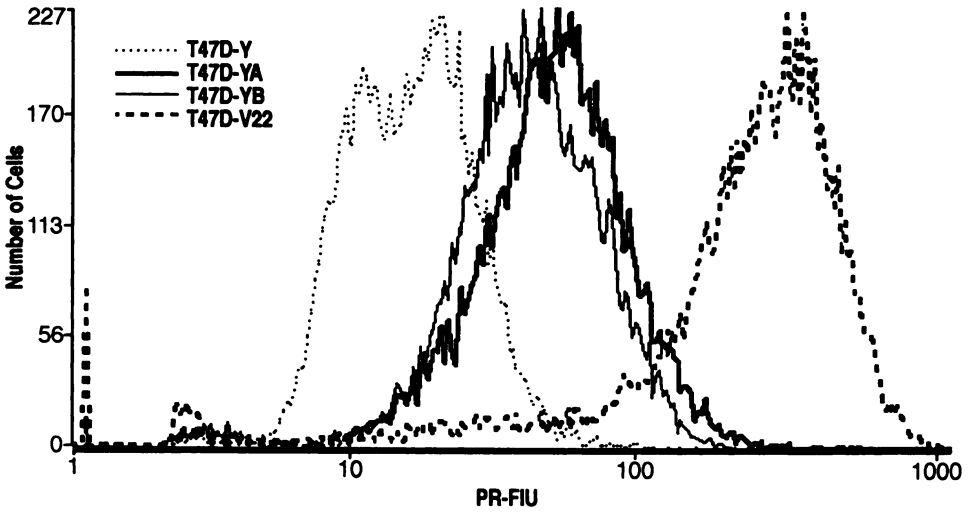

Fig. 4. Comparison of PR levels measured by flow cytometry in T47D-YA, T47D-YB, and wild-type T47D-V22 cells, with PR-negative T47D-Y cells. T47D cells containing endogenously or ectopically expressed receptors were harvested, fixed, and permeabilized. The cells were incubated with the anti-hPR mouse monoclonal antibody AB-52 and labeled with a fluorescein isothiocyanate-conjugated anti-mouse secondary antibody. Fluorescence intensity units (*FIU*) in 10,000 cells were quantitated by flow cytometry as described (27) and are displayed on a log scale on the *X*-axis. Cell number on a linear scale is shown on the *Y*-axis.

of the Y curve, we estimate that the two new PR-positive cell lines are heterogenous and may have a small PR-negative subpopulation. In this respect, they resemble many wild-type T47D sublines. This PR-negative subpopulation could be eliminated by recloning the cells.

An immunoblot (Fig. 5) demonstrates the equimolar mixture of B- and A-receptors that are present in wild-type T47D clone 11 cells (Fig. 5, *Lanes 1* and *2*), the absence of either receptor isoform in T47D-Y cells (Fig. 5, *Lanes 3* and *4*), and the unique presence of one or the other receptor isoform in YB and YA cells (Fig. 5, *Lanes 5–8*). The levels of each isoform in YB and YA cells are approximately the same as the levels of that isoform in clone 11 and other T47D (not shown) cells. The structure of the receptors in YB and YA cells is also analogous to that of the wild-type receptors. The triplet banding pattern of wild-type B-receptors (Fig. 5, *Lane 1*) is retained in the ectopically expressed B-receptors found in hormone untreated (−R) YB cells (Fig. 5, *Lane 5*), and the characteristic molecular weight upshifts of B- and A-receptors produced by hormone treatment (+R) are also retained in the new cell lines (Fig. 5, *Lanes 2, 6,* and *8*). Both structural features are due to receptor phosphorylation (32–34). Table 1 quantitates PR levels by ligand binding assay in several of the relevant T47D cell lines and in PR-negative MDA-231 cells whose origin is unrelated to that of T47D cells. Again, the YA and YB cells have lower levels of PR than wild-type T47D-V22 cells, which contain both receptors.

A gel mobility shift assay is shown in Fig. 6, demonstrating the ability of the receptors extracted from wild-type T47D cells and the Y derivatives to dimerize and bind a palindromic PRE. Like wild-type receptors in clone 11 cells (Fig. 6, *Lane 2*), the ectopically expressed receptors in YB and YA cells (Fig. 6, *Lanes 4* and *6*) cannot bind DNA *in vitro* if they are unactivated by hormone. R5020 activates the wild-type A- and B-receptors in clone 11 cells to a DNA-binding state (Fig. 6, *Lane 3*). The two endogenous PR isoforms produce the three expected dimeric species (17) in response to hormone (Fig. 6, *Lane 1*, which is equivalent to *Lane 3* but in which the film has been underdeveloped to demonstrate the three dimers). Fig. 6, *Lanes 2* to *9* were purposely overexposed to reveal the presence, if any, of minor dimeric PR species. In our hands, the gel mobility shift assay is the most sensitive method for detecting receptors that are below the resolution levels of immunoblotting. T47D-Y cells are PR-negative (Fig. 6, *Lanes 8* and *9*) by this method as well. Only B:B homodimers appear to be present in YB cells (Fig. 6, *Lane 5*), and only A:A homodimers are present in YA cells (Fig. 6, *Lane 7*). The smear under the B:B band (Fig.6, *Lane 5*) could represent ~3% of an A:B heterodimer. There are clearly no A:A homodimers. We conclude that in YB cells, no significant levels of A-receptors are produced.

Our intent in developing these new T47D cell lines is, among other things, to use them in studies of the long-term effects of progestin treatment on breast cancer cells. In order to be physiologically relevant, the synthetically expressed receptors should, as much as possible, reflect the kinetic behavior of naturally expressed receptors. One characteristic of long-term steroid hormone treatment is ligand-dependent receptor down-regulation (22, 35–41). The immunoblot in Fig. 7 shows receptor levels in hormone-untreated T47D-B11, YA, or YB cells and following 24 h of R5020 treatment. In hormone-treated cells, PR levels decreased by ~90% whether the receptors were natural or were synthetically expressed, suggesting that the ectopically expressed receptors and the natural receptors are similarly down-regulated and that the natural promoters (7) of the PR gene are not involved in this effect.

cAMP-induced Functional Synergism/Switching in YA and YB Cells. The functional differences in responses to antagonists between B- and A-receptors that were observed by transient expression methods (Fig. 3) and which prompted construction of the YA and YB cells can indeed be demonstrated in these new models. In the study shown

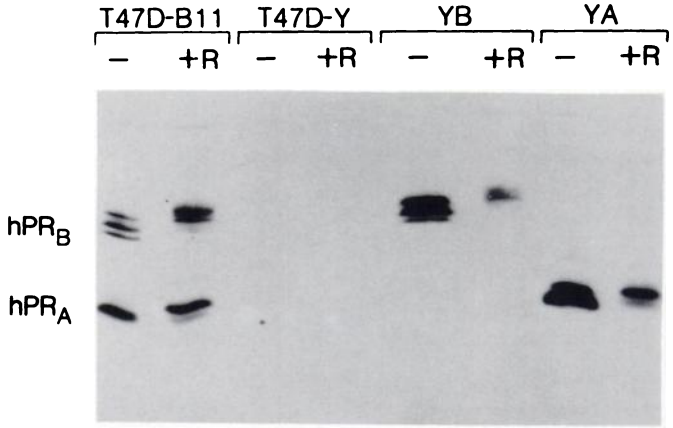

Fig. 5. PR content and structural analysis of wild-type T47D clone 11 cells and three new T47D cell lines analyzed by immunoblotting. The cell lines indicated were treated (+R) or not treated (–) with 100 nM of the agonist R5020 for 1 h and then harvested. Total cellular receptors were extracted by freeze-thawing with 0.4 M KCl, desalted, resolved by SDS-PAGE, and electroblotted to nitrocellulose. The nitrocellulose sheet was probed with the anti-PR antibody AB-52, and protein bands were detected by enhanced chemiluminescence and autoradiography.

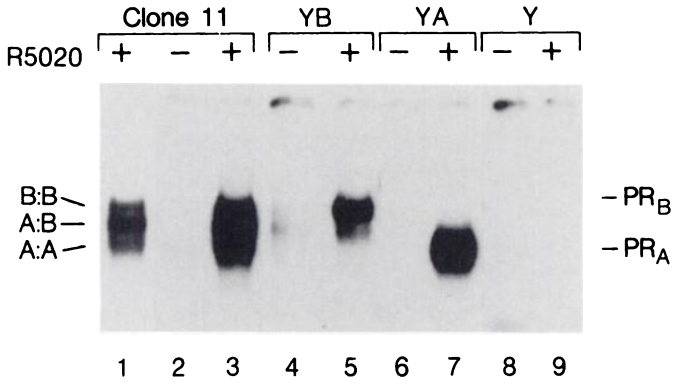

Fig. 6. PR content of wild-type T47D clone 11 and three new T47D cell lines analyzed by binding to a PRE *in vitro* using the gel mobility shift assay. The cell lines indicated were treated (+) or not treated (−) 4 h with 100 nM of R5020; receptors were extracted from the cells by freeze-thawing in 0.4 M KCl, and cell extracts were incubated with a ^{32}P-labeled perfect palindromic PRE. Receptor-DNA complexes were resolved by non-denaturing gel electrophoresis, following which the gel was dried and autoradiographed for 3.5 h (*Lanes 2–9*) or 1 h (*Lane 1*).

in Fig. 8, the cells were transfected with the MMTV-CAT reporter and then treated 24 h with R5020 or three antiprogestins in the presence or absence of 8-Br-cAMP. 8-Br-cAMP alone does not stimulate CAT synthesis in either cell line (Fig. 8, *Lane 2*; YA and YB). In YA cells, R5020 alone moderately stimulates CAT transcription from MMTV-CAT (Fig. 8, *Lanes 3* and *4*), but the agonist effect is synergistically (see also Fig. 9*A*) enhanced by the presence of 8-Br-cAMP (Fig. 8, *Lanes 5* and *6*). Thus, in this model like in the transient models, agonist-occupied A-receptors are relatively weak transactivators, the activity of which is strongly enhanced by cAMP. When only A-receptors are available as they are in YA cells, the three antiprogestins RU486, ZK98299, or ZK11299 have no intrinsic agonist-like activity (Fig. 8, *Lanes 7, 10,* and *13*), and 8-Br-cAMP does not activate them (Fig. 8, *Lanes 8* and *9*; *Lanes 11* and *12*; and *Lanes 14* and *15*).

However, the agonist and antagonists have quite different effects in the B-receptor-containing YB cells. R5020-regulated transcription from the MMTV-CAT reporter is very strong in these cells (Fig. 8, *Lanes 3* and *4*) so that the additional stimulation by 8-Br-cAMP is moderate (Fig. 8, *Lanes 5* and *6*; Fig. 9*B*). Thus, in YB cells, the cAMP-mediated synergism of agonist effects is more difficult to observe than in YA cells. By contrast, 8-Br-cAMP strongly enhances the transcriptional phenotype of the antagonists RU486 and ZK112993. Both of these antiprogestins are weak agonists on the MMTV-CAT promoter (Fig. 8, *Lanes 7* and *13*) but become strong agonists when 8-Br-cAMP is added (Fig. 8, *Lanes 8* and *9*; *Lanes 14* and *15*). The antagonist ZK98299 is entirely different since it has no intrinsic agonist activity alone (Fig. 8, *Lane 10*), and no transcriptional enhancement is produced by ZK98299-occupied B-receptors in the presence of 8-Br-cAMP (Fig. 8, *Lanes 11* and *12*). This resistance of ZK98299 in YB cells to the activating effects of 8-Br-cAMP is similar to the one we described in T47D-B11 cells that contain the natural mixture of both receptors (1).

Fig. 9 shows a quantitative summary of transcription data using either the MMTV-CAT reporter (Fig. 9, *A* and *B*) described in Fig. 8 or the PRE$_2$-TATA$_{tk}$-CAT reporter (Fig. 9, *C* and *D*) in either YA cells (Fig. 9, *A* and *C*) or YB cells (Fig. 9, *B* and *D*). Regardless of the promoter, agonist-like transactivation by the antagonists RU486 and ZK112993 in the presence of 8-Br-cAMP occurs only in the YB cells and appears to be B-receptor-specific. Antagonist-activated transcription in YB cells is as efficient as agonist-activated transcription in YA cells. By contrast, the synergism between the agonist R5020 and 8-Br-cAMP is most evident with A-receptors in the YA cells. Finally, ZK98299-occupied PR are transcriptionally inactive under all conditions tested.

DISCUSSION

Their constitutive high level production of PR (22) have made T47D cells the major model to study the actions of progesterone in breast cancer, unencumbered by the need for estradiol priming. In most progesterone target tissues, including PR-positive breast cancer cell lines, PR are estrogen-regulated proteins (42). However, in many T47D sublines, this estrogen regulatory step for PR has been bypassed (22), due perhaps to dominant estrogen receptor mutants likely to be present in these cells (43–45). T47D cells are the best source of human PR for protein purification (29); they were the cells from which the human PR cDNA was cloned (46), and they are commonly used recipient cells for transcription and growth regulation studies (Ref. 14 and references therein). Because of these special phenotypic properties of T47D cells and because factors other than receptors may be missing in persistently receptor-negative cells (47), we thought it prudent to retain the T47D cellular milieu in developing new models to study the two PR isoforms. For that we needed a PR-negative T47D subline. We developed a monoclonal line without selection pressure by outgrowth of PR-negative cells from a T47D subline (T47D-D) that contained mixed PR-positive/PR-negative cell populations identified by flow cytometry. The new PR-negative cells (T47D-Y) were then used as recipients for the independent stable transfection and expression of the two PR isoforms.

PR-negative T47D Cells. Despite the high constitutive levels of PR expression in wild-type T47D cells, there is extensive heterogeneity, characterized by the existence of subpopulations having widely differing amounts of the receptors (23). Even the "PR-negative" T47D clone 8 cell line described by Chalbos *et al.* (48) is actually a mixture of PR-negative and PR-positive cells (23). Because the PR levels in the T47D-D subline were spontaneously declining, we chose these cells from which to clone out a pure PR-negative cell line. Since flow cytometry at passage 485 suggested that more than 50% of T47D-D cells were PR negative, we were surprised that 91 clonal derivatives yielded only two stable PR-negative sublines. This suggests that, in most cells in the T47D-D population at the time of cloning, the PR-negative phenotype is transitory and that reversions occur commonly, due perhaps to genetic instability (44). The downward drift in PR levels has continued in this cell line, however, and more than a year later, they are functionally PR negative. The single cell from

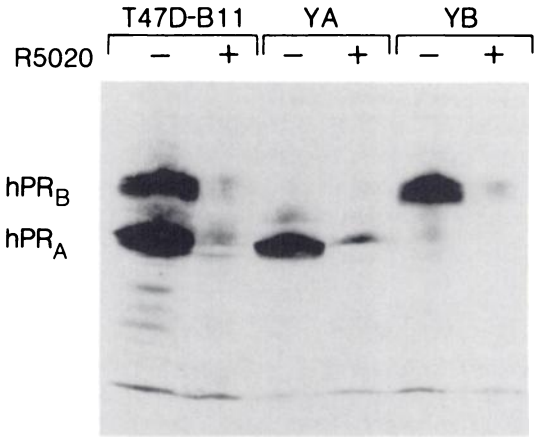

Fig. 7. Ectopically expressed A- or B-receptors are down-regulated like wild-type PR following ligand occupancy. T47D cells endogenously (T47D-B11) or ectopically (YA or YB cells) expressing PR were treated 24 h with (+) or without (−) 100 nM of R5020. Whole cell extracts (0.4 M KCl) were desalted, resolved by SDS-PAGE, and immunoblotted with AB-52 as described.

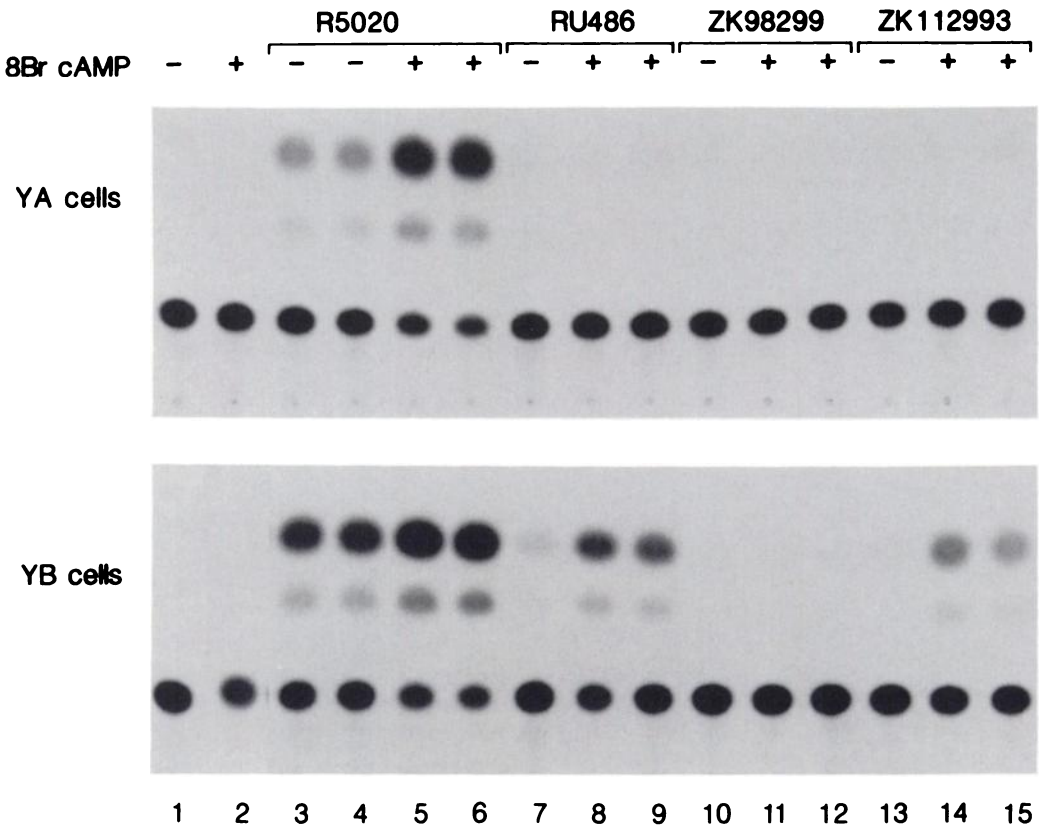

Fig. 8. When cAMP levels are elevated, RU486 and ZK112993 but not ZK98299 are strong transactivators in T47D-YB cells and not in T47D-YA cells. T47D-YA (*top*) and T47D-YB (*bottom*) cells were transiently transfected with the MMTV-CAT reporter and treated with the steroid hormones indicated in the presence (+) or absence (−) of 1 mM 8-Br-cAMP; CAT activity levels were measured by TLC. Hormone concentrations were 50 nM for R5020, RU486, and ZK112993 and 100 nM for ZK98299. YA cells contain only A-receptors; YB cells only B-receptors.

which the T47D-Y line was cloned was apparently not subject to this fluctuation in receptor levels, since it has been in continuous culture for at least 65 passage generations, and the PR-negative phenotype has persisted.

PR Isoform-specific Cell Lines. By stably reintroducing progesterone B- or A-receptor expression vectors into the constitutively PR-negative T47D-Y cell line, we have constructed models that allow the study of PR isoform-specific regulatory mechanisms in a breast cancer cell-specific setting. Immunoblots of YA and YB cells show expression of a protein having the appropriate size and immunoreactivity of authentic A- or B-receptors. The absence of one of the two PR isoforms in YA and YB cells strongly suggests that the expressed receptors arise from the transfected cDNAs and not from reactivation of the endogenous gene, since in natural human cells, one receptor isoform has not been seen alone.

The immunoblots of YB cells (Fig. 5) are of special interest because of the lack of A-receptors. In the past, origin of the A-receptors has been contentious. In the beginning, a strong argument was made (49) that A-receptors were proteolytic artifacts generated from B-receptors, an argument that can be laid to rest by the data in Fig. 5 and similar findings by others (7, 8, 11, 12). More recently, two other sources for A-receptors have been proposed, that they arise either by translation initiation from an internal AUG found in full-length PR messages (12) or that they arise by translation of different, isoform-specific messages (8, 11). Note that these two mechanisms are not mutually exclusive and both could be operative. However, our data are consistent with the second mechanism, since in YB cells, the internal AUG is present in the message transcribed from the B-specific hPR1 cDNA but is apparently not used to initiate translation as judged by the absence of A-receptors. Curiously, failure to produce A-receptors from the B-specific cDNA is especially evident in these stable receptor expression systems, since some synthesis of A-receptors is usually seen when hPR_B are transiently expressed from the same cDNA. However, the high levels of A-receptors synthesized by internal AUG use in cell-free systems are probably artifactual, and for other proteins, have been shown to result from deficiencies of a component(s) in reticulocyte lysates needed for faithful initiation from upstream start-sites (50).

Also of interest is the fact that these stable, ectopically expressed receptors are subject to homologous ligand-dependent down-regulation. Down-regulation (also called receptor processing), defined as a loss of ligand binding capacity or of immunoreactive receptor protein in response to hormone binding, was first described for ER following estradiol or tamoxifen treatment (35, 36, 42). Subsequently, other steroid receptors (38, 39, 51), including PR (13, 37), were shown to undergo down-regulation. Neither the function of, nor the mechanism for, this receptor processing step are known. For GR, it is argued that down-regulation "desensitizes" cells to glucocorticoids (40). For ER, ligand-induced down-regulation has been considered to be an active process in which changes in the rate of receptor synthetic and degradative steps lead to a new, albeit lower, steady-state level of ER (42). Recently, Burnstein *et al.* (40) and Alksnis *et al.* (41) showed that stably transfected GR undergo ligand-dependent down-regulation at the protein and message level. Our data confirm their observations for PR proteins and suggest, like theirs do, that the minimal genomic sequences present in the receptor cDNA or sequences in the message and not endogenous PR promoter sequences are responsible for receptor autoregulation. In fact, since hPR2 and hPR1 contain little or no 5′-untranslated sequences, respectively, any regulatory sites must be in the coding or 3′-untranslated regions. The ability of the syn-

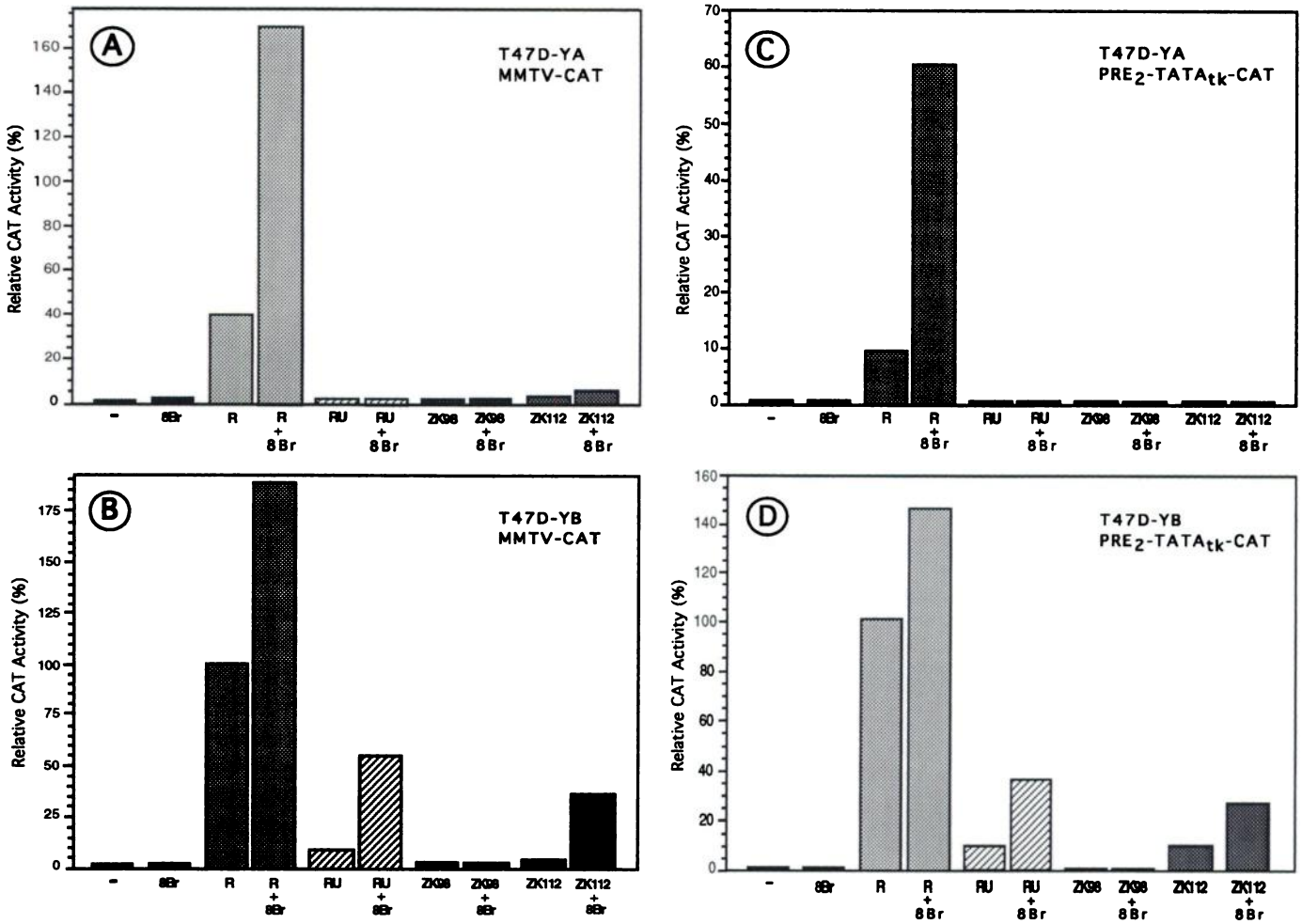

Fig. 9. cAMP-induced transcriptional synergism with antiprogestins is B-receptor specific but not promoter specific. T47D-YA cells (*A* and *C*) or YB cells (*B* and *D*) transiently transfected with the MMTV-CAT (*A* and *B*) or PRE_2-$TATA_{tk}$-CAT (*C* and *D*) reporters were treated with the progestins at the concentrations shown in Fig. 8 plus/minus 8-Br-cAMP. Cells were harvested and assayed for CAT activity normalized to β-galactosidase activity. *8Br*; 8-Br-cAMP; *R*, R5020; *RU*, RU486; *ZK98*, ZK98299; *ZK112*, ZK112993. CAT activity in R5020-treated YB cells for each promoter was set at 100%.

thetically produced YB and YA cells to down-regulate their PR in a manner analogous to the natural PR in T47D cells suggests that we can use these new cells to carry out physiologically relevant long-term experiments involving progestin treatment.

These models permit us to independently study the function of each PR homodimer, either B:B or A:A, in a stable breast cancer cell milieu. The studies described here confirm earlier conclusions based on transient assays (16–18) that B- and A-receptors are functionally different, especially when they are occupied by progesterone antagonists, and rule out "squelching" artifacts to explain the earlier results. However, the A:B heterodimer cannot be ignored. In wild-type, hormone-treated cells in which A- and B-receptors are present in equimolar amounts, 50% of the activated receptors exist in the heterodimeric state. Thus, it is as important to study the heterodimers uncontaminated by homodimers as it is to study each homodimer alone. In studies to be reported elsewhere,[5] we have succeeded in transiently expressing pure A:B heterodimers in cells by constructing chimeric PR that have the dimerization domain of c-Fos or c-Jun fused to the COOH-terminus of hPR_A or hPR_B. Based on these studies and studies like those in Tung *et al.* (18) and Vegeto *et al.* (52), we conclude that in the antagonist-occupied A:B heterodimer, the transcriptional phenotype of A-receptors is dominant, and under appropriate conditions, they repress transactivation by antagonist-occupied B-receptors in the presence of cAMP.

cAMP and Transcriptional Synergism/Switching. Using wild-type T47D cells stably expressing MMTV-CAT (B-11 cells), we recently reported that cAMP can amplify the transcriptional signals of agonist-occupied AR, GR, and PR (1). All three receptors bind to the same DNA response elements (GRE/PRE) on the MMTV promoter (20). In T47D-B11 cells, the levels of AR and GR are too low to activate the MMTV promoter in response to homologous hormone treatments, but sensitivity to androgens and glucocorticoids is restored if cellular cAMP levels are simultaneously elevated. Additionally, we showed that in the presence of cAMP, the progesterone antagonists RU486 and ZK112993 switch the function of PR to render them potent transactivators (1). We now show that the two PR isoforms behave dissimilarly in their cooperativity with cAMP: (*a*) with regard to R5020, the synergism between cAMP and agonist-occupied receptors is most pronounced in YA cells with A-receptors (Figs. 3, 8, and 9), which by themselves are relatively weak transactivators. We speculate that in YA cells, cAMP sensitizes the MMTV promoter to the weak signal transmitted by R5020-occupied A-receptors, just as it does in B11 cells, to the weak signals transmitted by hormone-occupied GR and AR. Since in YB cells agonist-occupied B-receptors alone are already strong transactivators, cAMP has only modest

[5] M. K. Mohamed, L. Tung, G. S. Takimoto, and K. B. Horwitz. The leucine zippers of c-Fos and c-Jun for progesterone receptor dimerization: mechanisms of A-dominance in the A/B heterodimer, manuscript submitted.

further effects on this isoform; (*b*) with regard to progesterone antagonists, the isoform specificity of the cAMP effect is even more interesting. Here we find absolutely no effect of cAMP on A-receptors, due perhaps to the fact that the antagonists (specifically RU486 and ZK112993) exhibit no agonist-like activity on these receptors in YA cells. Perhaps there is no minimal signal for cAMP to amplify? By contrast, the two antagonists appear to have some weak agonist-like activity when bound to B-receptors in YB cells; hence, cAMP strongly amplifies this signal, rendering the antagonist-occupied receptors potent transactivators. Therefore, it is significant that B-receptors occupied by the antiprogestin ZK98299 are not subject to this functional modulation by cAMP. Unlike RU486 and ZK112993, ZK98299-occupied PR do not bind to DNA *in vitro* (30, 53) or *in vivo* (30, 54).[6] We speculate that ZK98299-occupied PR are physically removed from cAMP control by their failure to bind DNA. This further implies that cooperativity between DNA-bound PR and a cAMP-regulated factor accounts for the transcriptional synergism. We find no evidence that cAMP directly phosphorylates PR *in vivo* (55).

The functional cooperativity between some progesterone antagonists and cAMP has important clinical implications. Acquisition of sensitivity to factors that raise the levels of cAMP may be one mechanism by which breast cancers develop "resistance" to steroid hormone antagonists during endocrine treatment and may explain the well-known tissue-specific differences in the actions of such antagonists. In both cases, antagonists have unexpected agonist-like effects (55). It follows that the antiprogestin ZK98299 may be especially useful clinically because it protects PR from these unintended effects. We also note that, with respect to the transactivating capabilities of RU486 and ZK112993, since antagonist-occupied A-receptors are dominant-negative repressors of the cAMP-induced B-receptor activation, the ratio of B- to A-receptors in a tumor or tissue could dictate the direction of transcription during treatment with these antiprogestins. Virtually nothing is known about the ratio of the two isoforms in normal or malignant human target tissues. Finally, the cooperativity between cAMP and progesterone antagonists like RU486 and ZK112993 extends beyond the transcriptional models described here. We find that the agonist R5020 stimulates growth by inducing a single round of DNA synthesis, seen as a doubling of the number of YA and YB cells in the S-phase of the cell cycle. RU486 blocks this effect in both cell lines. However, if cAMP levels are raised, the S-phase suppression by RU486 is prevented but only in YB cells.[4]

ACKNOWLEDGMENTS

We are grateful to P. Chambon for his gift of the PR expression vectors and MMTV-CAT and D. Henderson for the Schering AG antiprogestins. We thank H. Morse and the Cytogenetics Core Laboratory for karyotype analyses.

REFERENCES

1. Sartorius, C. A., Tung, L., Takimoto, G. S., and Horwitz, K. B. Antagonist-occupied human progesterone receptors bound to DNA are functionally switched to transcriptional agonists by cAMP. J. Biol. Chem., *268:* 9262–9266, 1993.
2. Beck, C. A., Weigel, N. L., Moyer, M. L., Nordeen, S. K., and Edwards, D. P. The progesterone antagonist RU486 acquires agonist activity upon stimulation of cAMP signaling pathways. Proc. Natl. Acad. Sci. USA, *90:* 4441–4445, 1993.
3. Nordeen, S. K., Bona, B. J., and Moyer, M. L. Latent agonist activity of the steroid antagonist, RU486, is unmasked in cells treated with activators of protein kinase A. Mol. Endocrinol., *7:* 731–742, 1993.
4. Schrader, W. T., and O'Malley, B. W. Progesterone-binding components of chick oviducts. Characterization of purified subunits. J. Biol. Chem., *247:* 51–59, 1972.
5. Horwitz, K. B., and Alexander, P. S. *In situ* photolinked nuclear progesterone receptors of human breast cancer cells: subunit molecular weights after transformation and translocation. Endocrinology, *113:* 2195–2201, 1983.
6. Krett, N. L., Wei, L. L., Francis, M. D., Nordeen, S. K., Gordon, D. F., Wood, W. M., and Horwitz, K. B. Human progesterone A-receptors can be synthesized intracellularly and are biologically functional. Biochem. Biophys. Res. Commun., *157:* 278–285, 1988.
7. Kastner, P., Krust, A., Turcotte, B., Stropp, U., Tora, L., Gronemeyer, H., and Chambon, P. Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. EMBO J., *9:* 1603–1614, 1990.
8. Wei, L. L., Gonzalez-Aller, C., Wood, W. M., Miller, L. A., and Horwitz, K. B. 5′-Heterogeneity in human progesterone receptor transcripts predicts a new amino-terminal truncated "C"-receptor and unique A-receptor messages. Mol. Endocrinol., *4:* 1833–1840, 1990.
9. Feil, P. D., Clarke, C. L., and Satyaswaroop, P. G. Progesterone receptor structure and protease activity in primary human endometrial carcinoma. Cancer Res., *48:* 1143–1149, 1988.
10. Horwitz, K. B., Francis, M. D., and Wei, L. L. Hormone-dependent covalent modification and processing of human progesterone receptors in the nucleus. DNA, *4:* 451–460, 1985.
11. Kastner, P., Bocquel, M-T., Turcotte, B., Garnier, J-M., Horwitz, K. B., Chambon, P., and Gronemeyer, H. Transient expression of human and chicken progesterone receptors does not support alternative translational initiation from a single mRNA as the mechanism generating two receptor isoforms. J. Biol. Chem., *265:* 12163–12167, 1990.
12. Conneely, O. M., Kettelberger, D. M., Tsai, J. J., Schrader, W. T., and O'Malley, B. W. The chicken progesterone receptor A and B isoforms are products of an alternate translation initiation event. J. Biol. Chem., *264:* 14062–14064, 1989.
13. Wei, L. L., Krett, N. L., Francis, M. D., Gordon, D. F., Wood, W. M., O'Malley, B. W., and Horwitz, K. B. Multiple human progesterone receptor messenger ribonucleic acids and their autoregulation by progestin agonists and antagonists in breast cancer cells. Mol. Endocrinol., *2:* 62–72, 1988.
14. Horwitz, K. B. The molecular biology of RU486. Is there a role for antiprogestins in the treatment of breast cancer? Endocr. Rev., *13:* 146–163, 1992.
15. Umesono, K., and Evans, R. M. Determinants of target gene specificity for steroid/thyroid hormone receptors. Cell, *57:* 1139–1146, 1989.
16. Tora, L., Gronemeyer, H., Turcotte, B., Gaub, M-P., and Chambon, P. The N-terminal region of the chicken progesterone receptor specifies target gene activation. Nature (Lond.), *333:* 185–188, 1988.
17. Meyer, M. E., Pornon, A., Ji, J., Bocquel, M-T., Chambon, P., and Gronemeyer, H. Agonistic and antagonistic activities of RU486 on the functions of the human progesterone receptor. EMBO J., *9:* 3923–3932, 1990.
18. Tung., L., Mohamed, K. M., Hoeffler, J. P., Takimoto, G. S., and Horwitz, K. B. Antagonist-occupied human progesterone B-receptors activate transcription without binding to progesterone response elements and are dominantly inhibited by A-receptors. Mol. Endocrinol., *7:* 1256–1265, 1993.
19. Jantzen, H-M., Strähle, U., Gloss, B., Stewart, F., Schmid, W., Boshart, M., Miksicek, R., and Shütz, G. Cooperativity of glucocorticoid response elements located far upstream of the tyrosine aminotransferase gene. Cell, *49:* 29–38, 1987.
20. Cato, A. C. B., Miksicek, R., Shütz, G., Arnemann, J., and Beato, M. The hormone regulatory element of mouse mammary tumour virus mediates progesterone induction. EMBO J., *5:* 2237–2240, 1986.
21. Keydar, I., Chen, L., Karbey, S., Weiss, F. R., Delarea, J., Radu, M., Chaitcik, S., and Brenner, H. J. Establishment and characterization of a cell line of human breast carcinoma origin. Eur. J. Cancer, *15:* 659–670, 1979.
22. Horwitz, K. B., Mockus, M. B., and Lessey, B. A. Variant T47D human breast cancer cells with high progesterone receptor levels despite estrogen and antiestrogen resistance. Cell, *28:* 633–642, 1982.
23. Graham, M. L., II, Smith, J. A., Jewett, P. B., and Horwitz, K. B. Heterogeneity of progesterone receptor content and remodeling by tamoxifen characterize subpopulations of cultured human breast cancer cells: analysis by quantitative dual parameter flow cytometry. Cancer Res., *52:* 593–602, 1992.
24. El-Ashry, D., Oñate, S. A., Nordeen, S. K., and Edwards, D. P. Structural determinants of a glucocorticoid receptor recognition element. Mol. Endocrinol., *4:* 1866–1873, 1989.
25. Cailleau, R., Young, R., Olive, M., and Reeves, W. J., Jr. Breast tumor cell lines from pleural effusions. J. Natl. Cancer Inst., *53:* 661–674, 1974.
26. Graham, M. L., II, Dalquist, K. E., and Horwitz, K. B. Simultaneous measurement of progesterone receptors and DNA indices by flow cytometry: analysis of breast cancer cell mixtures and genetic instability of the T47D line. Cancer Res., *49:* 3943–3949, 1989.
27. Graham, M. L., II, Bunn, P. A., Jr., Jewett, P. B., Gonzalez-Aller, C., and Horwitz, K. B. Simultaneous measurement of progesterone receptors and DNA indices by flow cytometry: characterization of an assay in breast cancer cell lines. Cancer Res., *49:* 3934–3942, 1989.
28. Banerji, J., Rusconi, S., and Schaffner, W. Expression of a β-globin gene is enhanced by remote SV40 DNA sequences. Cell, *27:* 299–308, 1981.
29. Estes, P. A., Wei, L. L., Toft, D. O., Sullivan, W. P., Horwitz, K. B., and Edwards, D. P. Immunologic analysis of human breast cancer progesterone receptors. I. Immunoaffinity purification of transformed receptors and production of monoclonal antibodies. Biochemistry, *26:* 6250–6262, 1987.
30. Takimoto, G. S., Tasset, D. M., Eppert, A. C., and Horwitz, K. B. Hormone-induced progesterone receptor phosphorylation consists of sequential DNA-independent and DNA-dependent stages: analysis with zinc finger mutants and the progesterone antagonist ZK98299. Proc. Natl. Acad. Sci. USA, *89:* 3050–3054, 1992.
31. Law, M. L., Kao, F. T., Wei, Q., Hartz, J. A., Greene, G. L., Zarucki-Schulz, T., Conneely, O. M., Jones, C., Puck, T. T., O'Malley, B. W., and Horwitz, K. B. The progesterone receptor gene maps to human chromosome band 11q13, the site of the

[6] K. B. Horowitz, unpublished observations.

mammary oncogene *int-2*. Proc. Natl. Acad. Sci. USA, *84:* 2877–2881, 1987.
32. Sheridan, P. L., Krett, N. L., Gordon, J. A., and Horwitz, K. B. Down-regulation of human progesterone receptors is independent of their nuclear phosphorylation. Mol. Endocrinol., *2:* 1329–1342, 1988.
33. Sheridan, P. L., Evans, R. M., and Horwitz, K. B. Phosphotryptic peptide analysis of human progesterone receptors: new phosphorylated sites formed in nuclei after hormone treatment. J. Biol. Chem., *264:* 6520–6528, 1989.
34. Sheridan, P. L., Francis, M. D., and Horwitz, K. B. Synthesis of human progesterone receptors in T47D cells: nascent A- and B-receptors are active without a phosphorylation-dependent post-translational maturation step. J. Biol. Chem., *264:* 7054–7058, 1989.
35. Horwitz, K. B., and McGuire, W. L. Nuclear mechanisms of estrogen action: effects of estradiol and antiestrogen on cytoplasmic and nuclear estrogen receptors and nuclear receptor processing. J. Biol. Chem., *253:* 8185–8191, 1978.
36. Horwitz, K. B., and McGuire, W. L. Actinomycin D prevents nuclear processing of estrogen receptors. J. Biol. Chem., *253:* 6315–6318, 1978.
37. Mockus, M. B., and Horwitz, K. B. Progesterone receptors in human breast cancer: stoichiometric translocation and nuclear receptor processing. J. Biol. Chem., *258:* 4778–4783, 1983.
38. Cidlowski, J. A., and Cidlowski, N. B. Regulation of glucocorticoid receptors by glucocorticoids in cultured HeLa S3 cells. Endocrinology, *109:* 1975–1982, 1981.
39. Svec, F., and Rudis, M. Glucocorticoids regulate the glucocorticoid receptor in the AtT-20 cell. J. Biol. Chem., *256:* 5984–5987, 1981.
40. Burnstein, K. L., Jewell, C. M., and Cidlowski, J. A. Human glucocorticoid receptor cDNA contains sequences sufficient for receptor down-regulation. J. Biol. Chem., *265:* 7284–7291, 1990.
41. Alksnis, M., Barkhem, T., Strömstedt, P-E., Ahola, H., Kutoh, E., Gustafsson, J-å., Poellinger, L., and Nilsson, S. High level expression of functional full length and truncated glucocorticoid receptor in Chinese hamster ovary cells: demonstration of ligand-induced down-regulation of expressed receptor mRNA and protein. J. Biol. Chem., *266:* 10078–10085, 1991.
42. Horwitz, K. B., and McGuire, W. L. Estrogen control of progesterone receptor in human breast cancer: correlation with nuclear processing of estrogen receptor. J. Biol. Chem., *253:* 2223–2228, 1978.
43. Horwitz, K. B. Is a functional estrogen receptor always required for progesterone receptor induction in breast cancer? J. Steroid Biochem., *15:* 209–217, 1981.
44. Graham, M. L., II, Krett, N. L., Miller, L. A., Leslie, K. K., Gordon, D. F., Wood, W. M., Wei, L. L., and Horwitz, K. B. Genetic instability and estrogen receptor mutations: possible elements in the progression of breast cancers to hormone resistance. Cancer Res., *50:* 6208–6217, 1990.
45. Wang, Y., and Miksicek, R. J. Identification of a dominant negative form of the human estrogen receptors. Mol. Endocrinol., *5:* 1707–1715, 1991.
46. Misrahi, M., Atger, M., d'Auriol, L., Loosfelt, H., Meriel, C., Fridlansky, F., Guiochon-Mantel, A., Galibert, F., and Milgrom, E. Complete amino acid sequence of the human progesterone receptor deduced from cloned cDNA. Biochem. Biophys. Res. Commun., *143:* 740–748, 1987.
47. Zajchowski, D. A., Sager, R., and Webster, L. Estrogen inhibits the growth of estrogen-receptor negative, but not estrogen receptor positive, human mammary epithelial cells expressing a recombinant estrogen receptor. Cancer Res., *53:* 5004–5011, 1993.
48. Chalbos, D., Vignon, F., Keydar, I., and Rochefort, H. Estrogens stimulate cell proliferation and induce secretory proteins in a human breast cancer cell line (T47D). J. Clin. Endocrinol. Metab., *55:* 276–283, 1982.
49. Logeat, F., Pamphile, R., Loosfelt, H., and Milgrom, E. One-step immunoaffinity purification of active progesterone receptor: further evidence in favor of the existence of a single steroid binding subunit. Biochemistry, *24:* 1029–1035, 1985.
50. Dorner, A. J., Semler, B. L., Jackson, R. J., Hanecak, R., Duprey, E., and Wimmer, E. *In vitro* translation of poliovirus RNA: utilization of internal initiation sites in reticulocyte lysate. J. Virol., *50:* 507–514, 1984.
51. Quarmby, V. E., Yarbrough, W. G., Lubahn, D. B., French, F. S., and Wilson, E. M. Autologous down-regulation of androgen receptor messenger ribonucleic acid. Mol. Endocrinol., *4:* 22–28, 1990.
52. Vegeto, E., Shahbaz, M. M., Wen, D. X., Goldman, M. E., O'Malley, B. W., and McDonnell, D. P. Human progesterone receptor A form is a cell- and promoter-specific repressor of human progesterone receptor B function. Mol. Endocrinol., *7:* 1244–1255, 1993.
53. Klein-Hitpass, L., Cato, A. C. B., Henderson, D., and Ryffel, G. U. Two types of antiprogestins identified by their differential action in transcriptionally active extracts from T47D cells. Nucleic Acids Res., *19:* 1227–1234, 1991.
54. Bocquel, M-T., Ji, J., Ylikomi, T., Benhanou, B., Vergezac, A., Chambon, P., and Gronemeyer, H. Type II antagonists impair the DNA binding of steroid hormone receptors without affecting dimerization. J. Steroid Biochem. Mol. Biol., *45:* 205–215, 1993.
55. Horwitz, K. B. Mechanisms of hormone resistance in breast cancer. Breast Cancer Res. Treat., *26:* 119–130, 1993.

New T47D Breast Cancer Cell Lines for the Independent Study of Progesterone B- and A-Receptors: Only Antiprogestin-occupied B-Receptors Are Switched to Transcriptional Agonists by cAMP

Carol A. Sartorius, Steve D. Groshong, Louise A. Miller, et al.

Cancer Res 1994;54:3868-3877.