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**Progesterone Receptor Isoforms, PRA and PRB, Differentially Regulate Expression of
the Breast Cancer Resistance Protein (BCRP) in Human Placental Choriocarcinoma
BeWo Cells**

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BCRP, breast cancer resistance protein; ABCG2, ATP-binding cassette transporter G2; MX, mitoxantrone; FTC, fumitremorgin C; PRA, progesterone receptor A; PRB, progesterone receptor B; PRE, progesterone response element; AGT, aminoglutethimide; RU-486, mifepristone; PBS, phosphate-buffered saline; FBS, fetal bovine serum; EMSA, electrophoretic mobility shift assay.

Abstract

BCRP plays a significant role in drug disposition and in conferring multidrug resistance in cancer cells. Previous studies have shown that steroid hormones such as 17β -estradiol and progesterone can affect BCRP expression in cancer cells. In this study, we investigated the molecular mechanism by which BCRP expression in human placental choriocarcinoma BeWo cells is regulated by progesterone. Transfection of the progesterone receptor (PR) isoforms, PRA and PRB, resulted in a similarly increased expression of PRA and PRB, respectively. However, progesterone significantly increased BCRP expression and activity only in PRB-transfected cells. This stimulatory effect of progesterone was abrogated by the PR antagonist RU-486. Consistently, transcriptional activity of the *BCRP* promoter was induced 2 – 6-fold by 10^{-8} – 10^{-5} M progesterone in PRB-transfected cells. Progesterone had little effect on BCRP expression and activity, and transcriptional activity of the *BCRP* promoter in PRA-transfected cells; however, co-transfection of PRA and PRB significantly decreased the progesterone-response compared with that in cells transfected with only PRB. Mutations in a novel progesterone response element (PRE) identified between -243 to -115 bp of the *BCRP* promoter region significantly attenuated the progesterone-response in PRB-transfected cells, and deletion of the PRE nearly completely abrogated the progesterone effect. Specific binding of both PRA and PRB to the *BCRP* promoter through the identified PRE was confirmed using the electrophoretic mobility shift assay. Collectively, progesterone induces BCRP expression in BeWo cells via PRB, but not PRA. PRA represses the PRB activity. Thus, PRA and PRB differentially regulate BCRP expression in BeWo cells.

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The breast cancer resistance protein (BCRP, gene symbol *ABCG2*) is the second member of the subfamily G of the large human ATP-binding cassette transporter superfamily (Allikmets et al., 1998; Doyle et al., 1998; Miyake et al., 1999). Overexpression of BCRP in cancer cells has been shown to confer multidrug resistance by pumping anticancer drugs out of the cell (Doyle et al., 1998; Litman et al., 2000). BCRP has a broad spectrum of substrates, ranging from chemotherapeutic agents to organic anions (Krishnamurthy and Schuetz, 2006; Mao and Unadkat, 2005). The role of BCRP in clinical drug resistance has also been implicated in various clinical studies (Robey et al., 2007).

BCRP is also present in normal tissues. It is highly expressed in the epithelium of the small intestine, in the liver canalicular membrane, and in the apical membrane of the placental syncytiotrophoblasts (Maliepaard et al., 2001). Consistent with this tissue distribution, BCRP has been shown to play a significant role in absorption, distribution, and elimination of BCRP substrate drugs (Jonker et al., 2000; Kruijtz et al., 2002; Zhang et al., 2006).

BCRP-mediated drug resistance and disposition may, therefore, be influenced by any factors that can affect BCRP expression. We, and others, have previously shown that steroid hormones such as 17 β -estradiol and progesterone can regulate BCRP expression in various cancer cell lines, including the human placental choriocarcinoma BeWo cells (Ee et al., 2004; Imai et al., 2005; Wang et al., 2006). To date, the molecular mechanism by which BCRP expression is regulated by progesterone is still not known.

The physiological effects of progesterone are mediated by interaction of the hormone with the progesterone receptor (PR) isoforms, PRA and PRB. PRA and PRB are expressed from a single gene as a result of transcription from two alternative promoters (Kastner et al., 1990) and translation initiation at two alternative AUG start codons (Conneely et al., 1989).

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PRA and PRB differ only in that PRB contains an additional 164 amino acids at the N-terminus that are missing in PRA. In transfected cell systems, the two PR isoforms have distinct transcriptional properties that are specific to both the cell type and target gene promoter used (Tora et al., 1988). In general, PRB acts as a stronger transcriptional activator, whereas the transactivational activity of PRA is cell- and gene-specific (Giangrande and McDonnell, 1999). PRA also functions as a transcriptional inhibitor of PRB as well as of other steroid receptors when PRA itself is transcriptionally inactive (Giangrande and McDonnell, 1999; Vegeto et al., 1993).

In the present study, we investigated the molecular mechanism by which BCRP expression in BeWo cells is regulated by progesterone. We showed that BCRP expression in BeWo cells and transcriptional activity of the *BCRP* promoter, were induced by progesterone through PRB, but not PRA. PRA represses the PRB activity on transcriptional activation of the *BCRP* gene. We also identified a novel progesterone response element (PRE) in the *BCRP* promoter region.

Materials and Methods

Materials. Progesterone (P-8783), aminoglutethimide (AGT), and mifepristone (RU-486) were purchased from Sigma (St. Louis, MO). AGT is a first generation aromatase inhibitor which blocks the synthesis of steroid hormones. Fumitremorgin C (FTC) was obtained from the National Cancer Institute, Bethesda, MD. The BeWo cell line was purchased from American Type Culture Collection (Manassas, VA). RPMI 1640 phenol-red free and GIBCO Opti-MEM were from Gibco (Grand Island, NY). Phosphate-buffered saline (PBS) was from Invitrogen (Carlsbad, CA). HPLC-grade DMSO was from Fisher Scientific (Pittsburgh, PA). Charcoal/dextran-stripped fetal bovine serum (FBS) was purchased from HyClone (Logan, UT).

Progesterone immunoassay. BeWo cells were maintained in RPMI medium supplemented with 5% charcoal/dextran-stripped FBS for at least 48 h before the experiments. To measure progesterone concentrations in the culture medium, the BeWo cells were cultured for 24 h in fresh medium containing no AGT (vehicle controls) or AGT at various concentrations (10^{-7} - 10^{-3} M). Cells were then switched to fresh medium containing AGT at the same concentrations. After an additional 24 h or 48 h of culture, progesterone concentrations in the medium were measured by an immunoassay using a progesterone ELISA kit (Cayman Chemical Co., Ann Arbor, MI), according to the manufacturer's instruction. The assay is based on the use of a specific antibody raised against progesterone.

Whole cell lysate preparation. To examine BCRP and PR protein expression, BeWo cells were seeded at a cell density of 1.5×10^6 cells/well in 10 cm dishes and grown for 16 – 18 h. Cells were then transfected with 0.4 - $3.2 \mu\text{g}/10^5$ cells of the PRA expression vector or 0.4 - $1.6 \mu\text{g}/10^5$ cells of the PRB expression vector, using Lipofectamine Plus according to the manufacturer's instruction (Invitrogen, Carlsbad, CA). Six h after transfection, cells were

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switched to the fresh medium, supplemented with 10^{-4} M AGT, and cultured for an additional 24 h to detect PR expression or treated with 10^{-6} M progesterone for an additional 48 h in the presence and absence of 10^{-5} M RU-486 to detect BCRP expression. Cells were then harvested and whole cell lysates prepared as previously described (Wang et al., 2006).

SDS-polyacrylamide gel electrophoresis and immunoblotting. To detect BCRP protein, the protein samples of whole cell lysates (20 μ g each lane) were subjected to immunoblotting using BXP-21 (1:500 dilution), a BCRP-specific monoclonal antibody (mAb) (Kamiya Biomedical, Seattle, WA), as previously described (Wang et al., 2006). To detect PR protein, the protein samples of whole cell lysates (30 μ g each lane) were subjected to immunoblotting by the use of an antibody (1:2,000 dilution) that recognizes both PRA and PRB (catalog no. sc-538x, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The donkey anti-rabbit IgG-HRP conjugate antibody (Santa Cruz Biotechnology, Inc.) was used as the secondary antibody at 1:3000 dilution for PR detection. Human β -actin was detected as an internal control as previously described (Wang et al., 2006). Relative BCRP protein levels were determined by densitometric analysis of the immunoblots using the NIH Scion Image software (Scion, Frederick, MD).

Intracellular mitoxantrone (MX) accumulation assay. Transport studies using [3 H]MX were performed to examine whether progesterone treatment affects MX efflux activity of the BeWo cells. Briefly, BeWo cells were seeded at a cell density of 1.8×10^5 per well in 6-well plates. Cells were transfected with 0.4 μ g plasmid/ 10^5 cells of the PRA or PRB expression vector. Six h after transfection, cells were cultured in medium supplemented with 10^{-4} M AGT for 24 h. Cells were switched to the fresh medium containing 10^{-4} M AGT and 10^{-6} M progesterone in the presence and absence of 10^{-5} M RU-486. After 48 h of treatment, cells grown as a monolayer were washed once with pre-warmed PBS and incubated in 1 ml per

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well of Opti-MEM for 30 min. In inhibition experiments, cells were first pre-incubated with 10 μ M FTC for 1 h. The experiments were then started by the addition of [3 H]MX (20 nM) in the presence and absence of 10 μ M FTC in 1 ml of Opti-MEM, and incubation was continued for 60 min. The MX efflux was then stopped by washing the cells three times with ice-cold PBS. The cell monolayer was suspended in 1 ml of 2% (w/v) SDS for whole cell lysate preparation. The whole cell lysates (900 μ l) were subjected to counting in a scintillation counter. Counts were normalized to the protein concentration that was measured by the Bio-Rad DC protein assay, using the remaining lysates. The intracellular MX concentrations were calculated on the basis of radioactivity associated with the cells and presented as picomoles of [3 H]MX per milligram protein. The difference in intracellular MX concentrations in the presence and absence of FTC was used as a measure of FTC-inhibitable MX efflux activity of the BeWo cells. This FTC-inhibitable MX efflux activity is attributable to BCRP expression. The experiments were performed in triplicate at 37°C in a humidified incubator and repeated twice.

Plasmids and cloning. Human PRA and PRB expression vectors were kindly provided by Dr. P. Chambon (INSERM, Universite Louis Pasteur, Paris, France). The *BCRP* promoter-luciferase reporter constructs with varying length of the *BCRP* promoter (5'-flanking region -1285/+362, -628/+362, -312/+362, -243/+362, and -115/+362) were described previously (Bailey-Dell et al., 2001). We identified two putative PREs, PRE1 (between -1143 and -1129 bp) and PRE2 (between -187 and -173 bp), using the NUBIScan program (University of Basel, Switzerland). PRE1 or PRE2 was deleted, one at a time, from the -1285/+362 construct using PCR mutagenesis as previously described (Lee et al., 2006). The -243/+362 construct was used as template for PCR mutagenesis to generate point (Mut1 and Mut2) or double (Mut3) mutations in PRE2 as well as deletion of PRE2. The primers used for deletion of PRE1 were

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5'-AGGCAGGGTTTCACCATGTCTGAAGTCTTGGCCTCAAGT-3' and
5'-ACTTGAGGCCAGGAGTTCGACATGGTGAAACCCTGCCT-3'. The primers used for
deletion of PRE2 were 5'-CTTGTCCCTGCGTGTCTAGCCCCGAGGGAGGG-3' and
5'-CCCTCCCTCGGGGCTAGACACGCAGGGACAAG-3'. The primers used for Mut1
were 5'-TGTCACGGTAGGGTGACCCTA G-3' and
5'-CTAGGGTCACCCTACCGTGACA-3'; for Mut2 were
5'-TGTCACGGCAGGGTTACCCTAG-3' and 5'-CTAGGGTAACCCTGCCGTGACA-3';
and for Mut3 were: 5'-TGTCACGGTAGGGTTACCCTAG-3' and
5'-CTAGGGTAACCCTACCGTGACA-3'. All constructs were verified by sequencing.

Luciferase reporter assay. To examine transcriptional activity of the *BCRP* promoter,
BeWo cells were seeded at a cell density of 3.5×10^4 cells per well in 24-well plates and
cultured for 18 – 20 h. Cells were then transiently transfected with 0.15 μ g of the *BCRP*
promoter luciferase reporter constructs, 0.4 - 3.2 μ g/ 10^5 cells of the PRA and/or PRB
expression vectors, and 0.1 μ g of the β -galactosidase transfection control plasmid using
Lipofectamine Plus (Invitrogen), according to the manufacturer's instructions. Six h after
transfection, cells were cultured in medium containing 10^{-4} M AGT and incubation was
continued for 24 h. Cells were then switched to fresh medium containing 10^{-4} M AGT
supplemented with various concentrations of progesterone in the presence or absence of 10^{-5}
M RU-486. After 48 h of treatment, the cells were harvested and analyzed for both the
luciferase and β -galactosidase activities using the assay kits from Promega (Madison, WI).
Relative luciferase activities were normalized for β -galactosidase activities for each sample.
The experiments were performed in triplicate and repeated at least twice.

Electrophoretic mobility shift assay (EMSA). Non-radioactive EMSA was performed
using the LightShift™ chemiluminescent EMSA kit (Pierce, Rockford, IL). Nuclear protein

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extracts were prepared from BeWo cells using the NE-PER nuclear extraction kit (Pierce). Non-labeled and 3'-biotinylated oligonucleotides were synthesized by Operon Biotechnologies, Inc. (Novato, CA). The sequence of oligonucleotides containing PRE1 or PRE2 was 5'-CATGTTGGCCAGGCTGGTCTCGAAC-3' or 5'-GTGTCACGGCAGGGTGACCCTAGCC-3', respectively. The binding reactions (20 μ l each) were carried out at room temperature for 25 min in the presence of 50 ng/ μ l poly(dI-dC), 0.05% Nonidet P-40, 5 mM MgCl₂, 10 mM EDTA, 2.5% glycerol, 30 fmol of biotin-end-labeled target DNA, and 4 μ g of nuclear protein extract in 1 \times binding buffer. For competition experiments, a 200-fold molar excess of the unlabeled oligonucleotide harboring PRE2, a 20- or 200-fold molar excess of the unlabeled oligonucleotide having PRE2 deleted, and a 200-fold molar excess of a non-specific unlabeled oligonucleotide that does not contain any known binding sequences were added in respective reactions. For supershift experiments, 2 μ g of the anti-human PR antibody or BXP-21 (used as a mouse IgG control) were added in the binding reaction. Non-denaturing 5% polyacrylamide gels (Bio-Rad Hercules, CA) were pre-electrophoresed for 60 min in 0.5 \times TBE buffer (0.089 M Tris-base, 0.089 M Boric Acid, 2 mM EDTA (disodium), pH 8.3 with boric acid) before loading the binding reaction samples. The samples were then electrophoresed in 0.5 \times TBE buffer and transferred onto a positively charged nylon membrane (HybondTM-N⁺). Transferred DNAs were cross-linked to the membrane at 120 mJ/cm² for 15 min and detected using horseradish peroxidase-conjugated streptavidin, according to the manufacturer's instructions (Pierce, Rockford, IL).

Statistical analysis. Data were analyzed for statistical significance using Student's *t*-test or one-way ANOVA analysis followed by Neuman Keuls test as indicated in figure legends. Differences with *p* values < 0.05 were considered statistically significant.

Results

AGT inhibited progesterone secretion from BeWo cells. It has been shown that human placental JEG-3 cells secrete progesterone and this progesterone production can be inhibited by the addition of AGT (Cheng et al., 2001). We thus examined progesterone secretion from BeWo cells. When BeWo cells were grown to a cell density of approximately 3.5×10^4 cells per well (0.5 ml of medium per well) after 24 h of culture, the progesterone concentrations in the medium were approximately 6.5×10^{-8} M. Progesterone production was inhibited by AGT in a dose-dependent manner, and the maximal inhibition was achieved by the addition of 10^{-4} M AGT, which inhibited progesterone production by 95% (Fig. 1A). After 48 h of culture, the progesterone production was slightly increased to 6.8×10^{-8} M and was also inhibited by 10^{-4} M AGT by 95% (Fig. 1A). The treatment of BeWo cells with AGT had no significant effect on cell viability estimated by counting viable cells using trypan blue (data not shown). Since the activity of a classical PR is likely already saturated at 6.5×10^{-8} M progesterone (the binding affinity of progesterone to PRA or PRB is approximately 0.5×10^{-9} M (Dijkema et al., 1998)), to investigate if BCRP expression in BeWo cells is regulated by progesterone through a classical PR, we included 10^{-4} M AGT in all subsequent experiments to inhibit production of endogenous progesterone. Inhibition of progesterone production by AGT resulted in a 30% decrease of the transcriptional activity of the *BCRP* promoter (Fig. 1B). The transcriptional activity was also significantly inhibited by adding 10^{-5} M RU-486 by approximately 60%, and a combination of RU-486 and AGT did not further decrease the transcriptional activity. The addition of 10^{-7} M progesterone completely reversed the decrease in transcriptional activity caused by AGT and even increased the activity approximately 2-fold compared with the vehicle control (Fig. 1B). These results suggest that progesterone could transactivate the *BCRP* gene via a classical PR that is endogenously expressed in BeWo cells.

Expression of PRA and PRB in BeWo cells. We next examined PRA and PRB expression in BeWo cells by immunoblotting. Both endogenous PRA and PRB were expressed in BeWo cells; however, the level of PRA was much lower than that of PRB (Fig. 2A). Transfection of PRA increased PRA expression, and the maximal level was reached with 1.6 μg plasmid/ 10^5 cells. Interestingly, transfection of PRA with 1.6 or 3.2 μg plasmid/ 10^5 cells slightly decreased endogenous expression of PRB (Fig. 2A). Transfection of PRB with 0.4 μg plasmid/ 10^5 cells increased PRB expression to a similar level as the maximal level of PRA, and transfection with 1.6 μg plasmid/ 10^5 cells did not further increase PRB expression (Fig. 2A).

Progesterone induced BCRP protein expression via PRB, but not PRA. We then evaluated the role of PRA and PRB in BCRP expression in BeWo cells. As shown in Fig. 2B, BCRP protein expression was increased approximately 3-fold by 10^{-6} M progesterone in cells with no transfection of either PR, and was strongly induced approximately 9-fold in cells transfected with 0.4 μg plasmid/ 10^5 cells of PRB. Transfection with 1.6 μg plasmid/ 10^5 cells of PRB did not further increase BCRP expression. The addition of a 10-fold molar excess of RU-486 significantly, but not completely, abrogated the progesterone effect on BCRP expression in both the PRB-transfected and non-transfected cells (Fig. 2B). However, transfection of PRA with 0.4 μg plasmid/ 10^5 cells showed little effect on BCRP expression compared with that in non-transfected cells (Fig. 2C). Transfection of PRA (1.6 μg plasmid/ 10^5 cells) even decreased, rather than increased BCRP expression. These results suggest that progesterone likely induces BCRP expression in BeWo cells via PRB, but not PRA. Progesterone induced BCRP expression even in non-transfected cells, which is likely due to endogenous expression of PRB in BeWo cells (Fig. 2A). In the above experiments,

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AGT (10^{-4} M) was added in culture medium to inhibit production of progesterone from BeWo cells. We found that the addition of 10^{-4} M AGT decreased BCRP expression in BeWo cells by approximately 40% as compared with that in cells treated with the vehicle alone and the treatment of cells with 10^{-3} M AGT did not further decrease BCRP expression (data not shown). This decrease in BCRP expression is likely caused by inhibition of progesterone production. A combination of 10^{-4} M AGT with 10^{-5} M RU-486 had no apparent effect on BCRP expression as compared with the AGT treatment alone (data not shown). In general, the effect of AGT treatment on BCRP expression is consistent with the effect on the transcriptional activity of the *BCRP* promoter as shown in Fig. 1B. We did not detect any effect of the AGT plus RU-486 treatment on BCRP expression as compared with the AGT treatment alone. Such effect would be expected from the *BCRP* promoter activity data in Fig. 1B. This is likely due to the fact that immunoblotting is not as sensitive as the promoter reporter assay.

Effect of PRA and PRB transfection on BCRP-mediated MX efflux activity. To examine if the effect of progesterone on BCRP expression in PRA- and/or PRB-transfected cells is reflected in BCRP efflux activity, we investigated the effect of progesterone treatment on MX efflux by the BeWo cells using a MX accumulation assay. MX, a high affinity BCRP substrate, has been used as a model substrate to measure BCRP transport activity (Gupta et al., 2004; Robey et al., 2001). Treatment of non-transfected cells with 10^{-6} M progesterone decreased MX accumulation by approximately 10 – 20% compared with the vehicle controls (Fig. 3A and 3C). Transfection of PRA with 0.4 μ g plasmid/ 10^5 cells did not influence the progesterone effect on MX accumulation (Fig. 3A) and transfection of PRA with 1.6 μ g plasmid/ 10^5 cells even reversed the decrease in MX accumulation caused by the progesterone treatment (Fig. 3C); however, transfection of PRB significantly decreased MX

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accumulation by approximately 20 – 40% (Fig. 3A and 3C). Co-transfection of PRA and PRB at 4:1 PRA/PRB ratio had no effect on MX accumulation (Fig. 3C). Since lower MX accumulation reflects higher BCRP expression, these activity data are consistent with the BCRP protein expression data shown in Fig. 2. To eliminate the possible contribution of endogenous efflux transporters such as P-glycoprotein, a specific BCRP inhibitor FTC was used to determine FTC-inhibitable MX efflux activity. Because 10 μ M FTC used in the assay is sufficient to fully inhibit BCRP, the portion of MX efflux that can be inhibited by 10 μ M FTC is attributable to BCRP expression. Treatment of non-transfected cells with 10^{-6} M progesterone resulted in an increase of FTC-inhibitable MX efflux by approximately 25%; however, the MX efflux was increased by approximately 110% by 10^{-6} M progesterone in PRB-transfected cells (Fig. 3B and 3C). The addition of 10^{-5} M RU-486 significantly abrogated the progesterone-mediated stimulation of MX efflux in PRB-transfected cells to the same level as in non-transfected control cells (Fig. 3B). Transfection of PRA with 0.4 μ g plasmid/ 10^5 cells showed no additional progesterone-stimulation of MX efflux compared with non-transfected and progesterone-treated cells (Fig. 3B), and transfection of PRB with 1.6 μ g plasmid/ 10^5 cells even completely diminished the progesterone-stimulation of MX efflux (Fig. 3D). As expected, co-transfection of PRA and PRB at 4:1 PRA/PRB ratio also completely abrogated the progesterone-stimulation of MX efflux (Fig. 3D). Collectively, the activity data are fully consistent with the BCRP protein expression results.

Progesterone increased transcriptional activity of the *BCRP* promoter via PRB, but not PRA. To further confirm that progesterone induces BCRP expression via PRB at the transcriptional level, we investigated transcriptional activation of the *BCRP* promoter by progesterone in PRA- and PRB-transfected cells. After normalized to the activities of the vehicle control cells, transcriptional activity of the *BCRP* promoter was significantly induced,

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2 – 6-fold, by 10^{-8} – 10^{-6} M progesterone in PRB-transfected cells in a dose-dependent manner, and the maximal effect was achieved at 10^{-6} M progesterone (Fig. 4A). Increase of progesterone concentration to 10^{-5} M did not further increase the activity. The addition of RU-486 nearly completely abolished the progesterone-response (Fig. 4A). In contrast, progesterone increased transcriptional activity of the *BCRP* promoter up to only 1.5-fold in PRA-transfected cells (Fig. 4A). These results indicate that progesterone increased transcriptional activity of the *BCRP* promoter primarily through PRB.

PRA represses the PRB activity. To further determine the interplay between PRA and PRB, BeWo cells were either transfected with PRA alone or cotransfected with PRA and PRB. Transcriptional activity of the *BCRP* promoter in cells with no transfection of either PR was stimulated approximately 1.5-fold by 10^{-6} M progesterone, and the addition of RU-486 fully inhibited this induction (Fig. 4B). In cells transfected with 0.4 μ g plasmid/ 10^5 cells of PRA, the transcriptional activity was slightly but significantly decreased by 18% compared with that in non-transfected cells, and the addition of progesterone again increased the activity by approximately 40%, and the addition of RU-486 completely inhibited the induction (Fig. 4B). In cells transfected with 1.6 or 3.2 μ g plasmid/ 10^5 cells of PRA, the transcriptional activity was further decreased by approximately 50%, and the addition of progesterone showed little stimulation. Thus, it appears that PRA is inactive in transcriptional activation of the *BCRP* promoter. Transactivation of the *BCRP* promoter by progesterone in non-transfected cells is likely mediated by endogenous PRB, and PRA appears to repress the PRB activity. To further confirm this conclusion, we examined the progesterone-response in cells co-transfected with PRA and PRB at different ratios. As expected, in cells transfected with only PRB, the transcriptional activity was strongly induced approximately 15-fold by 10^{-6} M progesterone (Fig. 4C). Note that the fold-induction values in Fig. 4A and 4C are not the same, as the

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activities were normalized to different controls. When cells were co-transfected with PRA and PRB, the progesterone-response was significantly decreased by approximately 70% at 1:1 PRA/PRB ratio, and was nearly completely inhibited at 4:1 or 8:1 PRA/PRB ratio (Fig. 4C). These results further support the conclusion that PRA represses the PRB activity on transactivation of the *BCRP* gene.

Localization of putative PREs in the *BCRP* promoter region. To localize the PREs in the *BCRP* promoter region, we next examined transcriptional activity of the *BCRP* promoter with varying length of the 5'-flanking region (Fig. 5A) in PRB-transfected cells. As expected, compared with the activity in non-transfected cells, transcriptional activity of the *BCRP* promoter in the -1285/+362 construct was induced over 10-fold (Fig. 5B). The transcriptional activity was decreased by approximately 70% with a deletion of the 5'-flanking region from -1285 to -628 bp. The activity was slightly increased with deletion to -312 bp, and was strongly induced 30-fold with further deletion to -243 bp; however, deletion from -243 to -115 bp completely abolished the transcriptional activity (Fig. 5B). These results suggest that there are two putative PREs in the *BCRP* promoter region, one between -1285 and -628 bp and another between -243 and -115 bp.

Identification of a novel PRE in the *BCRP* promoter region. To further identify the PREs, we analyzed the region -1285 to -115 bp of the *BCRP* promoter using the NUBIScan program (University of Basel, Switzerland). We identified two likely PREs, PRE1 and PRE2, in the regions between -1285 and -628 bp and between -243 and -115 bp, respectively (Fig. 6A). We then analyzed function of the two putative PREs. First, with standard molecular biological techniques, we deleted PRE1 and PRE2, one at a time, in the -1285/+362 construct,

and PRE2 in the -243/+362 construct, and then determined the progesterone-response of these deletions. Deletion of PRE1 in the -1285/+362 construct did not significantly affect the progesterone-response; however, deletion of PRE2 in the -1285/+362 construct resulted in a nearly complete loss of the progesterone-response (Fig. 6B). Likewise, deletion of PRE2 in the -243/+362 construct decreased the progesterone-response by more than 95% (Fig. 6B). The data suggest that PRE2 is a progesterone response element in the *BCRP* promoter, but PRE1 is not. To further confirm these results, we performed mutation analysis on PRE2 in the -243/+362 construct. The progesterone-response was reduced by approximately 50% when the constructs with a single mutation (Mut1 and Mut2) were used, compared with that associated with the original -243/+362 construct (Fig. 6C). The progesterone-effect was further decreased by approximately 65% when the construct with a double mutation (Mut3) was used. Again, deletion of PRE2 in the -243/+362 construct decreased the progesterone-response by approximately 95% (Fig. 6C).

Binding of PRA and PRB to the identified PRE. We then performed EMSA to examine if PRA and PRB can directly bind to the identified progesterone response element, PRE2. When the biotinylated oligonucleotide containing the PRE2 sequence was incubated with nuclear protein extracts, strong DNA-protein complexes were observed in the samples containing either PRA (Fig. 7A, lane 2) or PRB (Fig. 7B, lane 2). These complexes were supershifted upon the addition of the anti-PR antibody (lane 3), but not the mouse IgG (lane 4). The DNA-protein complexes were completely eliminated by adding a 200-fold molar excess of the unlabeled oligonucleotide containing the PRE2 sequence (lane 5). However, these complexes were not affected by the addition of a 20- or 200-fold molar excess of

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unlabeled oligonucleotide in which PRE2 is deleted (lanes 6 and 7) or a 200-fold molar excess of unlabeled nonspecific oligonucleotide that does not contain any known binding sequences (lane 8). When the biotinylated oligonucleotide containing the PRE1 sequence was incubated with nuclear protein extracts, no DNA-protein complexes were observed (lane 9). These results suggest that both PRA and PRB can specifically bind to the identified PRE, PRE2, in the *BCRP* promoter region.

Discussion

We have previously shown that BCRP expression in BeWo cells is significantly induced by progesterone only at 10^{-5} M, which is much higher than the binding affinity of progesterone to classical PRs (Wang et al., 2006). In this study, we demonstrated that after production of progesterone from BeWo cells was inhibited, the *BCRP* promoter could be transactivated by progesterone at nM concentrations (Fig. 1B). BCRP protein expression in BeWo cells was also induced by 10^{-6} M progesterone (Fig. 2B) which otherwise had little effect on BCRP expression when progesterone production was not inhibited (Wang et al., 2006). These results suggest that without inhibition of progesterone production, the activity of classical PRs is likely already saturated or close to saturation.

We then investigated the molecular mechanism by which BCRP expression is upregulated by progesterone. We, for the first time, showed that transcriptional activity of the *BCRP* promoter was strongly induced by progesterone through PRB, but not PRA (Fig. 4A). Furthermore, we identified a novel PRE in the *BCRP* promoter region between -187 and -173 bp upstream of the transcription start site (Figs. 5 and 6), and both PRA and PRB bind to this PRE (Fig. 7). The progesterone-response of PRB-mediated *BCRP* promoter activity was significantly reduced upon mutation or deletion of the PRE (Fig. 6), indicating that this PRE is involved in transactivation of the *BCRP* promoter. Thus, induction of *BCRP* gene expression by progesterone can be mediated by a classical mechanism through progesterone-activated PRB that directly binds to the identified PRE and interacts with specific co-activators and general transcriptional factors, leading to enhanced *BCRP* gene transcription. We note that the identified PRE is exactly the same as the estrogen response element published by Ee et al. (Ee et al., 2004). That progesterone and estrogen receptors share the same or similar response elements is possible, as earlier studies suggest that the

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regulatory elements for different steroids, including progesterone, 17 β -estradiol, and glucocorticoids, are either similar or at least share structural features (von der Ahe et al., 1985). Thus, progesterone and 17 β -estradiol could make an impact on each other in regulation of BCRP, when the two hormones are combined. The real situation could be very complex, as 17 β -estradiol can induce PRB expression (Flototto et al., 2004; Wang et al., 2006) and down-regulate BCRP expression through posttranscriptional modification (Imai et al., 2005), and on the other hand, PRA can repress the estrogen receptor activity (Giangrande and McDonnell, 1999). In BeWo cells, we previously showed that the 17 β -estradiol treatment alone down-regulated BCRP expression (Wang et al., 2006), presumably due to posttranscriptional modification as demonstrated by Imai et al. (Imai et al., 2005); however, the combined treatment of BeWo cells with 17 β -estradiol and progesterone significantly increased BCRP expression compared with progesterone treatment alone (Wang et al., 2006). We have hypothesized that this combined effect is likely due to induction by 17 β -estradiol of PRB in BeWo cells which then induces BCRP expression through progesterone (Wang et al., 2006). The data of the present study support this hypothesis. Although, as shown in this study, BCRP can be induced by progesterone via a classical PR mechanism, our previous study suggests that a non-classical membrane-bound PR could also be involved in upregulation of BCRP, particularly at high progesterone concentrations (Wang et al., 2006). This may explain why the addition of even a 10-fold molar excess of RU-486 could not completely inhibit progesterone-mediated induction of BCRP protein expression (Fig. 2B).

Transcriptional activity of the *BCRP* promoter was not completely eliminated upon deletion of the identified PRE (Figs. 6B and 6C). PRs can regulate transcription by direct binding to a PRE and/or by interaction with another transcriptional factor in a PRE-independent manner (van der Burg and van der Saag, 1996). Our data suggest that the

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BCRP promoter could also possibly be stimulated by progesterone through interaction of PR with other transcriptional factors that may stabilize PR interaction with the *BCRP* promoter. Alternatively, the identified PRE region may be comprised of several additive enhancer modules that also contribute to the basal promoter activity. In addition, we showed that deletion of the 5'-flanking region of the *BCRP* promoter from -1285 to -628 bp significantly decreased the progesterone-response (Fig. 5B). However, the predicted PRE1 between -1285 and -628 bp is shown not to be a PRE (Figs. 6B and 7). Therefore, the existence of positive regulatory element(s) other than PRE between -1285 and -628 bp is possible. The data shown in Fig. 5B also suggest that there seems to be a suppressive element(s) between -628 and -243 bp. Indeed, aberrant promoter methylation in the predicted CpG island between -599 and +329 bp of the *BCRP* promoter region has been shown to suppress transcription of the *BCRP* gene (To et al., 2006).

Various genes have been shown to be differentially regulated by PRA and PRB in a promoter- and tissue-specific manner (Brayman et al., 2006; Cheng et al., 2001). We found that PRB is a strong activator of transcription of the *BCRP* promoter, and PRA represses the PRB activity (Figs. 4B and 4C). Thus, PRA and PRB also differentially regulate *BCRP* gene expression in BeWo cells. The mechanism of this differential regulation is currently unknown. Even though both PRA and PRB can directly bind to the identified PRE (Fig. 7), it has been suggested that PRA transrepression of PRB activity is not dependent on DNA binding (Vegeto et al., 1993). However, we cannot rule out the possibility that coexpression of PRA and PRB leads to formation of nonfunctional PRA/PRB heterodimers that bind to the identified PRE. In addition, the fact that transfection of PRA decreased PRB expression (Fig. 2A) may also contribute to repression of PRB-mediated transcriptional activity of the *BCRP* promoter.

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Among human tissues, BCRP is most abundantly expressed in the apical membrane of the placental syncytiotrophoblast (Maliepaard et al., 2001), suggesting that BCRP plays a protective role for the fetus by limiting placental penetration of drugs/xenobiotics that are BCRP substrates. Jonker et al. (Jonker et al., 2000) showed that the fetus/maternal plasma ratio of topotecan was increased 2-fold in the pregnant mouse treated with the BCRP inhibitor GF-120918 compared with that in the vehicle-treatment control. We have also demonstrated that Bcrp1, the murine homolog of human BCRP, significantly limits fetal distribution of the BCRP/Bcrp1 substrates, nitrofurantoin and glyburide, in the pregnant mouse (data not shown). Progesterone is highly produced by the placenta during pregnancy, and the local progesterone concentration in the placenta can reach 8×10^{-6} M at term (Khan-Dawood and Dawood, 1984). Also, PR expression has been demonstrated in human placenta (Cudeville et al., 2000). Thus, placenta is very likely a target tissue for the action of progesterone. It is therefore reasonable to hypothesize that progesterone may induce BCRP expression in the placenta through PRB and augment the protective role of the transporter during pregnancy. We will test this hypothesis in future work. BCRP is also highly expressed in the liver (Mao and Unadkat, 2005). Although PR expression has been demonstrated in hepatocellular carcinoma of some patients (Cohen et al., 1998), few studies have shown PR expression in the non-tumoral liver; therefore, it remains to be determined if progesterone could affect hepatic BCRP expression and hence BCRP-mediated biliary excretion of drugs. BCRP has been shown to be strongly induced in mammary gland during lactation and is responsible for milk secretion of BCRP substrate drugs (Jonker et al., 2005). PR isoforms are expressed in mammary gland and play a key role in pregnancy-associated mammary gland morphogenesis and tumorigenesis (Conneely et al., 2003). However, since plasma progesterone levels rapidly decrease to the normal levels as in non-pregnant women within

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one week after delivery and during lactation (Neville et al., 2002), it seems unlikely that progesterone is involved in up-regulation of BCRP expression in lactating breast.

Hormonal therapy plays an integral role in the management of the majority of women with breast cancer expressing estrogen and progesterone receptors (Ingle, 2002). PR expression in breast cancer is an important indicator of likely responsiveness to endocrine agents. It has been shown that PRA and PRB are expressed in similar amounts in most breast tumors (Graham et al., 1996). Numerous endocrine agents are available to the clinician for the management of breast cancer, including progestins (Ingle, 2002). Thus, caution should be taken that such endocrine therapy may lead to enhanced drug resistance in PRB-positive cancers due to induction of BCRP by progestins. Since PRA represses the PRB activity, the combined effect of PRA and PRB on BCRP expression in tumors should also be considered.

In summary, our data show that progesterone induces BCRP expression in BeWo cells at the transcriptional level through PRB, but not PRA. PRA represses the PRB activity. These results provide new insight into the mechanistic understanding of regulation of BCRP expression by steroid hormones in human placenta during pregnancy as well as in cancer cells.

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Footnotes:

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Figure Legends

Figure 1. Inhibition of progesterone secretion from BeWo cells by AGT and the effect on

transcriptional activity of the *BCRP* promoter. **A**, BeWo cells were cultured in the absence (DMSO vehicle controls) or presence of varying concentrations (10^{-6} – 10^{-3} M) of AGT for 24 h. Cells were then switched to fresh medium containing AGT at the same concentrations, and cell culture was continued for an additional 24 h (solid bars) or 48 h (dashed bars). Progesterone production was measured by the progesterone immunoassay and shown as a percentage of the progesterone production in the vehicle controls (0.1% v/v DMSO). The progesterone concentration in the vehicle controls after 24 h of culture (approximately 6.5×10^{-8} M) was set as 100%. Data shown are mean \pm S.E. from three independent experiments. Significant differences: * $p < 0.01$ and ** $p < 0.001$ vs. the vehicle control; & $p < 0.05$ vs. the immediate adjacent group on the left by one-way ANOVA analysis followed by Neuman Keuls test. **B**, BeWo cells were transfected with the -1285/+362 *BCRP* promoter-luciferase and β -galactosidase transfection control plasmids, and then treated with 10^{-4} M AGT and/or 10^{-5} M RU-486 with or without 10^{-7} M progesterone (P) as indicated for 48 h. Cell lysates were prepared and assayed for luciferase and β -galactosidase activities. Luciferase activities were normalized for β -galactosidase activities for each sample. Relative promoter activities were expressed as fold-change relative to the vehicle controls (0.1% v/v DMSO) which were set as 1. Data shown are mean \pm S.E. of three independent experiments. Significant differences: * $p < 0.05$ and ** $p < 0.01$ vs. the vehicle control; # $p < 0.01$ vs. the AGT treatment alone by one-way ANOVA analysis followed by Neuman Keuls test.

Figure 2. Expression of PRA and PRB in BeWo cells, and the effect of progesterone on

BCRP protein expression in PR-transfected cells. BeWo cells were transfected with the PRA or PRB expression vector with the amount of plasmid DNA as indicated. After

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transfection, cells were cultured with 10^{-4} M AGT for 24 h. Cells were then switched to fresh medium containing 10^{-4} M AGT and cultured for an additional 24 h to detect PR protein (**A**), or for 48 h in the presence and absence of 10^{-6} M progesterone and 10^{-5} M RU-486 to detect BCRP protein in PRB-transfected cells (**B**) or in PRA-transfected cells (**C**). The relative BCRP levels normalized to β -actin (internal standard) in the vehicle (0.1% v/v DMSO) control cells with no PR-transfection and no progesterone treatment were set as 1.

Figure 3. Effect of progesterone on BCRP efflux activity in PR-transfected cells. In the MX efflux study, cells were transfected with PRA and/or PRB with the amount of plasmid DNA as indicated and treated with 10^{-6} M progesterone in the presence or absence of 10^{-5} M RU-486. The intracellular MX accumulation (**A** and **C**) or FTC-inhibitable MX efflux activities (**B** and **D**) of the vehicle controls were set as 100%. In **A** and **C**, the open and solid bars represent the intracellular MX accumulation in the absence and presence of 10 μ M FTC, respectively. FTC-inhibitable MX efflux activities in **B** and **D** were calculated from the data shown in **A** and **C**, respectively, by subtracting the intracellular MX accumulation in the absence of FTC from the corresponding intracellular MX accumulation in the presence of FTC. Data shown are means \pm S.E. from three independent experiments. Significant differences: * $p < 0.05$ and ** $p < 0.01$ vs. the respective vehicle control; # $p < 0.05$ and & $p < 0.01$ vs. the immediately adjacent group on the right; \$ $p < 0.05$ and \$\$ $p < 0.01$ vs. the immediately adjacent group on the left; ### $p < 0.01$ vs. the progesterone treatment group without PR transfection by one-way ANOVA analysis followed by Neuman Keuls test.

Figure 4. Effect of PRA and/or PRB transfection on the progesterone-response of transcriptional activity of the *BCRP* promoter. **A**, dose-dependent effect of progesterone on transcriptional activity of the *BCRP* promoter. BeWo cells were transfected with the

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-1285/+362 *BCRP* promoter-luciferase and β -galactosidase transfection control plasmids as well as the PRA (0.4 μ g plasmid/ 10^5 cells) or PRB (0.4 μ g plasmid/ 10^5 cells) expression vector. After transfection, cells were cultured with 10^{-4} M AGT for 24 h. Cells were then switched to fresh medium containing 10^{-4} M AGT and treated with the vehicle (0.1% v/v DMSO) or progesterone in the presence or absence of 10^{-5} M RU-486 for 48 h. The transcriptional activities of the vehicle control cells transfected with the 1285/+362 construct and the PRA or PRB expression vector were set as 1. Data shown are mean \pm S.E. from three independent experiments. Significant differences: * $p < 0.05$ and ** $p < 0.01$ vs. the vehicle control by one-way ANOVA analysis followed by Neuman Keuls test. **B**, effect of PRA transfection on the progesterone-response. BeWo cells were transfected with the -1285/+362 *BCRP* promoter-luciferase and β -galactosidase transfection control plasmids as well as the PRA expression vector (0.4 – 3.2 μ g plasmid/ 10^5 cells). Cells were then treated with the vehicle (0.1% v/v DMSO) or 10^{-6} M progesterone in the presence or absence of 10^{-5} M RU-486 for 48 h as described in **A**. The transcriptional activities of the vehicle control cells transfected with only the -1285/+362 construct were set as 1. Data shown are mean \pm S.E. from three independent experiments. Significant differences: * $p < 0.05$ and ** $p < 0.01$ vs. the vehicle control by Student's *t*-test. **C**, PRA represses the PRB activity. BeWo cells were transfected with the -1285/+362 *BCRP* promoter-luciferase and β -galactosidase transfection control plasmids as well as the PRB (0.4 μ g plasmid/ 10^5 cells) and PRA (0.4 – 3.2 μ g plasmid/ 10^5 cells) expression vectors at different ratios as indicated. Cells were then treated with the vehicle (0.1% v/v DMSO) or 10^{-6} M progesterone in the presence or absence of 10^{-5} M RU-486 for 48 h as described in **A**. The transcriptional activities of the vehicle control cells transfected with only the -1285/+362 construct were set as 1. Data shown are mean \pm S.E. from three independent experiments. Significant differences: * $p < 0.01$ vs. the immediate adjacent group on the left by Student's *t*-test. In all the experiments, luciferase

activities were normalized for β -galactosidase activities for each sample. Relative promoter activities were expressed as fold-induction relative to the vehicle controls.

Figure 5. Localization of putative PREs in the *BCRP* promoter region. **A**, schematic illustration of the *BCRP* promoter luciferase plasmids with varying length of the 5'-flanking region of the promoter. **B**, BeWo cells were transfected with the *BCRP* promoter luciferase and β -galactosidase transfection control plasmids with or without PRB transfection (0.4 μ g plasmid/ 10^5 cells). Cells were then treated with 10^{-4} M AGT and 10^{-6} M progesterone in the presence or absence of 10^{-5} M RU-486 for 48 h as described in Figure 3A. Luciferase activities were normalized for β -galactosidase activities for each sample. The transcriptional activities of the vehicle (0.1% v/v DMSO) control cells transfected only with the -1285/+362 construct were set as 1. Relative promoter activities were expressed as fold-induction relative to the vehicle controls. Data shown are mean \pm S.E. from three independent experiments. Significant differences: * $p < 0.01$ and ** $p < 0.001$ vs. the immediate adjacent group on the left by Student's *t*-test.

Figure 6. Identification of a novel PRE in the *BCRP* promoter region between -243 and -115 bp upstream of the transcriptional start site. **A**, schematic illustration of two likely PREs (PRE1 and PRE2). For reference, a consensus PRE (Jantzen et al., 1987) and a functionally analyzed palindrome (Strahle et al., 1987) are listed. In the putative PREs, bases identical to either of the reference PREs are underlined. Three mutations in the putative PRE2 are shown, and bases that were mutated are indicated in italic. **B**, BeWo cells were transfected with the -1285/+362 or -243/+362 construct with or without having the putative PREs deleted and the β -galactosidase transfection control plasmid as well as the PRB expression vector (0.4 μ g plasmid/ 10^5 cells). Cells were then treated with 10^{-4} M AGT and

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10^{-6} M progesterone in the presence or absence of 10^{-5} M RU-486 for 48 h as described in Figure 3A. Luciferase activities were normalized for β -galactosidase activities for each sample. The transcriptional activities of the vehicle (0.1% v/v DMSO) control cells transfected only with the -1285/+362 construct were set as 1. Relative promoter activities were expressed as fold-induction relative to the vehicle controls. Data shown are mean \pm S.E. from three independent experiments. Significant differences: * $p < 0.01$ and ** $p < 0.001$ vs. the immediate adjacent group on the left by Student's *t*-test. **C**, BeWo cells were transfected with the original -243/+362 construct or the -243/+362 construct with mutations in the PRE2 or having PRE2 deleted and β -galactosidase transfection control plasmid as well as the PRB expression vector (0.4 μ g plasmid/ 10^5 cells). Cells were treated with 10^{-4} M AGT and 10^{-6} M progesterone in the presence or absence of 10^{-5} M RU-486 for 48 h as described in Figure 3A. Luciferase activities were normalized for β -galactosidase activities for each sample. The transcriptional activities of the vehicle (0.1% v/v DMSO) control cells transfected only with the -243/+362 construct were set as 1. Relative promoter activities were expressed as fold-induction relative to the vehicle controls. Data shown are mean \pm S.E. from three independent experiments. Significant differences: * $p < 0.01$ and ** $p < 0.001$ vs. the immediate adjacent group on the left by Student's *t*-test.

Figure 7. Binding of PRA and PRB to the identified PRE in the *BCRP* promoter region.

Non-radioactive electrophoretic mobility shift assay was performed as described under “Materials and Methods” using nuclear protein extracts isolated from BeWo cells transfected with PRA (**A**) or PRB (**B**). Specific DNA-protein complexes were seen in samples incubated with the biotinylated oligonucleotide containing PRE2 (lane 2), but not PRE1 (lane 9). To detect supershift of the DNA-protein complexes, the antibody against human PR (lane 3) or mAb BXP-21 (used as control mouse IgG) (lane 4) was added. For competition analysis, a

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200-fold molar excess of unlabeled oligonucleotide containing PRE2 (lane 5), a 20- or 200-fold molar excess of unlabeled oligonucleotide having PRE2 deleted (lanes 6 and 7), or a 200-fold molar excess of unlabeled nonspecific oligonucleotide that does not contain any known binding sequences (used as a negative control) (lane 8) were added. Arrows indicate the PRE2-PRA, PRE2-PRB and supershifted DNA-protein complexes as well as non-specific DNA-protein binding.

Figure 1

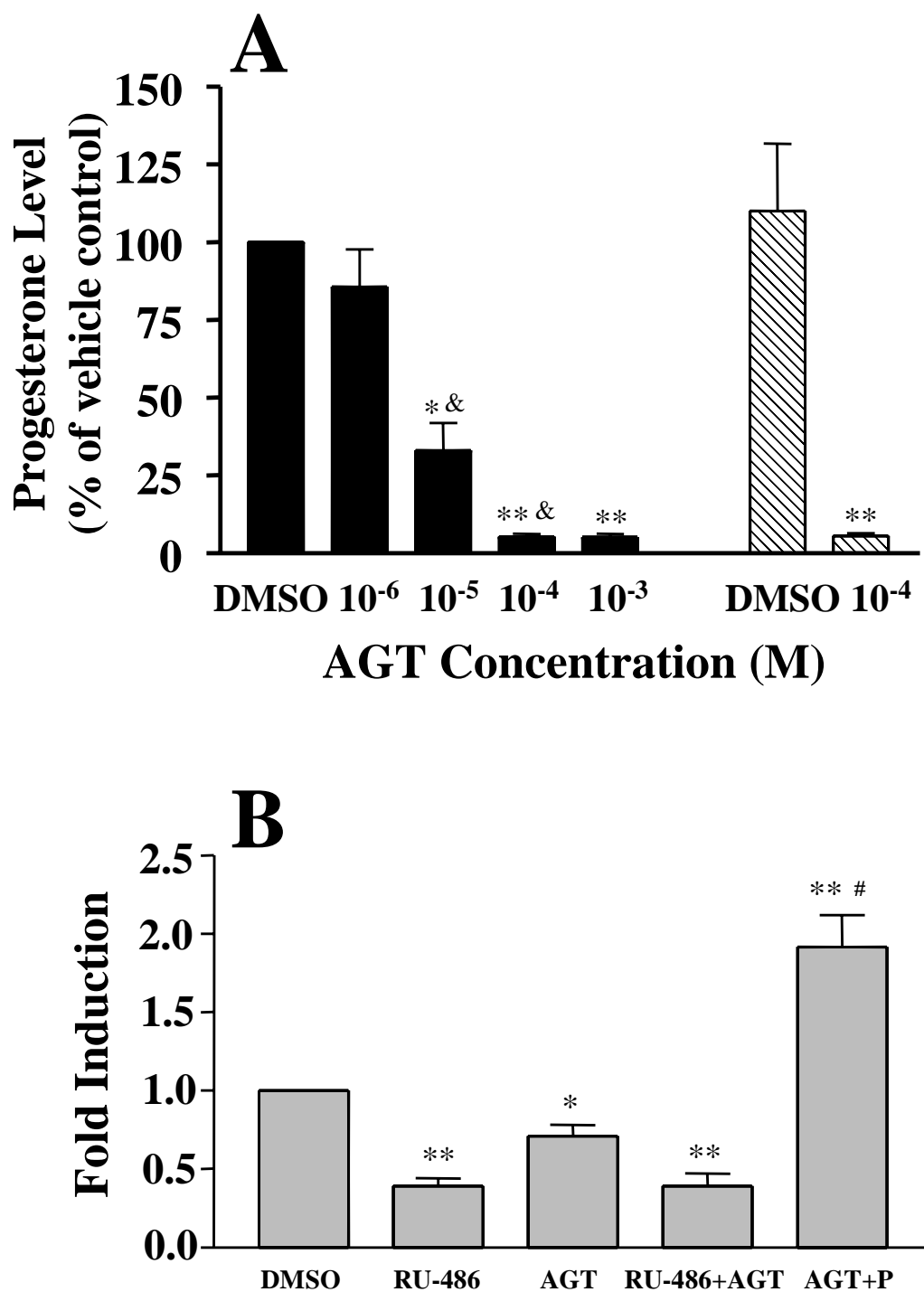


Figure 2

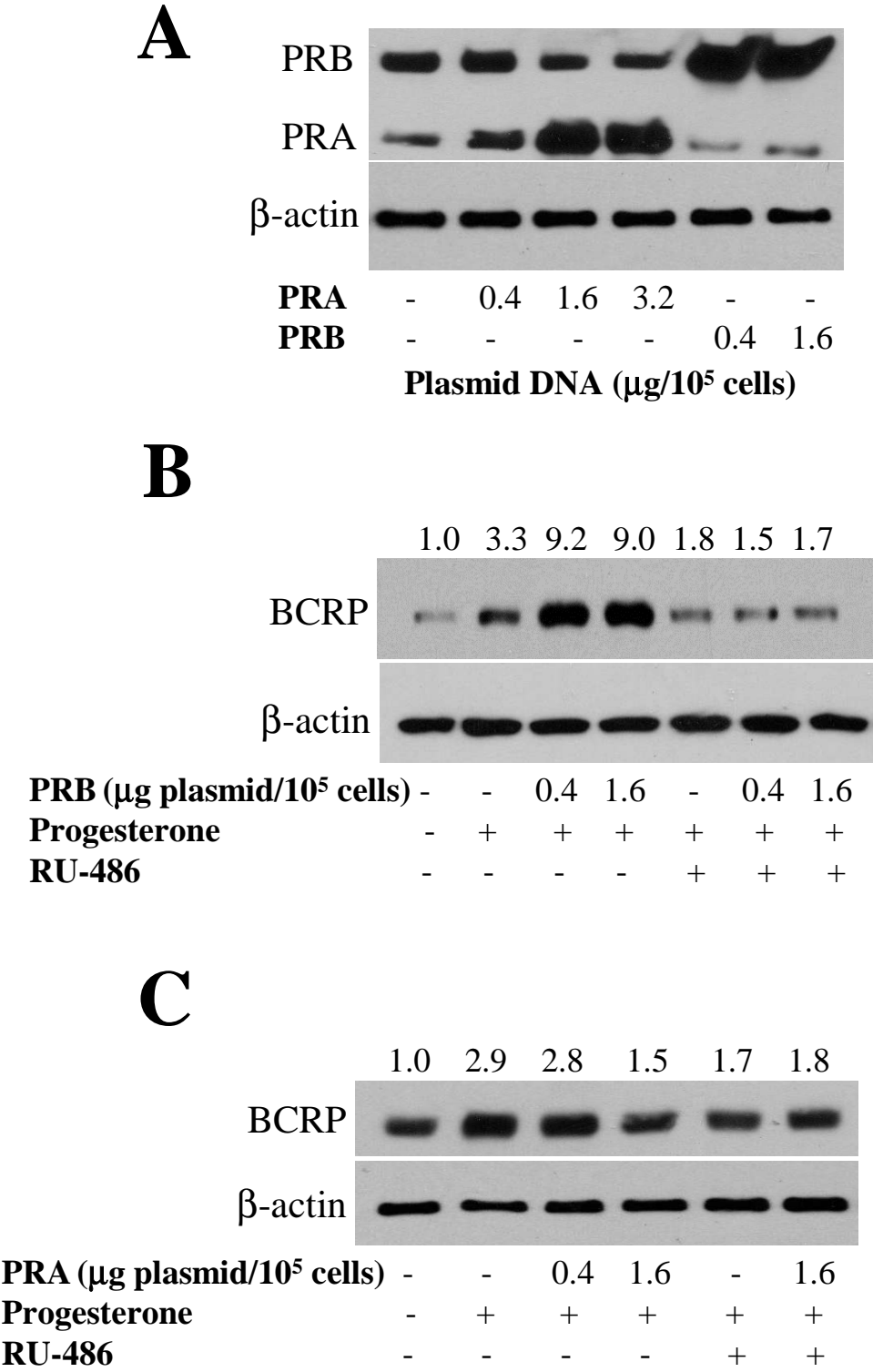
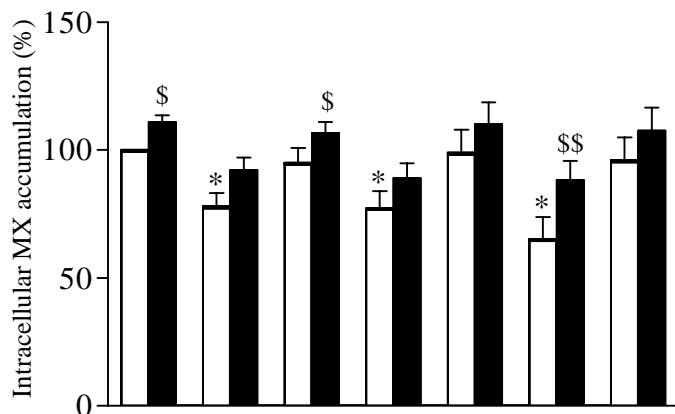


Figure 3

A



Progesterone

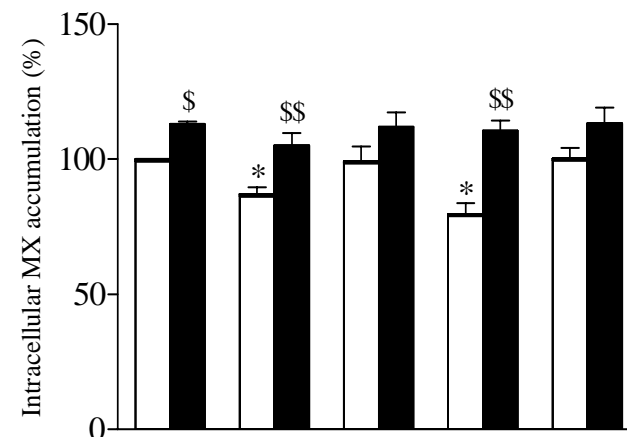
RU-486

PRA (0.4 µg plasmid/10⁵ cells)

PRB (0.4 µg plasmid/10⁵ cells)

-	+	+	+	+	+	+
-	-	+	-	+	-	+
-	-	-	+	+	-	-
-	-	-	-	-	+	+

C



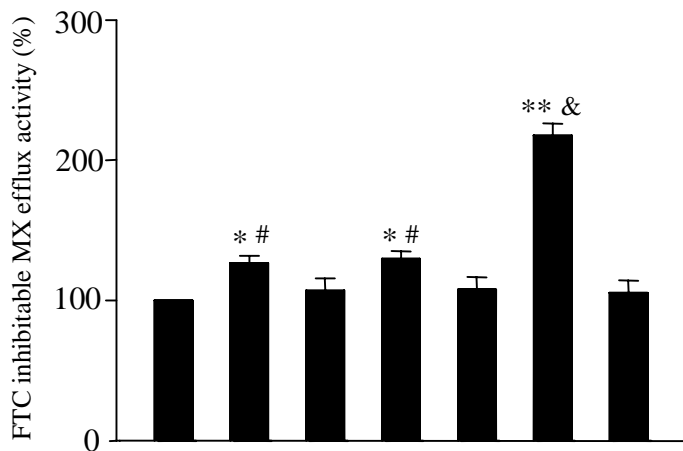
Progesterone

PRA (1.6 µg plasmid/10⁵ cells)

PRB (0.4 µg plasmid/10⁵ cells)

-	+	+	+	+
-	-	+	-	+
-	-	-	+	+

B



Progesterone

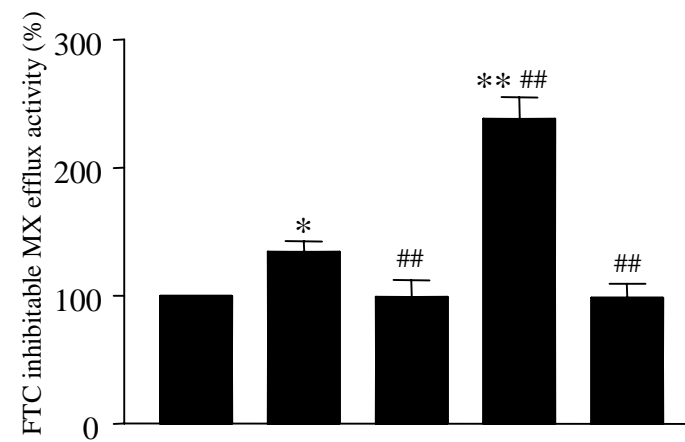
RU-486

PRA (0.4 µg plasmid/10⁵ cells)

PRB (0.4 µg plasmid/10⁵ cells)

-	+	+	+	+	+	+
-	-	+	-	+	-	+
-	-	-	+	+	-	-
-	-	-	-	-	+	+

D



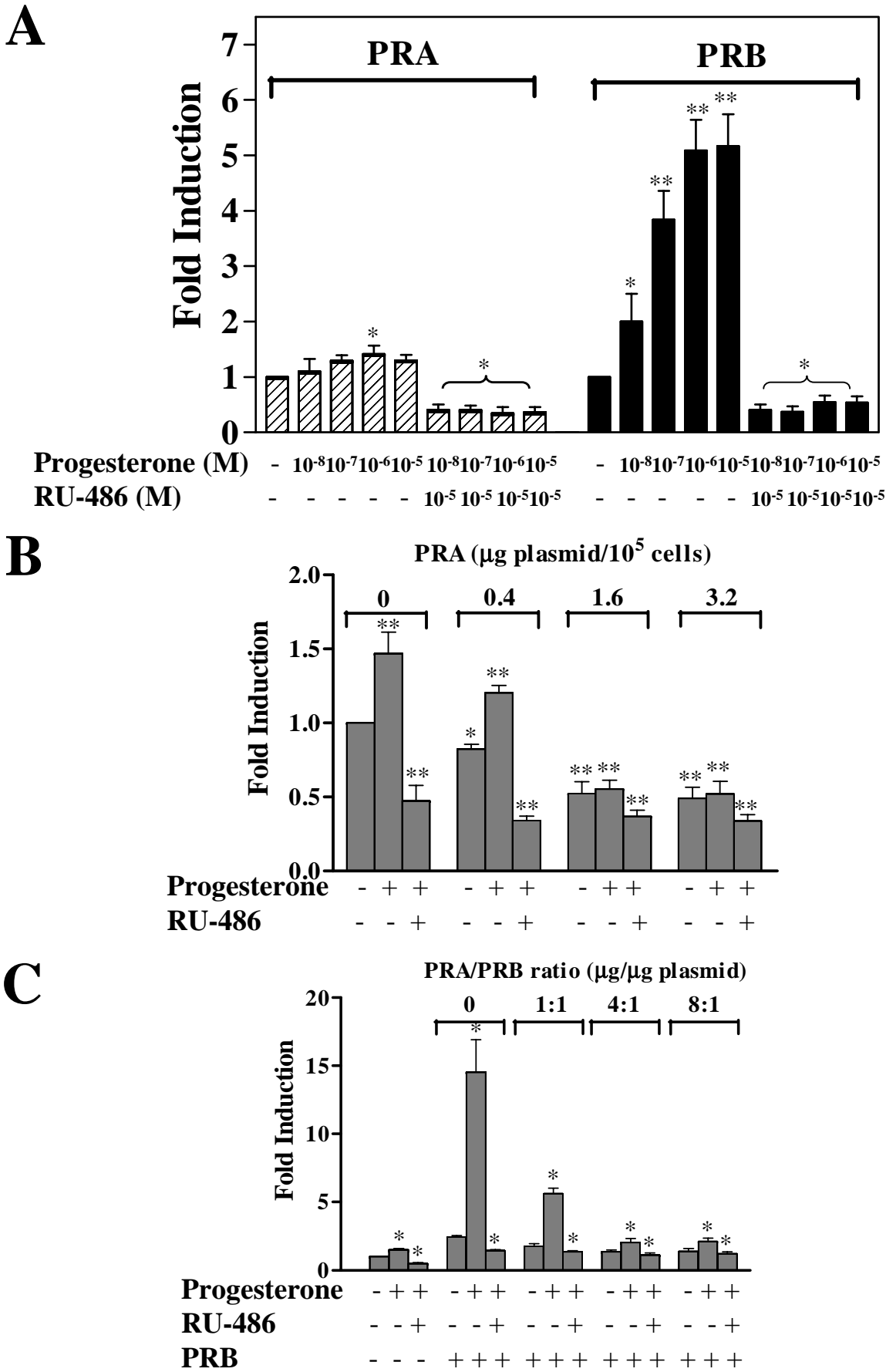
Progesterone

PRA (1.6 µg plasmid/10⁵ cells)

PRB (0.4 µg plasmid/10⁵ cells)

-	+	+	+	+
-	-	+	-	+
-	-	-	+	+

Figure 4



A

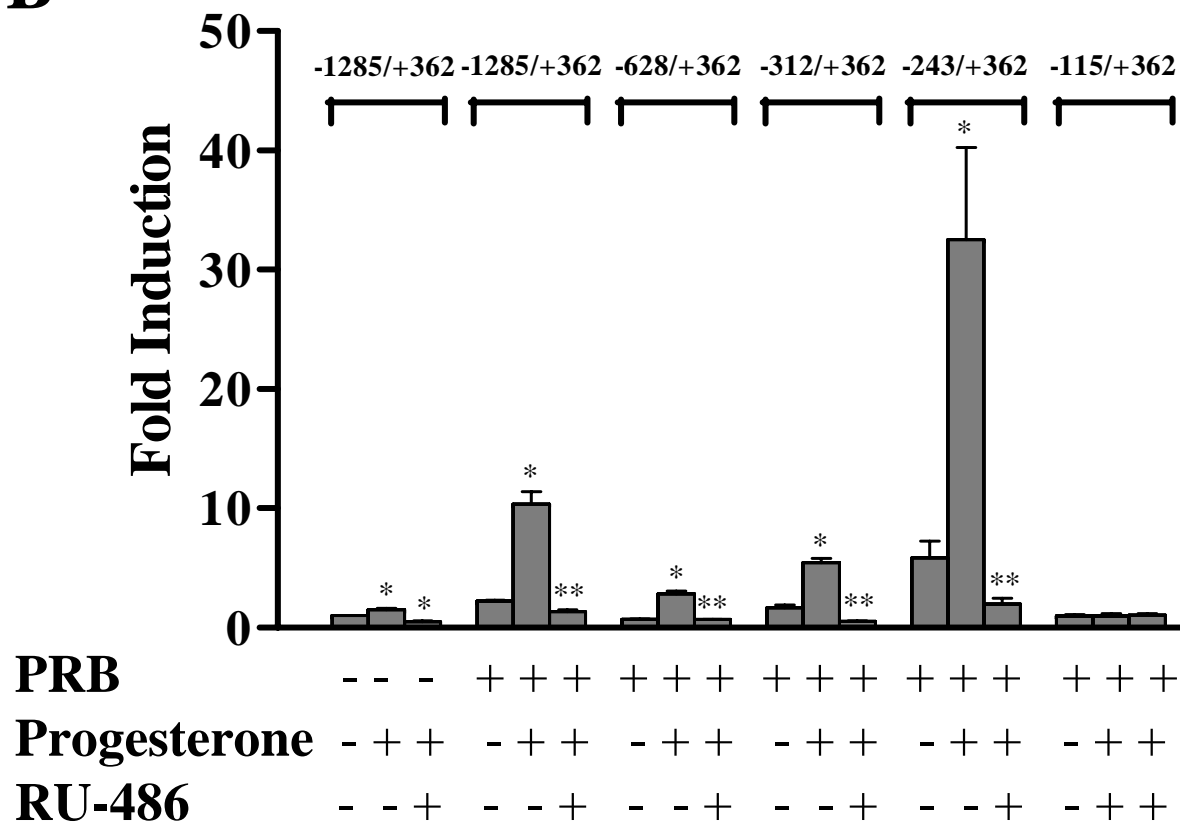
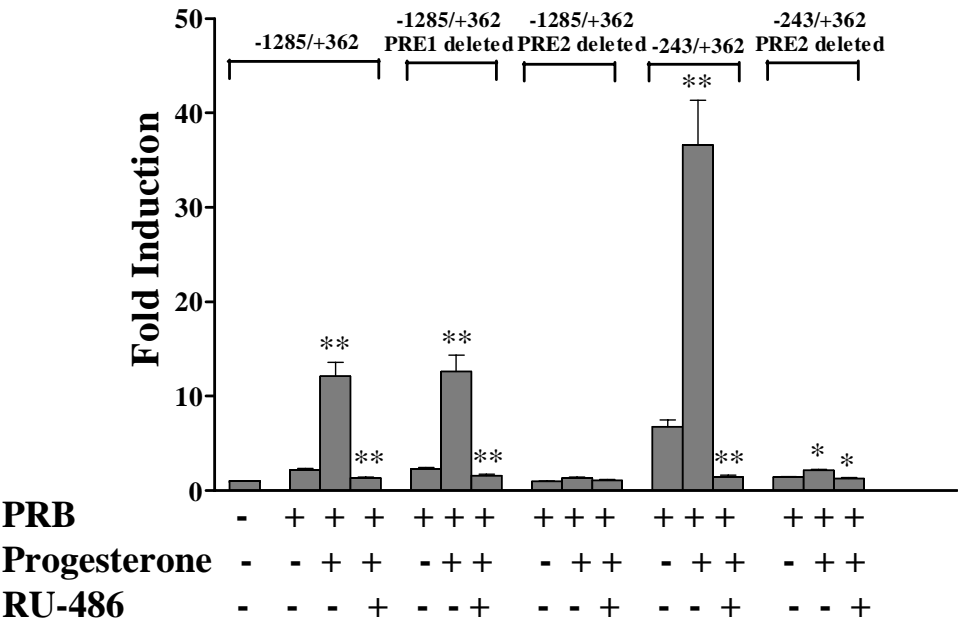


Figure 6

A

Consensus PRE		GGTACANNNTGTTCT	
Palindrome		AGAACANNNTGTTCT	
Putative PRE1	-1143	TGGCCAGGCTGGTCT	-1129
Putative PRE2	-187	ACGGCAGGGTGACCC	-173
Mut1 in PRE2	-187	ACGGTAGGGTGACCC	-173
Mut2 in PRE2	-187	ACGGCAGGGTTACCC	-173
Mut3 in PRE2	-187	ACGGTAGGGTTACCC	-173

B



C

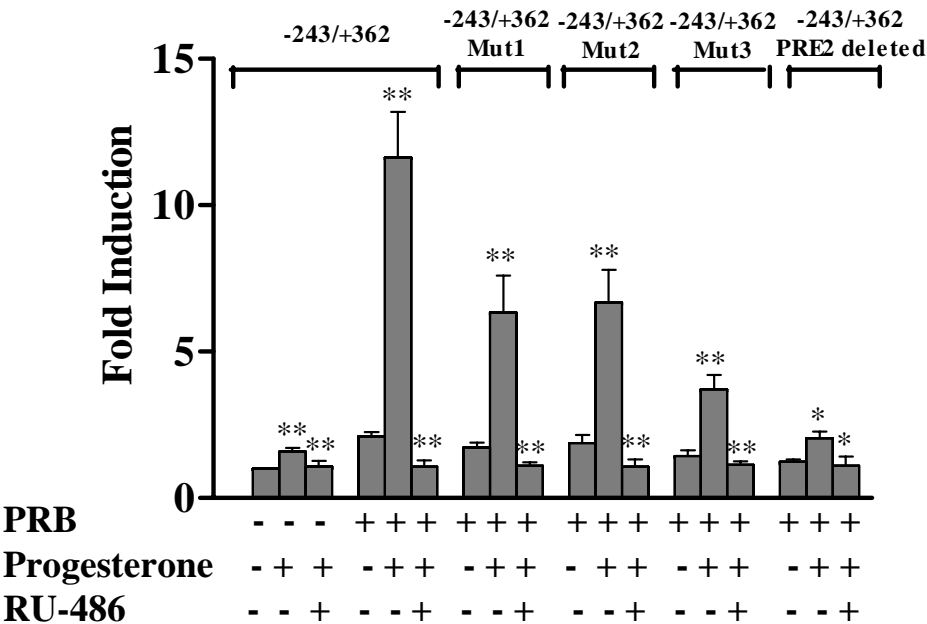


Figure 7

