

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

Honggang Wang, Eun-Woo Lee, Lin Zhou, Peter C. K. Leung, Douglas D. Ross, Jashvant D. Unadkat, and Qingcheng Mao

Department of Pharmaceutics, School of Pharmacy, University of Washington, Seattle, Washington (H.W., E.-W.L., L.Z., J.D.U., Q.M.), and Department of Life Science and Biotechnology, College of Natural Science, Donggeui University, Busan, Korea (E.-W.L.); Department of Obstetrics and Gynecology, University of British Columbia, Vancouver, British Columbia, Canada (P.C.K.L.); and University of Maryland Greenebaum Cancer Center and School of Medicine, and the Baltimore VA Medical Center, Baltimore, Maryland (D.D.R.)

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ABSTRACT

Breast cancer resistance protein (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 17 β -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 mifepristone (RU-486). Consistently, transcriptional activity of the *BCRP* promoter was induced 2- to 6-fold by 10⁻⁸ to 10⁻⁵ M progesterone in PRB-transfected cells. Progesterone had little effect on BCRP ex-

pression and activity and transcriptional activity of the *BCRP* promoter in PRA-transfected cells; however, cotransfection 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.

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).

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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 (Mao and Unadkat, 2005; Krishnamurthy and Schuetz, 2006). 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

ABBREVIATIONS: BCRP, breast cancer resistance protein; 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; EMSA, electrophoretic mobility shift assay; bp, base pair(s); ANOVA, analysis of variance; DMSO, dimethyl sulfoxide; PR, progesterone receptor; GF-120918, *N*-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide.

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 shown previously 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). 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 and of other steroid receptors when PRA itself is transcriptionally inactive (Vegeto et al., 1993; Giangrande and McDonnell, 1999).

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 that 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 Opti-MEM were from Invitrogen (Auckland, NZ). Phosphate-buffered saline (PBS) was from Invitrogen (Carlsbad, CA). High-performance liquid chromatography-grade DMSO was from Thermo Fisher Scientific (Waltham, MA). Charcoal/dextran-stripped fetal bovine serum was purchased from HyClone (Logan, UT).

Progesterone Immunoassay. BeWo cells were maintained in RPMI medium supplemented with 5% charcoal/dextran-stripped fetal bovine serum 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} to 10^{-3} M). Cells were then switched to fresh medium containing AGT at the same concentrations. After an additional 24 or 48 h of culture, progesterone concentrations in the medium were measured by an immunoas-

say using a progesterone enzyme-linked immunosorbent assay kit (Cayman Chemical Co., Ann Arbor, MI), according to the manufacturer's instructions. 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 to 18 h. Cells were then transfected with 0.4 to 3.2 $\mu\text{g}/10^5$ cells of the PRA expression vector or 0.4 to 1.6 $\mu\text{g}/10^5$ cells of the PRB expression vector using Lipofectamine Plus according to the manufacturer's instructions (Invitrogen). Six hours after transfection, cells were 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 were prepared as described previously (Wang et al., 2006).

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting. To detect BCRP protein, the protein samples of whole-cell lysates (20 μg each lane) were subjected to immunoblotting using BXP-21 (1:500 dilution), a BCRP-specific monoclonal antibody (Kamiya Biomedical, Seattle, WA), as described previously (Wang et al., 2006). To detect PR protein, the protein samples of whole-cell lysates (30 μg each lane) were subjected to immunoblotting by the use of an anti-PR polyclonal antibody that recognizes both PRA and PRB (Santa Cruz Biotechnology, Santa Cruz, CA), at a final concentration of 1 $\mu\text{g}/\text{ml}$. The donkey anti-rabbit IgG-horseradish peroxidase conjugate antibody (Santa Cruz Biotechnology) was used as the secondary antibody at 1:3000 dilution for PR detection. Human β -actin was detected as an internal control as described previously (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 Accumulation Assay. Transport studies using [^3H]mitoxantrone (MX) were performed to examine whether progesterone treatment affects MX efflux activity of the BeWo cells. In brief, BeWo cells were seeded at a cell density of 1.8×10^5 per well in six-well plates. Cells were transfected with 0.4 μg of plasmid per 10^5 cells of the PRA or PRB expression vector. Six hours 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 prewarmed PBS and incubated in 1 ml per well of Opti-MEM for 30 min. In inhibition experiments, cells were first preincubated with 10 μM FTC for 1 h. The experiments were then started by the addition of [^3H]MX (20 nM) in the presence and absence of 10 μM 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 μl) 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 (Bio-Rad, Hercules, CA). The intracellular MX concentrations were calculated on the basis of radioactivity associated with the cells and presented as picomoles of [^3H]MX per milligram of 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 (Institut National de la Santé et de la Recherche Médicale, Université 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, Basel, Switzerland). PRE1 or PRE2 was deleted, one at a time, from the -1285/+362 construct using polymerase chain reaction mutagenesis as described previously (Lee et al., 2006). The -243/+362 construct was used as template for polymerase chain reaction mutagenesis to generate point (Mut1 and Mut2) or double (Mut3) mutations in PRE2 and deletion of PRE2. The primers used for deletion of PRE1 were 5'-AGGCAGGGTTTACCATGTGCAAC-TCCTGGCCTCAAGT-3' and 5'-ACTTGAGGCCAGGAGTTCGACATGGTGAAACCTGCCT-3'. The primers used for deletion of PRE2 were 5'-CTTGTCCTGCGTGTCTAGCCCCGAGGGAGGG-3' and 5'-CCCTCCCTCGGGGCTAGACACGCAGGGACAAG-3'. The primers used for Mut1 were 5'-TGTCACGGTAGGGTGACCTA G-3' and 5'-CTAGGGTCACCTACCGTGACA-3'; for Mut2 were 5'-TGTCACGGCAGGGTTACCTAG-3' and 5'-CTAGGGTAACCTGCCGTGACA-3'; and for Mut3 were 5'-TGTCACGGTAGGGTTACCTAG-3' and 5'-CTAGGGTAACCTACCGTGACA-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/well in 24-well plates and cultured for 18 to 20 h. Cells were then transiently transfected with 0.15 μ g of the *BCRP* promoter luciferase reporter constructs, 0.4 to 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 hours 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. Nonradioactive electrophoretic mobility shift assay (EMSA) was performed using the Light-Shift chemiluminescent EMSA kit (Pierce, Rockford, IL). Nuclear protein extracts were prepared from BeWo cells using the NE-PER nuclear extraction kit (Pierce). Nonlabeled 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'-GTGT-CACGGCAGGGTGACCCTAGCC-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 $MgCl_2$, 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 nonspecific 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. Nondenaturing 5% polyacrylamide gels (Bio-Rad Hercules, CA) were pre-electrophoresed for 60 min in $0.5 \times$ Tris borate-EDTA buffer [0.089 M Tris base, 0.089 M boric acid, and 2 mM EDTA (disodium), pH 8.3 with boric acid] before loading the binding reaction samples. The samples were then electrophoresed in $0.5 \times$ Tris borate-EDTA buffer and transferred onto a positively charged nylon membrane (Hybond-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).

Statistical Analysis. Data were analyzed for statistical significance using Student's *t* test or one-way ANOVA followed by Neumann-Keuls test as indicated in the 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/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

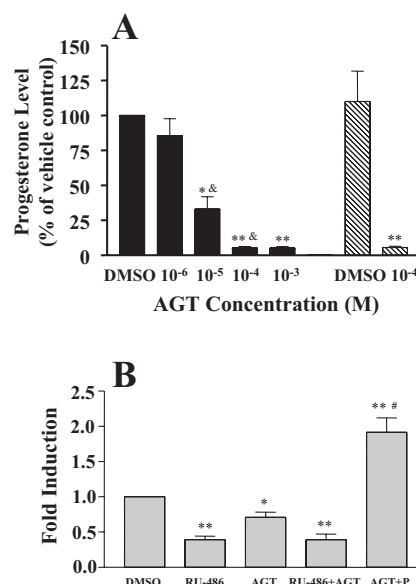


Fig. 1. Inhibition of progesterone secretion from BeWo cells by AGT and the effect on transcriptional activity of the *BCRP* promoter. A, BeWo cells were cultured in the absence (DMSO vehicle controls) or presence of varying concentrations (10^{-6} to 10^{-3} M) of AGT for 24 h. Cells were then switched to fresh medium containing AGT at the same concentrations, and cell culture was continued for an additional 24 h (■) or 48 h (▨). Progesterone production was measured by the progesterone immunoassay and is 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, versus the vehicle control; &, *p* < 0.05 versus the immediate adjacent group on the left by one-way ANOVA analysis followed by Neumann-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, versus the vehicle control; #, *p* < 0.01 versus the AGT treatment alone by one-way ANOVA analysis followed by Neumann-Keuls test.

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). Because the activity of a classic PR is probably 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 whether BCRP expression in BeWo cells is regulated by progesterone through a classic 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 classic 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 μ g of plasmid/ 10^5 cells. It is interesting that transfection of PRA with 1.6 or 3.2 μ g of plasmid/ 10^5 cells slightly decreased endogenous expression of PRB (Fig. 2A). Transfection of PRB with 0.4 μ g of plasmid/ 10^5 cells increased PRB expression to a similar level as the maximal level of PRA, and transfection with 1.6 μ g of 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 μ g of plasmid/ 10^5 cells of PRB. Transfection with 1.6 μ g of 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 nontransfected cells (Fig. 2B). However, transfection of PRA with 0.4 μ g of plasmid/ 10^5 cells showed little effect on BCRP expression compared with that in nontransfected cells (Fig. 2C). Transfection of PRA (1.6 μ g of plasmid/ 10^5 cells) even decreased rather than increased BCRP expression. These results suggest that progesterone probably induces BCRP expression in BeWo cells via PRB but not PRA. Progesterone induced BCRP expression even in nontransfected cells, which is probably due to endogenous expression of PRB in BeWo cells (Fig. 2A). In the above experiments, 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% 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 probably caused by the inhibition of progesterone production. A combination of 10^{-4} M AGT with 10^{-5} M RU-486 had no apparent effect on BCRP expression 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 compared with the AGT treatment alone. Such an effect would be expected from the *BCRP* promoter activity data in Fig. 1B, probably because immunoblotting is not as sensitive as the promoter reporter assay.

Effect of PRA and PRB Transfection on BCRP-Mediated MX Efflux Activity. To examine whether 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 an MX accumulation assay. MX, a high-affinity BCRP substrate, has been used as a model substrate to measure BCRP transport activity (Robey et al.,

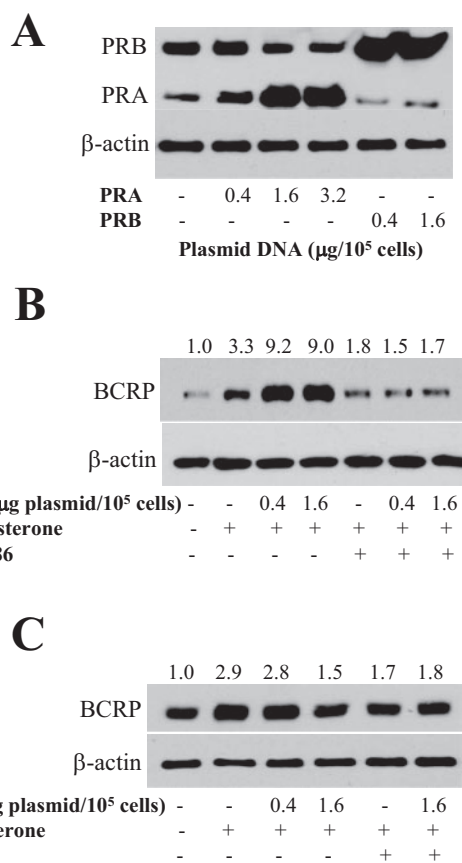


Fig. 2. Expression of PRA and PRB in BeWo cells, and the effect of progesterone on BCRP protein expression in PR-transfected cells. BeWo cells were transfected with the PRA or PRB expression vector with the amount of plasmid DNA as indicated. After transfection, cells were cultured with 10^{-4} M AGT 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.

2001; Gupta et al., 2004). Treatment of nontransfected cells with 10^{-6} M progesterone decreased MX accumulation by approximately 10 to 20% compared with the vehicle controls (Fig. 3, A and C). Transfection of PRA with 0.4 μ g of plasmid/ 10^5 cells did not influence the progesterone effect on MX accumulation (Fig. 3A), and transfection of PRA with 1.6 μ g of 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 accumulation by approximately 20 to 40% (Fig. 3, A and C). Cotransfection of PRA and PRB at 4:1 PRA/PRB ratio had no effect on MX accumulation (Fig. 3C). Because 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 nontransfected cells with 10^{-6} M progesterone resulted in an increase of FTC-inhibitable MX efflux by approximately 25%; however, the MX efflux was in-

creased by approximately 110% by 10^{-6} M progesterone in PRB-transfected cells (Fig. 3, B and C). 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 nontransfected control cells (Fig. 3B). Transfection of PRA with 0.4 μ g of plasmid/ 10^5 cells showed no additional progesterone stimulation of MX efflux compared with nontransfected and progesterone-treated cells (Fig. 3B), and transfection of PRB with 1.6 μ g of plasmid/ 10^5 cells even completely diminished the progesterone stimulation of MX efflux (Fig. 3D). As expected, cotransfection of PRA and PRB at 4:1 PRA/PRB ratio also completely abrogated the progesterone stimulation of MX efflux (Fig. 3D). Altogether, 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, 2- to 6-fold, by 10^{-8} to 10^{-6} M progesterone in PRB-transfected cells in a

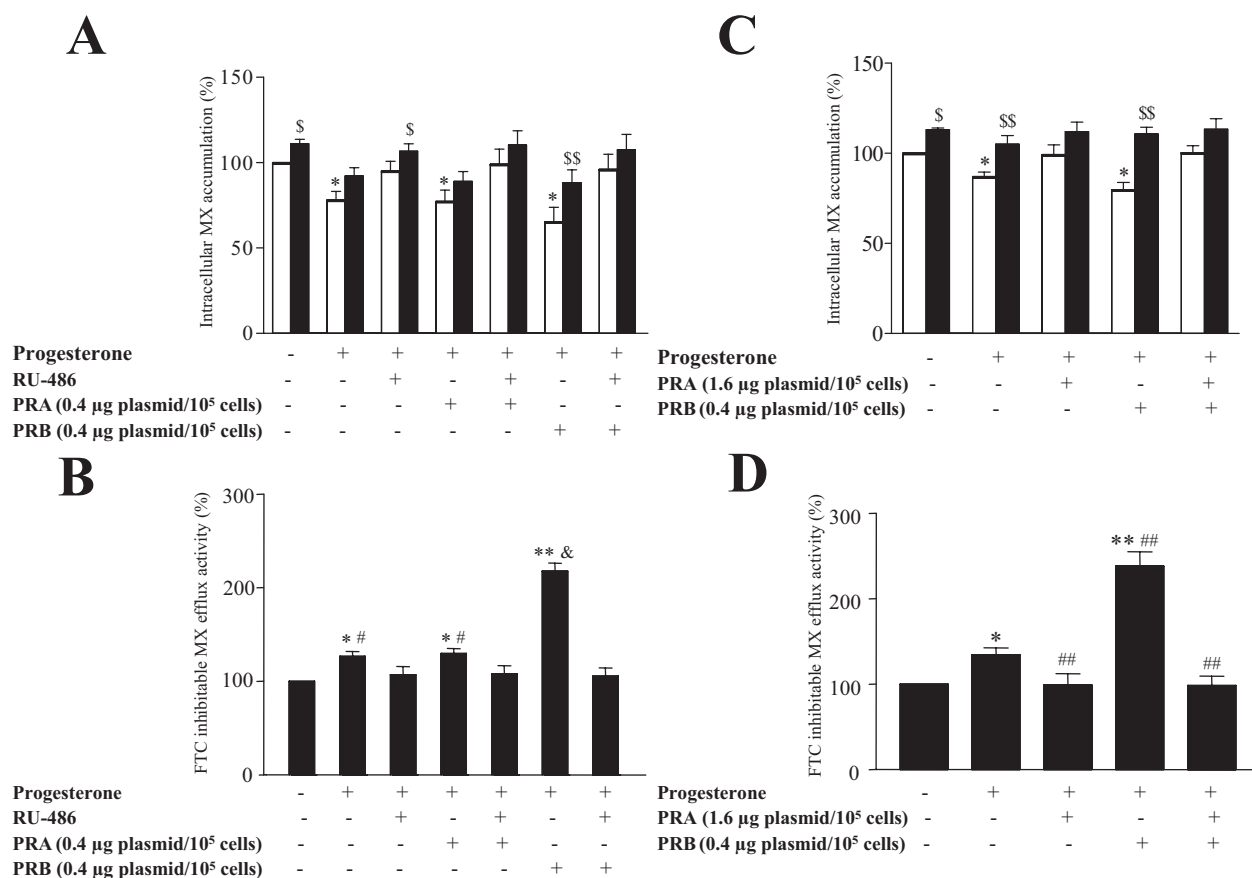


Fig. 3. Effect of progesterone on BCRP efflux activity in PR-transfected cells. In the MX efflux study, cells were transfected with PRA and/or PRB with the amount of plasmid DNA as indicated and treated with 10^{-6} M progesterone in the presence or absence of 10^{-5} M RU-486. The intracellular MX accumulation (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 **, $p < 0.01$, versus the respective vehicle control; #, $p < 0.05$, and &, $p < 0.01$, versus the immediately adjacent group on the right; \$, $p < 0.05$, and \$\$, $p < 0.01$, versus the immediately adjacent group on the left; ##, $p < 0.01$, versus the progesterone treatment group without PR transfection by one-way ANOVA analysis followed by Neumann-Keuls test.

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 were 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 of plasmid/ 10^5 cells of PRA, the transcriptional activity was slightly but significantly decreased by 18% compared with that in nontransfected cells, and the addition of progesterone again increased the activity by approximately 40%; the addition of RU-486 completely inhibited the induction (Fig. 4B). In cells transfected with 1.6 or 3.2 μ g of 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 seems that PRA is inactive in transcriptional activation of the *BCRP* promoter. Transactivation of the *BCRP* promoter by progesterone in nontransfected cells is probably mediated by endogenous PRB, and PRA seems to repress the PRB activity. To further confirm this conclusion, we examined the progesterone response in cells cotransfected 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. 4, A and C, are not the same, because the activities were normalized to different controls. When cells were cotransfected 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 nontransfected cells, transcriptional activity of the *BCRP* promoter in the -1285/+362 construct was induced more than 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 re-

gion -1285 to -115 bp of the *BCRP* promoter using the NUBIScan program (University of Basel). 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 whether 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 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 shown previously 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 classic 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 nanomolar 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

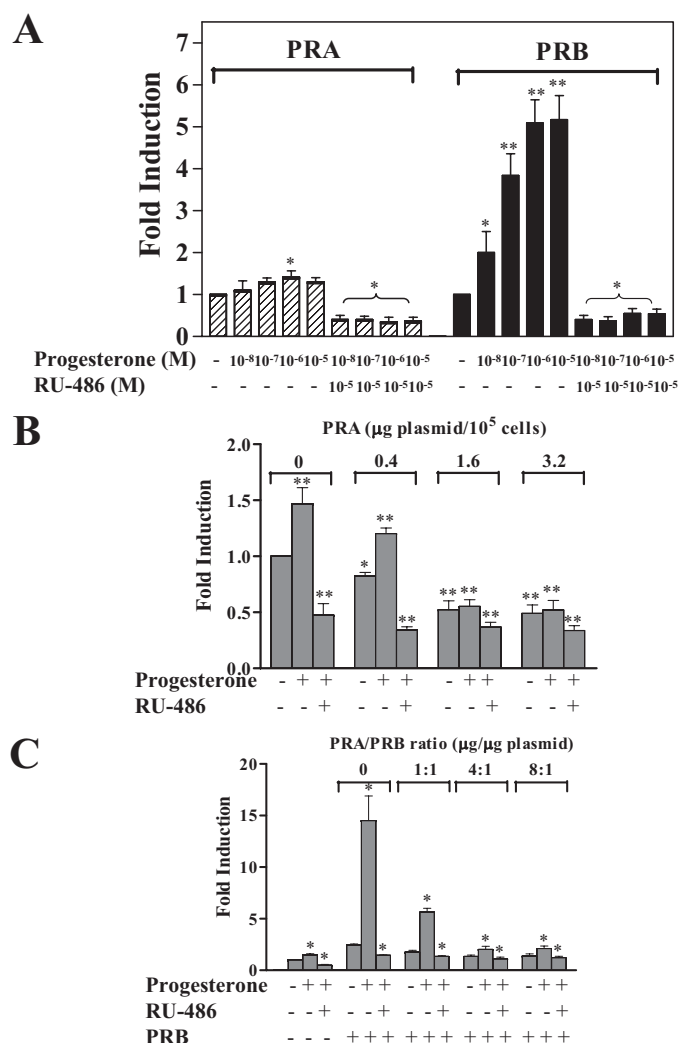


Fig. 4. Effect of PRA and/or PRB transfection on the progesterone response of transcriptional activity of the *BCRP* promoter. **A**, dose-dependent effect of progesterone on transcriptional activity of the *BCRP* promoter. BeWo cells were transfected with the -1285/+362 *BCRP* promoter-luciferase and β -galactosidase transfection control plasmids as well as the PRA (0.4 μ g of plasmid/10⁵ cells) or PRB (0.4 μ g of plasmid/10⁵ 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 **, $p < 0.01$, versus the vehicle control by one-way ANOVA analysis followed by Neumann-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 of plasmid/10⁵ 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 **, $p < 0.01$, versus 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 of plasmid/10⁵ cells) and PRA (0.4–3.2 μ g of plasmid/10⁵ 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

not inhibited (Wang et al., 2006). These results suggest that without inhibition of progesterone production, the activity of classic PRs is probably already saturated or close to saturation.

We then investigated the molecular mechanism by which *BCRP* expression is up-regulated 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

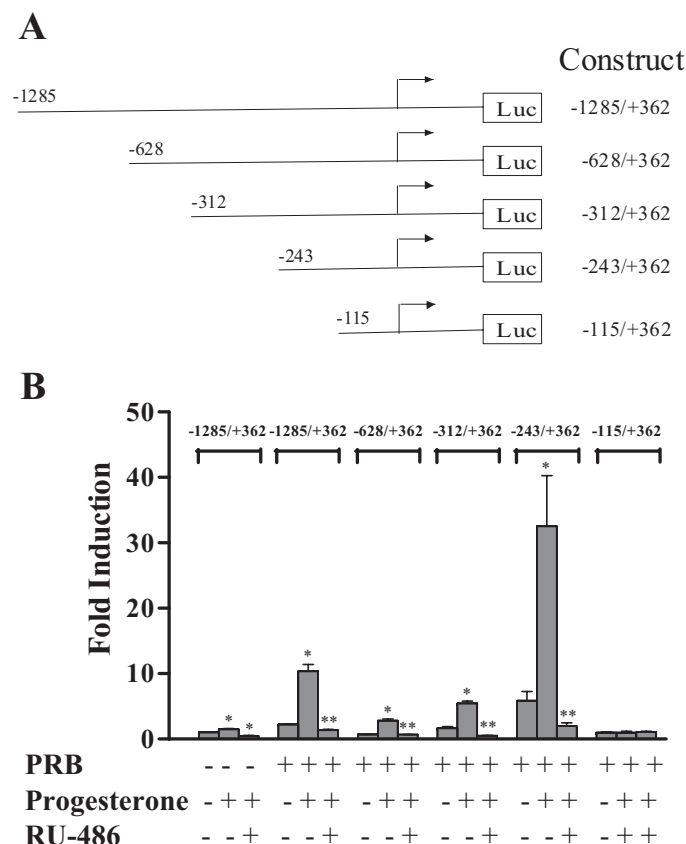


Fig. 5. Localization of putative PREs in the *BCRP* promoter region. **A**, schematic illustration of the *BCRP* promoter luciferase plasmids with varying length of the 5'-flanking region of the promoter. **B**, BeWo cells were transfected with the *BCRP* promoter luciferase and β -galactosidase transfection control plasmids with or without PRB transfection (0.4 μ g of plasmid/10⁵ 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 Fig. 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 **, $p < 0.001$, versus the immediate adjacent group on the left by Student's *t* test.

mean \pm S.E. from three independent experiments. Significant differences: *, $p < 0.01$ versus the immediate adjacent group on the left by Student's *t* test. In all of 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.

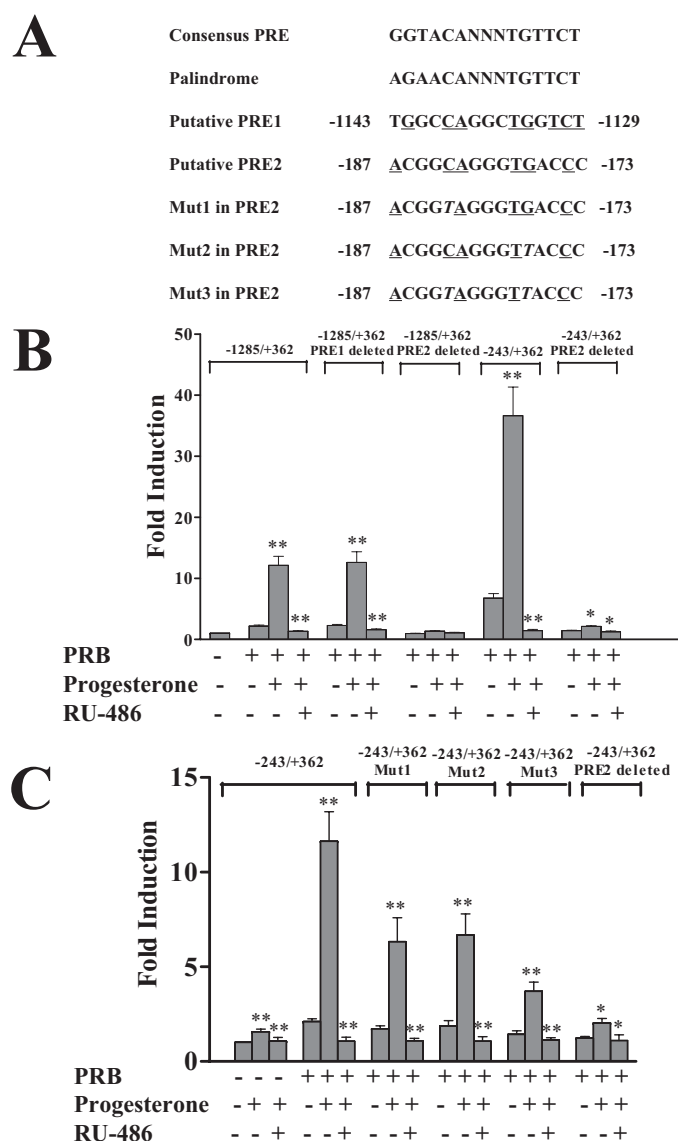


Fig. 6. Identification of a novel PRE in the *BCRP* promoter region between -243 and -115 bp upstream of the transcriptional start site. A, schematic illustration of two likely PREs (PRE1 and PRE2). For reference, a consensus PRE (Jantzen et al., 1987) and a functionally analyzed palindrome (Strahle et al., 1987) are listed. In the putative PREs, bases identical with 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 of 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 Fig. 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 **, $p < 0.001$, versus 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 of 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 Fig. 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

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 classic mechanism through progesterone-activated PRB that directly binds to the identified PRE and interacts with specific coactivators 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. (2004). That progesterone and estrogen receptors share the same or similar response elements is possible, because earlier studies suggest that the 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 affect each other in regulation of *BCRP*, when the two hormones are combined. The real situation could be very complex, because 17 β -estradiol can induce PRB expression (Flötotto et al., 2004; Wang et al., 2006) and down-regulate *BCRP* expression through posttranscriptional modification (Imai et al., 2005); on the other hand, PRA can repress the estrogen receptor activity (Giangrande and McDonnell, 1999). In BeWo cells, we showed previously that the 17 β -estradiol treatment alone down-regulated *BCRP* expression (Wang et al., 2006), presumably as a result of post-transcriptional modification, as demonstrated by 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 probably 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 classic PR mechanism, our previous study suggests that a nonclassic membrane-bound PR could also be involved in up-regulation 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 (Fig. 6, B and C). 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 *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. On the other hand, the identified PRE region may be composed 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

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 **, $p < 0.001$, versus the immediate adjacent group on the left by Student's t test.

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 (Cheng et al., 2001; Brayman et al., 2006). We found that PRB is a strong activator of transcription of the *BCRP* promoter, and PRA represses the PRB activity (Fig. 4, B and C). 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.

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. (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). In addition, 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 nontumoral liver; therefore, it remains to be determined whether progesterone could affect hepatic BCRP expression and hence BCRP-mediated biliary excretion of drugs. BCRP has been shown to be strongly induced in mammary glands during lactation and is responsible for milk secretion of BCRP substrate drugs (Jonker et al., 2005). PR isoforms are expressed in mammary glands and play a key role in pregnancy-associated mammary gland morphogenesis and tumorigenesis (Conneely et al., 2003). However, because plasma progesterone levels rapidly decrease to the normal levels as in nonpregnant women within 1 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. Because 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 hor-

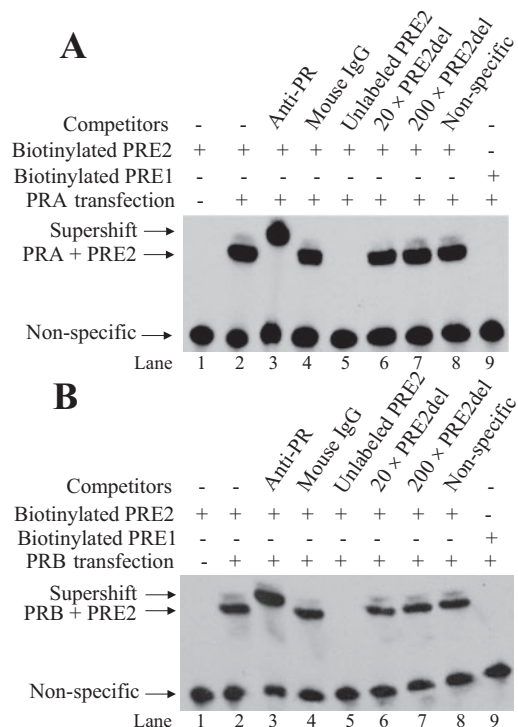


Fig. 7. Binding of PRA and PRB to the identified PRE in the *BCRP* promoter region. Nonradioactive electrophoretic mobility shift assay was performed as described under *Materials and Methods* using nuclear protein extracts isolated from BeWo cells transfected with PRA (A) or PRB (B). Specific DNA-protein 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 monoclonal antibody BXP-21 (used as control mouse IgG) (lane 4) was added. For competition analysis, a 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 nonspecific DNA-protein binding.

mones in human placenta during pregnancy and in cancer cells.

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Address correspondence to: Dr. Qingcheng Mao, Department of Pharmaceutics, School of Pharmacy, Box 357610, University of Washington, Seattle, WA 98195-7610. E-mail: qmao@u.washington.edu