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Preferential Stimulation of Human Progesterone Receptor B Expression by Estrogen in T-47D Human Breast Cancer Cells*

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Human progesterone receptor (PR) expression is controlled by two promoter regions giving rise to transcripts encoding PR A and B proteins. It is unknown whether estrogen and progesterone, the major physiological modulators of PR expression, exert their effects equally on the PR promoters. The aim of this study was to analyze estrogen and progestin effects on PR promoters, PR-encoding transcripts, and PR A and B proteins in T-47D human breast cancer cells. The progestin ORG 2058 caused a prolonged decrease in transcription of the PR gene and also abrogated estrogen stimulation of PR transcription. Estradiol (E2) treatment increased the activity of the B but not the A promoter transfected into T-47D cells. ORG 2058 had no effect on the basal or E2-stimulated activity of either promoter. E2 caused a preferential increase in transcripts derived from promoter B, whereas progestins decreased the levels of all PR transcripts. E2 preferentially increased the concentration of the PR B protein and caused a decrease in the PR A/B ratio. This demonstration that estrogen and progestin independently control the synthesis of transcripts arising from the PR promoters and that estrogen alters the cellular PR A/B ratio provides possible mechanisms underlying the cell and tissue specificity of PR regulation.

Progesterone plays a major role in mammalian reproductive biology, including development of the normal mammary gland and expression of its differentiated function during pregnancy, and promotion of uterine differentiation and preparation for implantation in pregnancy (1). Progesterone effects are mediated via the nuclear progesterone receptor (PR)[1] and control of progesterone action is achieved largely although not exclusively by control of the concentration of PR. The major physiological modulators of PR concentration are the ovarian hormone 17β-estradiol (E2), and progesterone itself, which binds to PR in order to exert progestational effects but also participates in regulation of its own receptor.

Understanding of PR regulation derives largely from detailed and elegant studies in the mammalian uterus and in breast cancer cells, which led to the generally accepted view that estrogen increases and progestins decrease PR expression (2, 3). However, the advent of monoclonal antibodies to the steroid hormone receptors and the examination of PR expression at an individual cell level have shown that PR regulation may be more complex than previously suspected. Regulation of PR in the uterus is a cell-specific event, and PR regulation in the normal breast *in vivo* may be different from its regulation in the uterus and in breast cancer cells. In the endometrium, immunohistochemical evidence supports the view that the cyclical effects of estrogen and progesterone are mediated by estrogen stimulation of PR and progesterone down-regulation of both PR and estrogen receptor (ER) (4–7). However, progesterone down-regulation of PR is not a uniform effect in the uterus, as myometrial and stromal PR levels are not decreased by progesterone and persist during the luteal phase of the menstrual cycle (4–7). Furthermore, circulating progestins cause a decrease in ER concentration in the normal breast during the menstrual cycle, as observed in the endometrium, but no decrease of PR (8–11). PR is expressed in the breast at similar concentrations throughout the menstrual cycle in normal women, and there is no evidence that its synthesis is under estrogen control in that tissue (9–11). It is possible that differences in the regulation of PR by estrogen and progestins in the breast and endometrium reflect the differential requirement for persistence of progesterone action in those tissues (1) and may be a consequence of expression of distinct PR isoforms.

The complexity of PR regulation *in vivo* is paralleled by evidence of complexity in the molecular mechanisms underlying PR expression. The expression of human PR is controlled by two promoters that direct the synthesis of mRNA transcripts originating from two clusters of transcription start sites and coding for the A and B PR proteins (12). The functional activities of PR A and B differ in a cell type-, promoter-, or ligand-specific manner (13–15). In some cases, the N-terminally truncated PR A can act as a repressor of the activity of PR B (16, 17) and more generally of the activity of other members of the nuclear receptor family (18, 19). PR A and B also differ in their ability to inhibit the activity of ER, in a way that is both promoter- and cell type-specific (19–22). The functional implications of N-terminal isoforms are not restricted to PR; androgen receptor mRNA isoforms differing within the region coding for the N-terminal portion of the protein have been described which are either developmentally regulated or are expressed in differentiated tissues in *Xenopus laevis* (23). In *Drosophila melanogaster*, three isoforms of the ecdysone receptor with

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[1] The abbreviations used are: PR, progesterone receptor; E2, estradiol; ER, estrogen receptor; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; SFCS, charcoal-stripped fetal calf serum; PRLR, prolactin receptor.

different N-terminal regions have been documented, which are expressed in different combinations during metamorphosis and may be required to elicit different metamorphic responses (24).

Regulation of PR concentration by estrogen and progestins is accompanied by increases and decreases, respectively, in PR mRNA levels, which are reflected in alterations in cellular PR levels (25–33), but the promoter specificity of these effects on human PR is not known. In the rabbit, estrogenic stimulation and progestin inhibition of PR gene expression take place via the same region in the 5′-untranslated region of the rabbit gene (34). An estrogen-responsive element has been defined in this region, which binds to ER in gel shifts and mediates E2 induction in transfections. However, PR does not bind to this region even though it suppresses E2 induction in the presence of the progestin R5020 (35).

Given the complexity of human PR regulation in target tissues *in vivo* and the complexity of regulation of PR gene expression via its two promoters, as well as the emerging evidence of marked functional differences in PR A and B activity, it is essential to gain a deeper insight into the mechanisms that control the regulation of PR by its major physiological modulators, estrogens and progestins. It is not known whether these steroids exert equivalent effects on the two PR promoters and lead to equivalent alterations in the levels of PR A and B proteins in progestin target cells, or whether progestin abrogation of estrogen stimulation of PR expression is mediated via the same mechanism. This question is critical to understanding the *in vivo* complexity of PR expression and regulation in the breast and endometrium. This study addresses the PR isoform specificity of the actions of estrogen and progestin in human breast cancer cells, which are physiological targets of estrogen and progestin action.

EXPERIMENTAL PROCEDURES

Materials—Materials were obtained from the sources previously listed (36). [α-^{32}P]UTP was an Amersham Australia (North Ryde, Sydney, Australia) product. Plasmids bearing the progestin-responsive mouse mammary tumor virus long terminal repeat linked to chloramphenicol acetyltransferase (pMSG-CAT) and bacterial β-galactosidase sequences (pCH110) were obtained from Pharmacia LKB Biotechnology (North Ryde, Sydney, Australia); pSG5-hPR1, pAER encoding human ER, PR(+464,+1105), and PR(−711,+31) in minimal pBLCAT vectors were gifts from Dr. Pierre Chambon, Strasbourg, France; and fatty acid synthetase cDNA was a gift from Dr. Henri Rochefort, Montpellier, France. The prolactin receptor (PRLR) cDNA and effects of progestins on PRLR have been described previously (37). Human fibroblast β-actin (38), α-tubulin (bα1) (39), and 36B4 (40) cDNAs have been described previously. The hPR(+464,+742)-pGEM construct was made by excision of the *Bgl*II-*Bsp*HI fragment of PR(+464,+1105)-CAT and blunt-end ligation into the *Sma*I site of pGEM-7Zf(+) (Promega Corp., Ryde, Australia). The hPR(+814,+1194)-pGEM construct was made in a similar manner using a *Sau*3AI fragment excised from pSG5-hPR1. Both constructs were verified by DNA sequencing. Non-overlapping probes were constructed due to the multiplicity of transcripts of similar sizes arising from both promoters observed when a single probe was employed in S1 nuclease protection assays (not shown).

Cell Culture—T-47D cells (41) were supplied by E. G. and G. Mason Research Institute, Worcester, MA and were cultured as described previously (36, 42, 43). Cells were negative for mycoplasma contamination as determined using the Gen-Probe rapid detection system (Gen-Probe Inc., San Diego, CA). CHO cells were a gift of Dr. Nigel Morrison, Garvan Institute of Medical Research (New South Wales, Australia), and were maintained in DMEM-Ham's medium supplemented with 10% fetal calf serum in vented flasks in a 5% CO_2 incubator. HeLa cells were a gift of Dr. Roger Reddel, Children's Medical Research Institute (New South Wales, Australia) and were maintained in DMEM medium supplemented with 10% fetal calf serum in vented flasks in a 5% CO_2 incubator.

T-47Dsd cells were prepared by maintaining T-47D cells for several weeks in phenol red-free RPMI 1640 medium containing 10% charcoal-stripped fetal calf serum (SFCS) and supplemented as described (42, 43), except that 1 μg/ml insulin was used. T-47Dsd cells proliferated with doubling times that were significantly slower than the parent line and demonstrated increased sensitivity to estradiol as measured by the lower estradiol concentrations required to increase gene expression of estrogen-sensitive genes (44).

Isolation and Analysis of RNA—RNA was isolated by the guanidinium isothiocyanate-cesium chloride method, and Northern analysis was carried out as described (36) except that cDNA probes were labeled by random priming using the Amersham Multiprime DNA labeling system. Autoradiograms were analyzed as described previously (36).

Nuclear RNA for S1 nuclease protection assays was prepared from nuclei that were isolated from freshly harvested T-47Dsd cells using Nonidet P-40 lysis buffer as described for nuclear run-on analysis (45) and solubilized in guanidinium isothiocyanate. RNA was isolated by the guanidinium isothiocyanate-cesium chloride method as described for total RNA.

Transcriptional Analysis—PR gene transcription was measured using modifications of the method of Greenberg and Ziff (45) as described previously (46). Labeled run-on transcripts were hybridized to nitrocellulose filters onto which 5 μg each in duplicate of pSG5-hPR1, PRLR, α-tubulin, and pUC12 cDNAs had been blotted and fixed by UV radiation. Volumes of labeled transcripts were chosen such that each sample per time point contained equal radioactivity. Filters were washed (45), dried, and exposed to x-ray film. Autoradiograms were analyzed densitometrically, and the mean heights of duplicate PR RNA signals on filters bearing treated samples were normalized for the mean heights of the duplicate α-tubulin signals on the same filter then expressed as a percentage of the similarly normalized PR RNA signal on control filters.

Gene Transfection—T-47D cells were plated into 150-cm^2 flasks (2.5–3.0 $\times$ 10^6 cells/flask) in RPMI 1640 phenol red-free medium containing 5% FCS 3 days prior to transfection. CHO cells were plated the day prior to transfection in 25-cm^2 flasks (5 $\times$ 10^5 cells/flask) in DMEM-Ham's medium supplemented with 10% SFCS. HeLa cells were were plated the day prior to transfection in 25-cm^2 flasks (3 $\times$ 10^5 cells/flask) in DMEM- medium supplemented with 10% FCS and changed on the day of transfection to DMEM medium supplemented with 5% SFCS. Cells were transfected using the calcium phosphate precipitation method (47) as described previously (46), with PR B-CAT (T-47D cells, 20 μg/flask; CHO cells, 5–20 μg/flask; HeLa cells, 5 μg/flask), PR A-CAT (T-47D cells, 20 μg/flask; CHO cells, 5–20 μg/flask; HeLa cells, 5 μg/flask), pAER (T-47D cells, 20 μg/flask; CHO cells, 5 μg/flask; HeLa cells, 5 μg/flask), and pCH110 (T-47D cells, 10–20 μg/flask; CHO cells, 1 μg/flask; HeLa cells, 3 μg/flask). Where indicated pMSG-CAT (40 μg/flask) and pCH110 (10–20 μg/flask) were co-transfected into separate flasks of T-47D cells. T-47D and CHO cells were subjected to osmotic shock 3–4 h after transfection, and exposure of T-47D cells to the DNA continued for 18 h thereafter as described (46), whereupon transfected T-47D cells were harvested from the 150-cm^2 flasks and replated (1 $\times$ 10^6 cells/flask) into triplicate 25-cm^2 flasks for treatment. DNA precipitates were removed from CHO cells after osmotic shock and from HeLa cells after 4 h. Triplicate 25-cm^2 flasks of transfected cells were treated, harvested, and enzyme activities measured as described previously (46).

Preparation of ^{32}P-Labeled Antisense RNA Probes—The hPR (+464,+742)-pGEM construct, which hybridizes to PR B promoter initiated transcripts only, and hPR(+814,+1194)-pGEM, which detects all PR mRNA species, were linearized at the 5′ end of each insert using *Eco*RI. Antisense RNA probes were synthesized from 1 μg of each linearized template with SP6 RNA polymerase in the presence of [α-^{32}P]UTP using a Riboprobe Gemini II kit (Promega Corp., Ryde, Australia). The control probe 36B4 (40) was prepared by transcription from *Hin*dIII-linearized template using T7 RNA polymerase. After transcription, the DNA templates were digested using RQI DNase I (Promega Corp) and probes were purified by phenol-chloroform extraction and ammonium acetate precipitation to remove unincorporated nucleotides.

S1 Nuclease Protection—T-47Dsd cells were plated as described in the legend to Fig. 5 and treated for various times with steroids or vehicle then harvested for preparation of nuclear RNA. The S1 nuclease protection assay was carried out essentially as described previously (48). Briefly, 5 $\times$ 10^5 cpm PR-B promoter-specific or PR total mRNA probe plus 2 $\times$ 10^5 cpm 36B4 control probe were co-precipitated with 20 μg of nuclear RNA, rinsed with ethanol, and dried under vacuum. The samples were redissolved in hybridization buffer, denatured at 80 °C for 5 min, and then incubated overnight at 52 °C. After hybridization samples were treated with 4000 units of S1 nuclease for 1 h at 42 °C. Digestion was stopped, and samples were purified by phenol-chloroform extraction, followed by two rounds of ammonium acetate precipitation. Final precipitates were dried under vacuum and resuspended in 10 μl

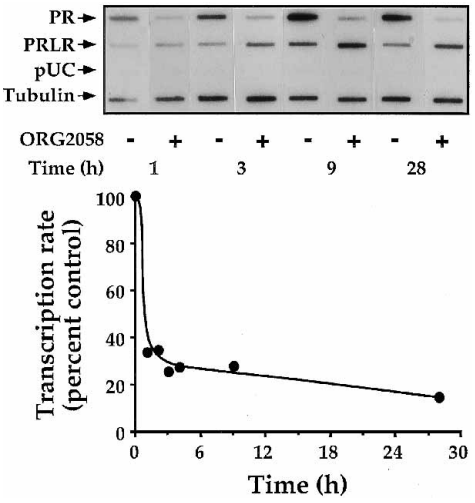

FIG. 1. **Time course of progestin effect on PR gene transcription rate**. T-47D cells were passaged twice in RPMI 1640 + 5% charcoal treated fetal calf serum (SFCS), then changed to RPMI 1640 + 1% SFCS 24 h before treatment. The cells were treated with either 10 nM ORG 2058 (+) or vehicle (−) for the indicated times. PR, PRLR, and α-tubulin gene transcription rates were estimated in duplicate using the nuclear run-on technique. The plasmid pUC12 was used as a negative control. The effect of ORG 2058 on PR gene transcription rate was quantified by densitometry and is shown as a percentage of control, corrected for α-tubulin transcription rate. The data between 0 and 9 h are representative of four to five experiments. The 28-h time point represents a single determination in duplicate.

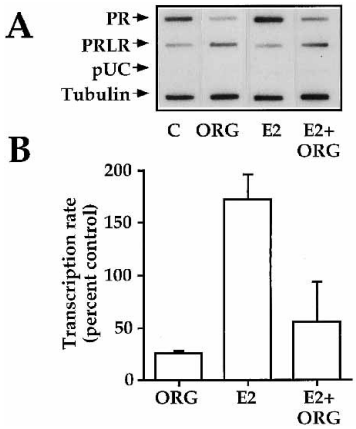

FIG. 2. **Effect of estrogen and progestin co-treatment on PR gene transcription.** T-47D cells cultured as described in Fig. 1 were treated with 10 nM 17β-estradiol, 10 nM ORG 2058, their combination, or vehicle for 1 or 3 h prior to measurement of PR, PRLR, and α-tubulin gene transcription rates by the nuclear run-on technique. The effects of these agents on PR gene transcription rate was the same at 1 h and 3 h, so the data were quantified by densitometry, 1-h and 3-h data pooled, and the results described in *panel B* as a percentage of control, corrected for α-tubulin gene transcription rate. Data are expressed as the mean ± S.D. (*E2*) of 3 or range (*ORG*, *ORG+E2*) of two experiments. *p* value, E2 *versus* control, 0.007.

of loading buffer containing 90% formamide, then separated on 10% polyacrylamide gels containing 8 M urea and visualized by autoradiography. PR mRNA concentrations were estimated by densitometric analysis and corrected for loading using 36B4.

Immunoblot Analysis of PR—T-47Dsd cells were harvested, after treatment for 24 h with E2 (0.001–10 nM) or vehicle, and stored as described (26), then thawed on ice in PEMTG buffer (26) containing 0.4 M KCl and a protease inhibitor mixture, which resulted in the indicated final concentrations of the inhibitors (all obtained from Sigma): phenylmethylsulfonyl fluoride (0.5 mM), pepstatin A (1.4 μM), bacitracin (100 μg/ml), benzamidine (25 mM), leupeptin (86 μM), and aprotinin (77 μg/ml). Cytosol extracts were prepared and transferred to nitrocellulose as described (26), except that the blocking buffer also contained 5% (w/v) skim milk. Blots were incubated with monoclonal antibodies against human PR (hPRa 6 and 7, Ref. 49) and goat anti-mouse immunoglobulins linked to horseradish peroxidase (Bio-Rad), after which PR immunoreactivity was revealed using a chemiluminescent method (ECL, Amersham). The relative intensity, in the linear range of the film, of the immunoreactive bands was calculated after densitometric scanning of x-ray films, followed by integration of the areas under the peaks corresponding to PR A and B. Results are expressed as the percentage of the immunoreactivity in control samples.

RESULTS

Progestin and Estrogen Modulation of PR Transcription Rate—Nuclear run-on transcription assays included both positive (tubulin) and negative (pUC) controls as well as a control gene (prolactin receptor, PRLR) whose regulation by estrogen and progestins was known to be different from PR; PRLR expression is stimulated by progestins but unaffected by estrogen in T-47D cells (37). Initial experiments focussed on a detailed analysis of the time course of PR transcription rate inhibition by the synthetic progestin ORG 2058. The transcription rate of the PR gene was markedly decreased upon short term progestin exposure (Fig. 1), as previously shown (26). There was an apparent small increase in basal transcription rate over time, as evidenced by the increased transcription rate of PR, PRLR, and tubulin (Fig. 1, vehicle-treated samples). This is probably due to two factors: culture conditions and nuclear run-on experimental design. Cells underwent a change to medium containing stripped fetal calf serum 24 h prior to treatment and for PR at least this leads to a slow increase in basal expression over time.[2] In addition, although run-on samples were normalized to contain the same radioactivity within each time point, there were some differences between time points; the 9-h and 28-h time points in particular contained more radioactivity than the 1-h and 3-h points. Nevertheless, as transcription rate was determined by normalization to the tubulin signal within each time point, these factors did not affect the overall results.

A detailed analysis of the long term effects of the progestin on PR transcription rate revealed that inhibition of >60% was apparent within 1 h, had reached 72% at 9 h of treatment (Fig. 1), and was sustained to 28 h. Exposure to estradiol significantly increased PR transcription rate (171 ± 24% (mean ± S.D.), $n = 3$, $p = 0.007$, Fig. 2), and this effect was detectable within 1 h of treatment. Progestin was able to abrogate the estradiol-mediated increase in PR transcription rate to levels that were below control (56 ± 38% (mean ± range), $n = 2$) but not as low as progestin treatment alone (25 ± 3% (mean ± S.D.), $n = 3$) (Fig. 2).

Differential Effect of Estrogen on PR Promoter-CAT Constructs—Having shown that estrogen increased PR transcription rate and that progestin caused a prolonged inhibition of PR gene transcription and abrogated the stimulatory estradiol effect, it was important to investigate whether these transcriptional effects were equally mediated via the two PR promoter regions. PR-B (−711/+31) and PR-A (+464/+1105) promoters in minimal pBLCAT vectors were transiently expressed in T-47D cells, and the effects of estradiol, ORG 2058, and their combination on reporter gene activity examined. Endogenous ER levels in T-47D cells were supplemented by transfection of the pAER plasmid encoding human ER and estrogen responsiveness of transfected cells demonstrated in control experiments using the estrogen-responsive pA2-tk-CAT construct (not shown).

The basal CAT activity arising from both PR promoter constructs was similar, as measured by the ratio of CAT activity derived from PR-B and PR-A in vehicle-treated samples (A/B ratio 0.91 ± 0.55 (mean ± S.D.), $n = 3$, data not shown). E2

[2] E. McGowan and C. L. Clarke, unpublished observations.

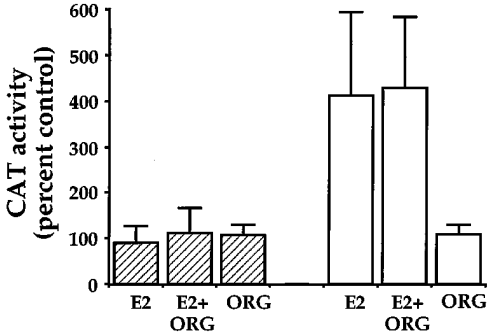

FIG. 3. **Estradiol and ORG 2058 effects on PR promoter activity in T-47D cells.** The PR A (*hatched bars*) and PR B (*open bars*) promoters linked to CAT were transiently transfected with the β-galactosidase expression vector pCH110 and a plasmid encoding the human estrogen receptor (pAER) into T-47D cells. Twenty-four hours later, cells were treated with 17β-estradiol (10 nM), ORG 2058 (10 nM), their combination, or vehicle and harvested 44 h thereafter for measurement of CAT and β-galactosidase activity. CAT activity is expressed as a percentage of control and is corrected for β-galactosidase activity. The data shown are the mean ± S.D. of four (PR B promoter) or three (PR A promoter) experiments. CAT activity in E2-treated *versus* control PR B samples: $p = 0.014$.

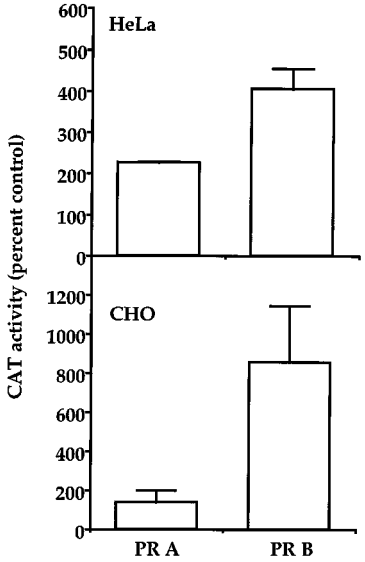

FIG. 4. **Regulation of PR promoter activity in CHO and HeLa cells.** CHO and HeLa cells were transfected with either the PR A or PR B promoter CAT construct, plus pAER and pCH110 as described under "Experimental Procedures." Transfected cells were treated with 10 nM 17β-estradiol or vehicle and harvested 40 h later to measure CAT and β-galactosidase activity. The effect of estradiol on CAT activity from the two promoters in each cell line is shown as a percentage of the vehicle-treated control. Results for CHO cells are the mean ± S.D. of four separate experiments: p value, E2 *versus* control, 0.002. The HeLa results are calculated from duplicate determinations and are representative of two separate experiments.

treatment caused a statistically significant increase in PR-B-CAT activity (411 ± 182% control (mean ± S.D.), $n = 4$, $p = 0.014$) but had little or no effect on PR-A-CAT activity (90 ± 36% control (mean ± S.D.), $n = 3$) (Fig. 3) at any plasmid concentration used (data not shown). The E2-mediated increase in PR-B-CAT activity was abolished upon co-transfection of PR-A-CAT (data not shown), in agreement with PR-A inhibition of ER activity on other estrogen-responsive sequences (19–21). There was little or no effect of the steroids used on transfection efficiency or β-galactosidase activity in T-47D cells (data not shown). There was no effect of the steroids used on CAT activity of the pBLCAT8+ vector alone (data not shown).

These data obtained in T-47D cells were at odds with the demonstration in HeLa cells that both promoter constructs were inducible by estrogen (12). Therefore, promoter constructs were co-expressed with ER in CHO and HeLa cells and the effect of E2 treatment measured. The basal expression of PR-A promoter was greater than PR-B promoter in CHO cells, measured as indicated above (A/B ratio 4.17 ± 0.08, $n = 3$, data not shown) and E2 treatment increased activity of both promoters; E2 treatment caused a significant increase in PR-B-CAT activity (858 ± 284% (mean ± S.D.), $p = 0.002$, $n = 4$) and a modest induction of PR-A-CAT (140 ± 58% (mean ± S.D.), $p = 0.217$, $n = 4$) (Fig. 4). The difference in the estrogen effect on A and B promoters was significant ($p = 0.003$). In HeLa cells, the basal activity of both promoters was similar and was increased by estrogen treatment (PR-B-CAT: 406 ± 48% control; PR-A-CAT: 227 ± 3% control (mean ± range), $n = 2$) (Fig. 4), in agreement with previous observations in HeLa cells (12). Taken together, these data showed that the PR-B promoter was inducible by estrogen in all cells tested, but that there was a cell-specific effect of estrogen on the PR-A promoter, which was estrogen-induced in CHO and HeLa cells but unaffected by estrogen in progestin-responsive T-47D cells.

Progestin Effect on PR Promoter Constructs—ORG 2058 had no effect on the basal expression in T-47D cells of either promoter construct (PR-B-CAT, 108 ± 20%; PR-A-CAT, 107 ± 21% (mean ± S.D.), $n = 4$), nor did it abrogate the E2 effect on PR-B-CAT (428 ± 156% (mean ± S.D.), $n = 4$) (Fig. 3). To eliminate the possibility that the failure to see progestin effects on the promoter constructs was due to the transfection procedure having affected progestin responsiveness in these cells, known end points of progestin action were measured in transfected cells. Transfection of the progestin-responsive plasmid pMSG-CAT and treatment with ORG 2058 resulted in strong progestin inducibility of this plasmid (not shown), indicating that cells transfected with equivalent amounts of DNA to that used in the experiments with the promoter constructs were still progestin-responsive. More generally, Northern analysis of total RNA showed that progestin-mediated down-regulation of PR and up-regulation of the progestin-dependent fatty acid synthetase mRNA were equivalent in transfected and control cells (not shown). Although transfected cells would form a minority of cells from which total RNA was isolated, these experiments indicated that general progestin responsiveness of T-47D cells was not diminished by the transfection procedure, demonstrated that PR was fully functional in transfected cells, and supported the conclusion that ORG 2058 had no effect on the PR promoter constructs in T-47D cells.

Estrogen Preferentially Increased Levels of Promoter B-derived PR Transcripts—The above data, obtained using transfection of minimal promoters in PR+ cells, showed that sequences mediating response to estrogen reside within promoter B and not promoter A. However, effects seen using minimal promoters may not reflect responses in the intact gene, and accordingly the effect of estrogen and progestin on transcripts derived from each promoter was determined by nuclease protection of nuclear RNA. These experiments were carried out in T-47Dsd cells, which were selected by culture in steroid-depleted medium and displayed increased sensitivity to estrogen (44). Two non-overlapping antisense RNA probes that recognized promoter B-derived transcripts(probe B: +464/+742) and all transcripts (probe T: +814/+1194), respectively, were prepared (Fig. 5*A*) and used in nuclease protection assays of nuclear RNA prepared from treated T-47Dsd cells. Both probes were verified by sequencing. Probe B was also verified by hybridization with blots of poly(A)$^+$ RNA; probe B recognized only transcripts (not shown) with mobilities corresponding to

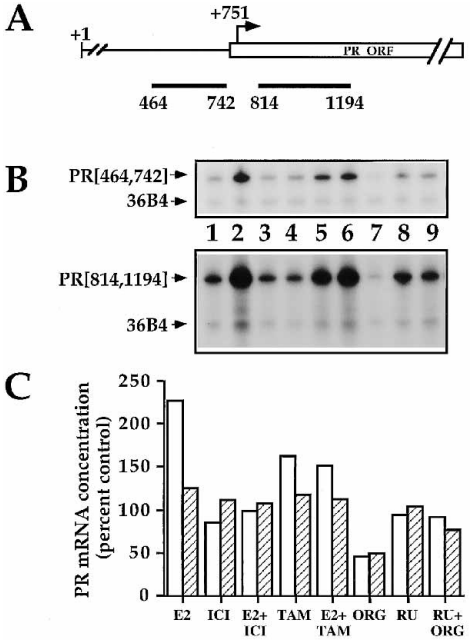

FIG. 5. **Regulation of PR promoter-specific transcripts by estrogen, progestin and their antagonists.** *Panel A*, relative positions on the PR gene of PR B promoter-specific probe PR(+464,+742), and PR(+814,+1194), which detects all PR mRNA species, are shown. The position of the first of the downstream cluster of transcription start sites at +751 with respect to the upstream transcription start is also indicated. *ORF*, open reading frame. *Panel B*, T-47Dsd cells were cultured in RPMI + 5% SFCS, changed to RPMI + 1% SFCS (+1 μg/ml insulin) and treated 1 day later with 100 nM ICI 164384 (*lanes 3* and *4*), 100 nM Tamoxifen (*lanes 5* and *6*), or vehicle (*lanes 1* and *2*) in the presence (*lanes 2*, *3*, and *5*) or absence (*lanes 1*, *4*, and *6*) of 0.1 nM 17β-estradiol for 24 h prior to isolation of nuclear RNA. Cells were also treated with 10 nM ORG 2058 (*lane 7*), 100 nM RU 38486 (*lane 8*), or their combination (*lane 9*). RNA fragments protected by the PR(+464,+742), PR(+814,+1194), and 36B4 antisense RNA probes were visualized by S1 nuclease protection assay. *Panel C*, PR B promoter-specific RNA (*open bars*) and total PR RNA (*hatched bars*) concentrations were measured by densitometry. The data are expressed as a percentage of control, corrected for 36B4 RNA concentration.

those previously described as arising from promoter B (12) in contrast with the recognition of all PR transcripts by the more downstream probe.

In nuclease protection assays, probe B protected a 278-base pair fragment and probe T a 380-base pair fragment detected on acrylamide gel electrophoresis. Relative transcript expression was determined after correction for recovery using the internal standard 36B4. Promoter B-derived transcripts accounted for 63% (calculated using control samples after correction for differences in the specific activity of antisense probes) of total detected transcripts and marked estradiol stimulation of these transcripts was observed (Fig. 5*B*). The magnitude of the effect of estradiol on B-derived transcripts (227%) was greater than that on total transcripts (125%), indicating that the effect of estradiol was primarily on promoter B activity (Fig. 5*C*). In the absence of probes specific for promoter A-derived transcripts, it could not be determined directly whether there was also an effect of estrogen on promoter A. However, the magnitude of the estrogen effect on total transcripts and the proportion of promoter B-derived transcripts in the total suggested that effect on promoter A, if present, was likely to be minor.

For human PR there are a number of mechanisms giving rise to mRNA size heterogeneity, which have been shown to result in transcripts of similar sizes arising from the two promoters (50). This makes it difficult to clearly distinguish promoter B-from promoter A-derived transcripts on Northern analysis and may explain the failure to note transcript specific effects of estrogen on PR mRNA when analyzed previously by this method (27, 29, 33, 51, 52). The use of non-overlapping probes in nuclease protection assays circumvented these limitations and allowed the quantitation of promoter B-derived transcripts and description of the preferential effect of estrogen on these transcripts.

The pure anti-estrogen ICI 164384 had little or no effect on PR transcript expression, but totally abrogated the estradiol stimulation of promoter B-derived transcripts (Fig. 5), whereas the triphenylethylene anti-estrogen tamoxifen was estrogenic both alone and in combination with estradiol: the effect of tamoxifen was predominantly on promoter B-derived transcripts (162% control *versus* 118% control for all transcripts), in agreement with the estradiol effect.

Progestin Equally Decreased Transcripts Derived from Both PR Promoters—The progestin ORG 2058 decreased the concentration of promoter B-derived and total transcripts equally (to 46% and 49% of control, respectively) (Fig. 5) and also abrogated the estradiol stimulation of PR transcripts (data not shown). The magnitude of the effects of ORG 2058 on levels of basal and estradiol-stimulated PR transcripts was very similar to that observed for the effects of this agent on PR transcription rate (Fig. 2). The anti-progestin RU 38486 had little or no effect alone, but totally abrogated the effect of ORG 2058 on promoter B-derived and total transcripts (Fig. 5). The ability of progestin to decrease all PR transcripts when expression of PR from the endogenous gene was measured contrasted with the inability of progestin to affect basal and estrogen-induced expression of the minimal PR promoters in transfection experiments (Fig. 3).

Estrogen Preferentially Increased PR B Protein Concentration in T-47Dsd Cells—Having shown promoter-specific effects of estradiol on PR transcripts, it was important to document whether this resulted in a selective effect on the cellular concentration of the PR A and PR B proteins. Estradiol is known to increase PR protein levels as measured by immunoblot (51–54), but individual effects on PR B and PR A proteins have not been documented. PR protein expression was measured by immunoblot analysis of T-47Dsd cells after treatment for 24 h with a range of estradiol concentrations. Both PR A and PR B were detectable in control cells (Fig. 6*A*), although the concentration of PR A exceeded that of PR B (Fig. 6*B*). Estradiol caused an increase in the concentration of PR protein, which was primarily due to an increase in PR B (Fig. 6*B*), and resulted in a change in the relative amount of PR A and PR B: the PR A/B ratio decreased from around 0.8 in control cells to 0.4 at a maximally effective concentration of estradiol (Fig. 6*C*).

Estrogen augmentation of PR levels increased until 48 h after treatment, when a decrease in induction, due to an increase in PR A and B levels in untreated cells (not shown), was noted. The preferential effect of estrogen on PR B levels observed in Fig. 7 was also noted in the time course and resulted in a decrease in PR A/B ratio over time. The estrogen effect on PR A/B ratio was rapid and essentially maximal 24 h after treatment (Fig. 7*C*). The PR A/B ratio began to recover after 72 h (Fig. 7*C*), due to the increase in PR levels in untreated cells noted above.

Although the estrogen-mediated increase in PR levels was primarily through an increase in PR B, there was also an increase in PR A of more modest magnitude. This was despite the fact that transfection studies had shown no effect of E2 on promoter A and nuclease protection had shown a preferential E2 effect on promoter B-derived transcripts. The mechanism underlying the estrogen augmentation of PR A protein levels is not known, but may be due to minor estrogen stimulation of

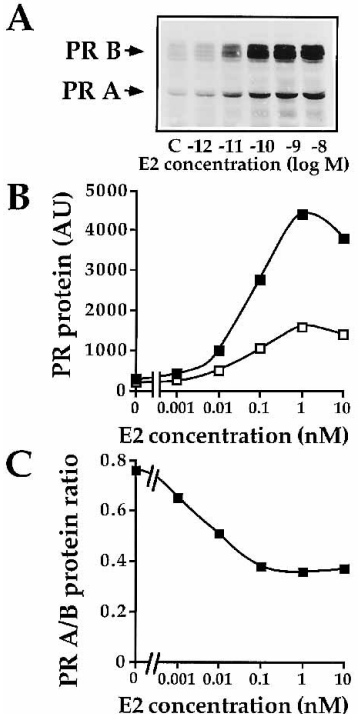

FIG. 6. **Concentration-dependent estradiol regulation of PR protein expression.** T-47Dsd cells were cultured in RPMI + 5% SFCS (+1 μg/ml insulin) and changed to RPMI + 1% SFCS on day 4 after plating. On day 6 cells were treated with the range of 17β-estradiol concentrations shown or vehicle and harvested 24 h later. *Panel A*, cytosols were prepared and PR A and B proteins were visualized by immunoblot as described under "Experimental Procedures." *Panel B*, PR A (□) and B (■) were measured densitometrically and expressed as arbitrary units. *Panel C*, the ratio of PR A to B was calculated at each concentration of estradiol used.

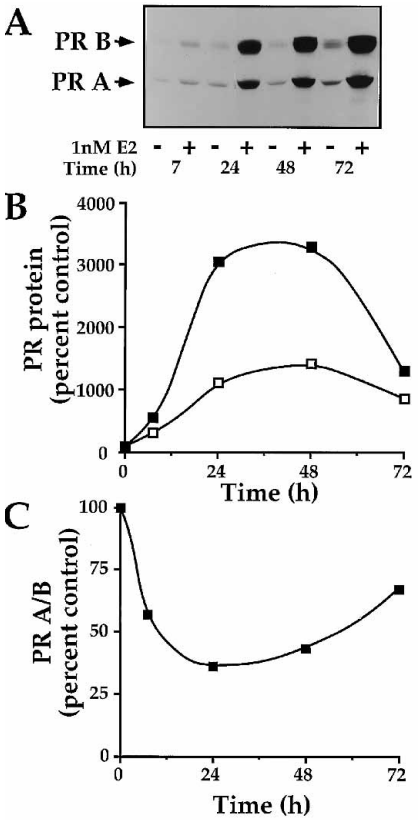

FIG. 7. **Time dependence of estradiol effect on PR protein expression in T-47Dsd cells.** T-47Dsd cells were cultured in RPMI + 5% SFCS (+1 μg/ml insulin) and changed to RPMI + 1% SFCS on day 3 after plating. Cells were treated 1 day later with 1 nM 17β-estradiol or vehicle and harvested at the times indicated. *Panel A*, cytosols were prepared and PR A and B proteins were visualized by immunoblot as described under "Experimental Procedures." *Panel B*, PR A (□) and B (■) were measured densitometrically at each treatment time and expression is shown as a percentage of the time-matched vehicle-treated control. *Panel C*, PR A/B ratio in estradiol-treated samples is expressed as a percentage of PR A/B in time-matched controls.

promoter A-derived transcripts, which would be difficult to detect clearly on nuclease protection, given that probes specific to promoter A-derived transcripts are not feasible. Increases in PR A may also be due to low concentrations of PR A arising from translation of promoter B-derived transcripts; although full-length human PR expression vectors transfected into HeLa or COS-1 cells express only PR B (55), it is not known whether promoter B-derived transcripts arising from the endogenous PR gene in target cells such as T-47D breast cancer cells express only PR B or also some PR A. Plasmids encoding the full-length chicken PR express low concentrations of PR A in addition to PR B (55).

DISCUSSION

Estrogen and Progestin Modulation of PR Gene Transcription—Progestin treatment of T-47D cells caused a prolonged suppression of PR transcription, demonstrating that the profound and sustained loss of receptor protein and mRNA previously described in breast cancer cells (25–27, 29, 56) is due to progestin-mediated transcriptional inhibition. This prolonged transcriptional repression of PR by its homologous ligand is in contrast with the transient drop in the transcription rate of the estrogen receptor upon exposure to estrogen (57). Estradiol increased the transcription rate of the PR gene in T-47D cells, as shown previously in MCF-7 cells (12), although the effect was clearly more modest and in keeping with the high basal expression of PR in T-47D cells. Nevertheless, the magnitude of the increase was sufficient to allow visualization of the effect of ORG 2058, which markedly decreased the estrogen-mediated increase in transcription rate. However, these data provided no information about whether the transcriptional effects documented here were mediated equally by the two PR promoters.

Preferential Modulation of PR B by Estrogen—In order to examine more closely the promoter specificity of the actions of estrogen and progestin, individual PR promoter constructs were transfected into T-47D cells. This showed that although basal expression of both promoters was similar, promoter B was stimulated by estrogen treatment, whereas promoter A was affected little, if at all. This differential effect of estradiol was confined to breast cancer cells, which are progestin-responsive target cells, and contrasted with the inducibility of both promoters by estrogen in CHO and HeLa cells. However, even in CHO and HeLa cells, the magnitude of the estrogen inducibility of promoter A was less than that of promoter B. It is not clear why the behavior of the PR promoter constructs should vary depending on the cell type into which they are transfected but it is possible that critical factors present in CHO and HeLa cells but absent in T-47D cells may have facilitated the estrogenic stimulation of PR-A-CAT in CHO and HeLa cells documented in this study and shown previously for HeLa cells (12). If the preferential effect of estrogen on promoter B noted in breast cancer cells in this study is also seen in target cells and tissues *in vivo*, it indicates that the primary transcriptional effect of estrogen on PR may be stimulation of promoter B of the PR gene.

The differential estradiol stimulation of the PR promoters observed in transfection experiments was unexpected and raised the question of whether such effects were operative in the endogenous PR gene. Nuclease protection assays were em-

ployed to examine the estrogen stimulation of promoter B in the endogenous gene; transcript levels were measured in nuclear RNA, in order to measure transcriptional events distinct from cellular events such as cytoplasmic mechanisms controlling mRNA stability. Promoter B-derived transcripts accounted for over half of total transcripts, and a marked estrogen-mediated increase in their level was noted. The percentage increase in promoter B-derived transcripts was greater than that observed for all transcripts, indicating that estrogen augmented PR mRNA levels primarily through an effect on promoter B and confirming the transfection results. Interestingly, tamoxifen, which has known estrogen agonist activity with respect to increasing PR concentration (58), also preferentially increased the concentration of promoter B-derived transcripts, whereas the pure anti-estrogen ICI 164384 was ineffective.

The observations that estradiol preferentially increased promoter B-derived transcripts in human PR are in contrast with evidence that estrogen stimulation of rabbit PR gene expression is confined to an estrogen response element within the open reading frame spanning the translation start site for PR B (34) and corresponding to the position of promoter A in the human receptor. The data in the human also contrast with the demonstration that the more proximal of the two rat PR promoters is preferentially stimulated by estrogen (59), which may be consistent with the observation that the rodent PR exists predominantly as the A form (51, 60). Recent studies have demonstrated four additional weak estrogen-responsive regions within the rat PR gene, which *in vitro* confer or contribute to estrogen responsiveness of both proximal and distal rat PR promoters (61). It is not known why estrogen control of PR expression should vary in the rabbit, rat, and human, particularly as there is extensive homology in the sequence of the PR gene in these species. Nevertheless, it is clear from this and previous studies (12) that the human PR promoter B is inducible by estrogen in all cells tested to date, and this study has shown that this is accompanied by increases in promoter B-derived transcripts and cellular levels of PR B protein. Clearly, control of PR expression by estrogen is complex and likely to be species-specific.

The differential effect of estradiol on promoter B-derived transcripts was supported by the observation that the PR B protein concentration was increased by estradiol to a greater extent than the PR A protein, leading to an alteration in the A/B ratio in favor of PR B. This supports previous observations that PR B levels declined more rapidly than PR A upon withdrawal of estrogen in endometrial carcinoma grown in nude mice (53) and suggests that in breast cancer cells and in endometrial carcinoma estradiol may stimulate PR expression by a common mechanism involving a preferential increase in the level of PR B. However, alterations in the PR A/B protein ratio may be confined to PR stimulation, as down-regulation of PR by progestins and other agents such as retinoic acid takes place without any effect on the relative concentrations of PR A and B (36).

Progestin Regulation of PR Expression—The progestin ORG 2058 decreased PR transcription rate and the concentration of PR transcripts. The magnitude of the effect on promoter B-derived transcripts was the same as that seen for all transcripts, indicating that the progestin equally decreased the concentration of all PR transcripts. Furthermore, progestin caused an abrogation of the estradiol effect on both PR transcription rate and levels of promoter B-derived transcripts. The failure to see a progestin effect on the basal or estrogen-stimulated promoter B-CAT construct transfected into T-47D cells, despite the demonstration that the progestin was active when other end points of progestin action were measured in transfected cells, indicates that the site of action of progestins lies outside promoter B.

Taken together, the progestin effects on PR transcription rate, PR promoters and PR RNA showed that: 1) progestins decreased both basal and estradiol-stimulated PR gene expression; 2) progestins equally decreased promoter A- and B-derived transcripts and therefore abrogated estrogen action independent of estrogen stimulation of promoter B and suggesting that the progestin effect was not mediated through the same sequence(s) as the estrogen effect; and 3) the progestin effect was not mediated directly through sequences contained within the PR promoters. This discordance between the site of estrogen and progestin action on human PR contrasts with the demonstration that progestin inhibition of estrogenic effects on rabbit PR are mediated through the same short sequence within the PR gene (35). However, it is consistent with the demonstration that constructs containing estrogen-responsive elements can still be inhibited by progestin when the progestin-responsive element is located as far as 2 kilobases upstream from the estrogen-responsive element (21). More generally, it is also consistent with the emerging view that progestin inhibition of ER activity on estrogen-responsive sequences is mediated not by binding of PR to DNA but indirectly, by quenching of transcription factors required for ER activity (19–22). Such a mechanism would explain progestin abrogation of estrogen induction of PR gene transcription and promoter B-derived transcript expression. Although no quenching of estrogen effects on minimal PR promoters in transfection studies was observed in this study, the endogenous levels of PR in T47D cells may not have been sufficiently high to quench the activity of high levels of exogenously transfected PR promoters and ER.

Functional Consequences of Preferential PR Isoform Expression—The functional implications of altered ratios of PR A and B upon estrogen treatment are not clear. Alterations in the ratio of chicken PR forms during the reproductive cycle and during different seasons have been reported (62, 63), suggesting the possibility that such changes in ratio may be associated with altered states of progestin responsiveness. The PR isoforms have different capacities to activate target genes (15) and consequently altering the ratio of A and B PR proteins may alter the overall cellular response to progestins. Furthermore, PR A has been shown to repress the function of PR B in a dominant manner (16, 17), suggesting that high levels of PR A may be associated with diminished progestin responsiveness in some systems. In some cases, PR A can act as a repressor more generally of the activity of other members of the nuclear receptor family (18, 19); therefore, expression of the N-terminally truncated PR A may affect responsiveness not just to progestins but to other steroidal ligands.

The inhibitory activity of PR A has been explored *in vitro*, and there is little evidence to date of this activity *in vivo*. Furthermore, the issue is clearly complex, as the rodent uterus is progesterone-responsive despite the fact that rodent PR exists predominantly as the A form of the receptor (51, 60) and ratios of rodent PR A/B fluctuate little during the year, with levels of PR A always exceeding levels of PR B (60). Nevertheless, *in vivo* studies in the mammalian uterus and the chick oviduct have suggested that estrogen augmentation of PR levels precedes the acquisition of progestin responsiveness (64, 65); the *in vivo* evidence, combined with the demonstration in this study of preferential estradiol stimulation of PR B, indicates that PR B may be an important mediator of the physiological effects of progestins in some progesterone-responsive tissues. In this regard, it is of interest that a subset of ER+PR+ breast tumors express very low levels of PR B and consequently very high ratios of PR A/B (66).

This study has shown that estrogen and progestin, the major physiological modulators of human PR expression, independently control the synthesis of transcripts arising from the two PR promoters in human breast cancer cells. Estrogen preferentially increased the concentration of transcripts derived from promoter B, whereas progestin decreased all transcripts equally. Promoter-specific control by estradiol of PR gene expression was reflected at the cellular level by a selective increase in the concentration of the PR B protein and a consequent change in the ratio of PR A and B proteins. Such independent control of gene expression and the resulting flexibility in the control of the cellular PR proteins, which mediate the physiological actions of progestins, are likely to provide a rational framework within which the complexity of cell- and tissue-specific regulation of progestin responsiveness can be considered.

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