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Progesterone Receptor A and B Messenger Ribonucleic Acid Levels in the Anterior Pituitary of Rats Are Regulated by Estrogen[1]

Marta Szabo,[2] Signe M. Kilen, Shane J. Nho, and Neena B. Schwartz

Department of Neurobiology and Physiology, Northwestern University, Evanston, Illinois 60208

ABSTRACT

In target tissues of most mammalian and avian species, progesterone receptors (PR) are expressed as structurally related, but functionally distinct, isoforms A and B, and they are regulated by estrogen (E) as well as by their cognate ligand, progesterone (P_4). The objectives of the present work were to identify mRNA expression for the A and B isoforms of PR in the anterior pituitary of the rat, to examine its regulation by gonadal steroids, and to compare this regulation with that in the primary target organ, the uterus. Messenger RNAs for the PR isoforms, determined by two separate reverse transcription-polymerase chain reaction protocols, one that detects PR A and PR B equally and the other specific for PR B, were identified in anterior pituitary of female and male rats. In anterior pituitary of cycling female rats, steady-state mRNA levels for both PR A+B and PR B were highest at 0900 h on proestrus, declined rapidly to nadir values at 0900 h on metestrus (PR A+B) or 0900 h on estrus (PR B), and remained below proestrous values through 2100 h on diestrus. Administration of E to intact proestrous female rats caused significant increases in mRNA for both PR A+B and PR B on estrus and metestrus. Blockade of P_4 action by administration of the antiprogestins RU-486 and ZK-98299 on proestrus had no effect on PR mRNA levels on the morning of estrus. Ovariectomy two and ten days after surgery markedly reduced mRNA levels for both PR A+B and PR B. Whereas treatment of 10-day-ovariectomized rats with E led to marked induction of mRNA for PR A+B and PR B two days later, treatment with P_4 one day after treatment had no effect on basal or E-stimulated PR mRNA. Regulation of PR mRNA expression in the pituitary differed from that in the uterus, in which P_4 treatment of ovariectomized rats antagonized the E-induced rise in mRNA for PR B, and antiprogestins increased mRNA for both isoforms. In addition to induction of PR mRNA in the pituitary of female rats by E in vivo, we also demonstrated induction by E in primary culture of anterior pituitary cells in vitro. We conclude that in the anterior pituitary of female rats, both the A and B isoforms of PR are expressed and regulated by E.

INTRODUCTION

Previous in vivo studies from our laboratory demonstrating estrogen (E)-dependent suppression of FSH secretion by the antiprogestin RU-486 suggested a key role for an E-inducible form(s) of hypothalamic or pituitary progesterone receptors (PR) in regulation of FSH secretion in the gonadotrophs of female rats [1–3]. Subsequent work demonstrating E-dependent suppression of basal and activin-stimulated FSH secretion by antiprogestins in anterior pituitary cell culture in vitro [4] clearly established the pituitary as the site of this regulation. The mechanism(s) whereby activation of PR may influence the synthesis and release of FSH, as well as the regulation of PR mRNA expression in the anterior pituitary of rats in vivo have not been elucidated. Although E- and progesterone (P_4)-regulated PR protein has long been identified in the anterior pituitary of rats by functional binding studies [5–9], until recently, information on PR mRNA expression and its regulation by gonadal steroids was available only in monkey pituitary [10]. Recent work by Turgeon and colleagues [11] has filled this gap by demonstrating that in cultured anterior pituitary cells of ovariectomized (OVX) rats, PR mRNA was up-regulated by E and acutely down-regulated by P. These investigators further demonstrated colocalization of PR protein with the β subunit of LH in the gonadotroph, confirming earlier demonstration of this phenomenon in the primate [12], and lending additional support to a role of PR in direct regulation of gonadotropin secretion.

In most species studied to date, PR are expressed as structurally related but functionally distinct [13] A and B isoforms, encoded by a single gene but transcribed into separate mRNAs [14]. One possible explanation of our earlier finding of estrous cycle stage-dependent suppression of serum FSH by the antiprogestin RU-486 was differential changes of the two PR isoforms across the estrous cycle by the rising and falling levels of serum E and P_4, known regulators of PR transcription in other classical target tissues and cells [15,16]. Previous studies examining mRNA levels in the monkey [10] and rat [11] pituitary relied on Northern blot or in situ hybridization analyses, using probes that detect the A and B isoforms of PR equally, and thus do not provide information on whether mRNA expression of the two isoforms is differentially regulated. To address this issue, in the present study we used two reverse transcription (RT)-polymerase chain reaction (PCR) protocols, which enabled us to selectively analyze mRNA levels for PR B and total (PR A+B) receptors.

MATERIALS AND METHODS

Animals

Adult female Crl:CD(SD)BR-CD rats, obtained from Charles River (Portage, MI), were housed at our animal facility under a 14L:10D schedule, with lights-on at 0500 h. They were provided with standard rat chow and tap water ad libitum. Their estrous cycle stage was monitored by daily vaginal cytology; only rats that had exhibited two consecutive 4-day estrous cycles were used in the study. Bilateral ovariectomy was performed under metophane anesthesia at random stages of the estrous cycle. Rats were killed by decapitation at the times indicated below. Trunk

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[2]Correspondence: Marta Szabo, Department of Neurobiology and Physiology, Northwestern University, 2153 North Campus Drive, Evanston, IL 60208-3520. FAX: 847 491 5211; e-mail: msz729@nwu.edu

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blood was collected for RIA of serum hormone levels; pituitary and uterus were rapidly removed, frozen on dry ice, and stored at −80°C until RNA extraction. Anterior pituitaries of adult male rats of the same strain, intact or castrated for 48 h, were extracted for comparison. Protocols were approved by the animal care and use committee of Northwestern University. Animals were maintained in accordance with the National Institutes of Health guide for the Care and Use of Laboratory animals.

RT-PCR Analysis

Total RNA was isolated from whole anterior pituitary, from a segment of one uterine horn weighing approximately 50 mg, or from a monolayer of primary pituitary cell culture by a single-step acid guanidinium thiocyanate-phenol-chloroform extraction (TRI Reagent; Molecular Research Center Inc., Cincinnati, OH). To eliminate interference by contaminating genomic DNA, RNA isolated from a pituitary cell monolayer was subjected to treatment with RQ1 deoxyribonuclease (DNase; 1 U/μg RNA; Promega, Madison, WI) at 37°C for 20 min and extracted with phenol-chloroform, 5:1, pH 4.7. Pituitary and uterine RNA were sufficiently pure for RT-PCR without DNase treatment. Approximately 4 μg of total RNA was reverse-transcribed into cDNA with M-MLV reverse transcriptase (5 U; Promega) and random hexamers to prime the reaction, at 42°C for 75 min. Approximately 1 μg reverse-transcribed RNA was used in each PCR amplification; RNA incubated under identical conditions, but without reverse transcriptase, served as a negative control. All amplifications were performed in duplicate. Two separate PCR protocols were employed to detect PR A+B and PR B (see Fig. 1A). The first amplified a 326-basepair (bp) sequence from the ligand-binding domain (LBD) common to the A and B isoforms; the second amplified a 221-bp fragment from the 5′ untranslated region (5′ UTR) of the rat PR cDNA, unique to the B isoform. The sequences of the oligonucleotide primers used in PR A+B-specific PCR, reported originally by Park and Mayo [17], were 5′-CCCACAGGAGTTTGTCAAGCTC-3′ (sense) and 5′-TAACTTCAGACATCATTTCCGG-3′ (antisense). Those used in the newly developed PR B-specific PCR were 5′-GTGTGAGGATTCTGCCTTTC-3′ (sense) and 5′-CGCTCTCAGGACTTCTTACG-3′ (antisense); their sequences were based on the structure of the rat PR gene published by Park-Sarge and Mayo [18] and retrieved from GenBank under accession nos. L16921 and L16922. Full homology of the 221-bp product generated in this PCR to the 5′ UTR region of PR B was verified by sequencing with the Sequenase DNA Sequencing Kit (USB Corp., Cleveland, OH). Primers that amplified a 514-bp cDNA for α-tubulin [19] were included to control for reaction efficiency and variations in concentrations of mRNA in the RT reaction. Alternatively, in studies involving the uterus, in which administration of E caused equal or greater induction of α-tubulin mRNA expression than that of PR, primers that amplified a 500-bp cDNA for ribosomal protein L19 (RPL19) [20], not susceptible to regulation by E, were used as internal control. PCR amplifications were performed in two steps to obtain similar intensities for the product of interest and the more abundant internal control. In the first step, the reaction mixture contained 2 mM $MgCl_2$, 0.2 mM of each of the four dNTPs, 12.5 pmol of each primer, 0.5 μCi [^{32}P]dCTP (Amersham, Arlington Heights, IL), single-strength *Taq* polymerase buffer, and 1.25 U *Taq* DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) in a final volume of 25 μl, overlaid with 45 μl mineral oil. After the appropriate number of cycles with the first set of primers, 20 μl of a cocktail containing 12.5 pmol of the second set of primers and 1.25 U additional *Taq* DNA polymerase was added in single-strength PCR buffer with 2 mM $MgCl_2$ for the remaining cycles. After initial denaturation at 94°C for 4 min, the reaction mixture was subjected to successive cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec, and extension at 72°C for 45 sec, followed by extension at 72°C for 10 min after the final cycle. The optimal number of cycles, determined experimentally to yield a linear relationship between signal intensity and input cDNA, and an exponential one with respect to cycle number, was 25 in PCR for the LBD, and 26 (uterus) or 27 (pituitary, pituitary cell monolayer) in PCR for the 5′ UTR. Amplifications with the primers for α-tubulin and RPL19 were performed for 23 cycles. Radiolabeled PCR products were separated by electrophoresis on 6% nondenaturing polyacrylamide gels in Tris borate-EDTA buffer. Signal intensity was quantified by phosphor imaging (Molecular Dynamics, Sunnyvale, CA).

Experimental Protocols

Experiment 1: Analysis of PR A+B and PR B mRNA levels across the 4-day estrous cycle. Rats were killed by decapitation at the following times: 0900 h proestrus, 1830 h proestrus, 2100 h proestrus, 0900 h estrus, 0900 h metestrus, 0900 h diestrus, 2100 h diestrus (n = 4–5/time point). Total RNA from anterior pituitary and uterus was isolated for RT-PCR analysis in each of the two PCR protocols. Serum levels of gonadotropins and gonadal steroids were used in addition to uterine intraluminal fluid content and vaginal cytology to confirm the estrous cycle stage.

Experiment 2: Effect of treatment with E on proestrus on PR A+B and PR B mRNA levels on estrus and metestrus. In a previous study from our laboratory, we demonstrated that RU-486 is capable of suppressing serum FSH only when administered on proestrus, when serum E is high, but not on estrus, when serum E is low unless it has been raised by a prior injection of estradiol benzoate (EB) [2]. Accordingly, EB, 50 μg/0.2 ml benzyl benzoate-sesame oil (1:4, v:v), or vehicle, was injected s.c. at 1700 h on proestrus. One half of the rats were killed at 1230 h on estrus and the other half at 0900 h on metestrus. The protocols in this and subsequent in vivo studies were chosen on the basis of our previous study to allow examination of the status of the PR at times when antiprogestin administration was effective in suppressing serum FSH. RT-PCR and serum hormone determinations were performed as above. In this and subsequent in vivo experiments, all mRNA values are expressed as a fraction of the 0900-h-proestrus control. Four 0900-h-proestrus control samples were included in the RT-PCR for normalization in each experiment.

Experiment 3: Effects of treatment with antiprogestins on proestrus on PR A+B and PR B mRNA levels on estrus. As in another previous in vivo study comparing the effects of the antiprogestins RU-486 (RU) and ZK-98299 (ZK) on FSH secretion [3], the drugs were dissolved in benzyl benzoate-sesame oil and were administered s.c. at a dose of 6 mg/kg at 1230 h on proestrus (n = 4–5/treatment). This treatment was shown to effectively block P_4 action on the evening of proestrus. Rats were killed and autopsied, and blood and tissues were collected at 0900 h on estrus.

Experiment 4: Effects of ovariectomy and replacement with E and P_4 on PR A+B and PR B mRNA levels. Rats at random stages of the estrous cycle were bilaterally OVX through dorsolateral incisions under metophane anesthesia at 0900 h. The day of surgery was designated as Day 0. At

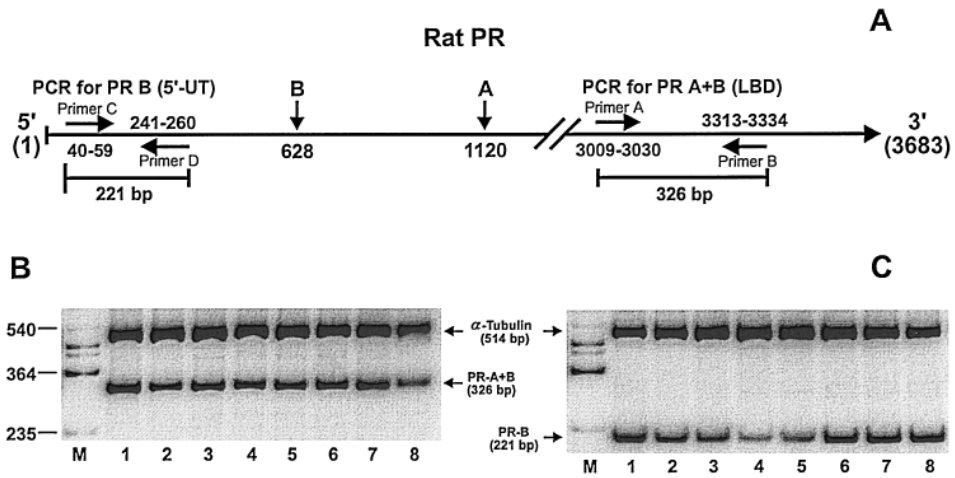

FIG. 1. **A**) Schematic representation of the full-length rat PR cDNA (Genbank accession nos. L16921 and L16922) indicating the location of the primer sets used to amplify a 326-bp sequence common to the A and B isoforms in the LBD and a 221-bp sequence unique to the B isoform in the 5′ UTR (5′ UTR). The base A at the transcription start site for PR B is labeled as (1); the translation start sites at 628 and 1120 for PR B and A, respectively, are indicated by vertical arrows. The exact location of the promoter and transcription start site(s) for rat PR A are not known. **B**) A representative gel showing the 326-bp PCR product for PR A+B amplified in the LBD PCR for 25 cycles. Reverse-transcribed RNA from pituitaries of rats collected at various times of the estrous cycle were amplified: 1) 0900 h proestrus, 2) 1830 h proestrus, 3) 2100 h proestrus, 4) 0900 h estrus, 5) 0900 h metestrus, 6) 0900 h diestrus, 7) 2100 h diestrus, 8) 0600 h proestrus. **C**) The same samples were amplified concurrently in the PR B-specific 5′-UTR PCR, yielding a 221-bp product, for 26 cycles. The internal control, α-tubulin, amplified for 23 cycles in both reactions, yielded a 514-bp product. M, Molecular weight markers (in base pairs).

0900 h on Day 10, animals received injections of vehicle (benzyl benzoate-sesame oil, 1:4) or EB (50 μg/rat) s.c. At 1600 h on Day 11, the vehicle- and EB-primed rats received a second injection of vehicle or P_4 (5 mg/rat) (n = 4–5/treatment). At 0900 h on Day 12, rats were killed by decapitation; blood and tissues were collected as in the preceding experiments.

Experiment 5: Induction of PR by E in primary anterior pituitary cell culture in vitro. Anterior pituitaries were collected from 6 to 14 rats at 0900–1000 h on metestrus and dissociated enzymatically as described [21]. The final cell pellet was suspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% charcoal-stripped fetal bovine serum supplemented with vehicle (absolute ethanol) or 10 nM estradiol-17β (E_2) as indicated. Cells were plated in 12-well culture plates at a density of 2–2.5 $\times$ 10^6 cells/3.8-cm^2 well in 2 ml medium with vehicle or E_2, and were incubated in a humidified atmosphere of 94% air and 6% CO_2 at 37°C for 48 h. Media were collected and replaced with fresh medium containing vehicle or E_2, and incubation was continued for an additional 48 h. Incubation with vehicle or E_2 was performed in quadruplicate, and the entire experiment was repeated for confirmation. At the end of the incubation, total RNA was rapidly isolated from the cell monolayer for RT-PCR analysis.

Hormone Assays

FSH and LH concentrations in the serum and incubating medium were determined by double-antibody RIAs as described previously [22] with reagents supplied by the NIDDK. Serum P_4 and E_2 were measured using kits from ICN Biomedicals (Irvine, CA) and Diagnostic Products Corp. (Los Angeles, CA), respectively.

Statistical Analysis

Data obtained in the in vivo experiments are presented as the mean ± SE, normalized as a fraction of the 0900-h-proestrus values. In vitro data are the mean ± SE of four incubations from one of two experiments with similar results. Statistical significance for multiple groups was evaluated by one-way ANOVA; post-hoc pair-wise comparisons were made by the Neuman-Kuels test. Comparison of two groups (e.g., OVX vs. intact; E vs. vehicle treatment in vitro) was by unpaired *t*-test; $P < 0.05$ was considered significant.

RESULTS

PR A+B and PR B mRNA Expression in Rat Anterior Pituitary

Both the formerly established RT-PCR protocol for PR A+B and the newly developed one for PR B detected their specific sequences of the expected size (326 bp in the LBD PCR and 221 bp in the 5′ UTR PCR) in reverse-transcribed RNA from anterior pituitary of female rats at various times across the estrous cycle (Fig. 1, B and C); products of the same size were also generated by PCR amplification of RT RNA from uterus. These sequences were undetectable, however, in negative control tissues such as the liver or muscle. Because two independent PCR protocols with possibly different efficiencies were used to amplify RT-RNA, it was not possible to directly compare the mRNA results for PR B with those for total receptor. Nonetheless, by careful equalization of the amplification conditions, certain approximations could be made about the distribution of mRNAs between the two isoforms. The 5′ UTR PCR had to be performed for more amplification cycles (26 or 27) than the LBD PCR (25) in order to obtain comparable signal intensity. When reverse-transcribed RNA from tissues obtained at 0900 h on proestrus was amplified for an equal number of cycles (25) in the two protocols, the PR B signal represented approximately one third and one half that of PR A+B in the anterior pituitary and the uterus, respectively. Taken together, these results suggest lower abundance of the message for PR B than for total receptor in both tissues. Comparison of the PR signal intensities in the two tissues revealed that whereas for the same quantity of

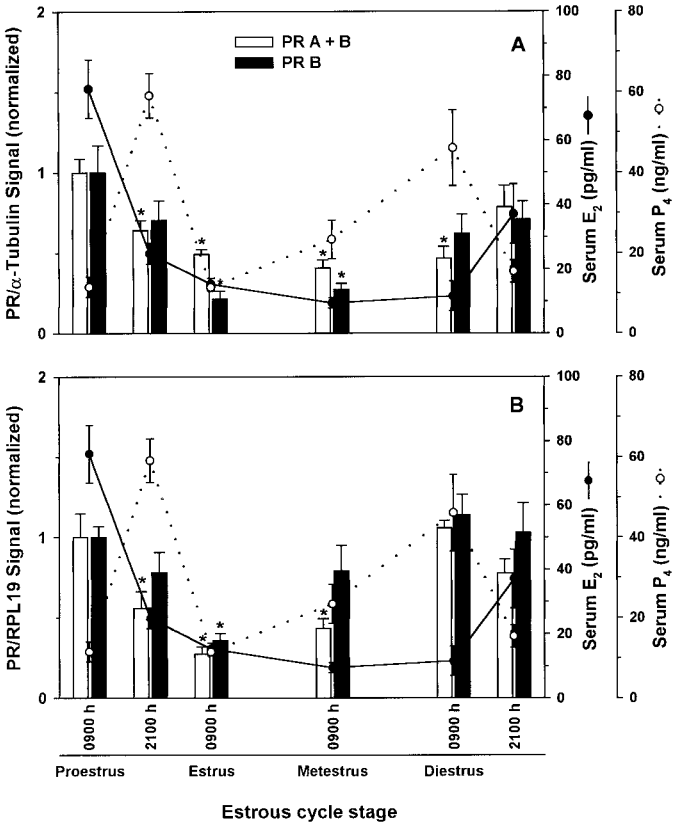

FIG. 2. Temporal changes in PR A+B and PR B mRNA expression in anterior pituitary (**A**) and uterus (**B**), and in serum E_2 and P_4 concentrations across the 4-day estrous cycle. Receptor mRNA data are expressed as a fraction of the PR signal/internal control signal ratio at 0900 h on proestrus, which was arbitrarily defined as 1.00 for both reactions. Data from four rats is shown as means ± SE; amplifications were performed in duplicate. *Values significantly different ($P < 0.05$) from the 0900-h-proestrus control.

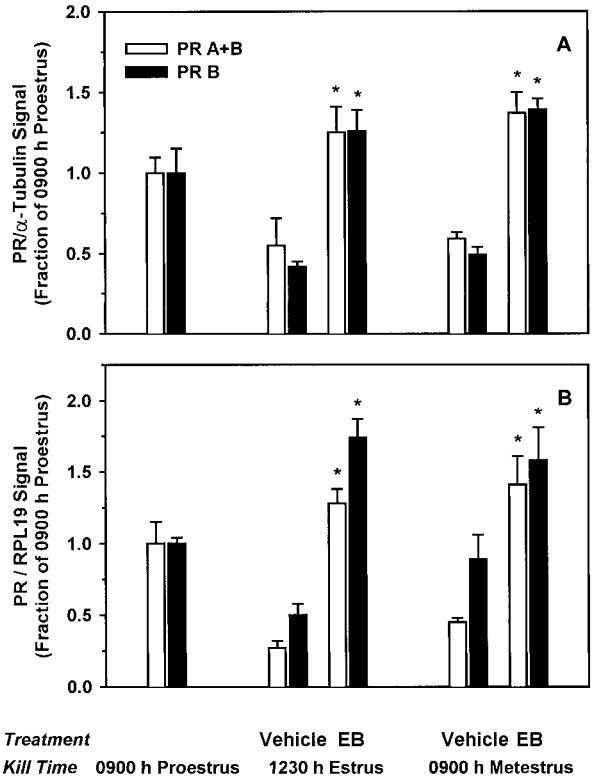

FIG. 3. Effects of s.c. administration of 50 μg EB at 1700 h on proestrus on PR mRNA levels, depicted as in Figure 2, in the pituitary (**A**) and uterus (**B**) at 1230 h on estrus and at 0900 h on metestrus. In the pituitary, the fold increase in mRNA for PR A+B and PR B, respectively, was 2.3 and 3.1 on estrus and 2.3 and 2.8 on metestrus. In the uterus, the corresponding fold inductions were 4.6 and 3.1 on estrus and 3.5 and 1.8 on metestrus. *Values significantly different ($P < 0.05$) from the corresponding vehicle-treated control.

input RNA the PR A+B signals were approximately equal in the pituitary and the uterus, the PR B signal was substantially lower in the pituitary than in the uterus.

PR A+B and PR B mRNA were also identified in the anterior pituitary of male rats (Table 1), in addition to the anterior pituitary of female rats, though at a much lower abundance than in the proestrous female rat. Whereas ovariectomy performed 48 h previously significantly reduced mRNA expression for PR A+B and more so for PR B, orchiectomy of the same duration had no effect on PR mRNA levels.

Experiment 1: PR A+B and PR B mRNA Expression Across the 4-Day Estrous Cycle

To selectively examine PR A+B and PR B mRNA expression as a function of rising and falling levels of E_2 and P_4 across the 4-day estrous cycle of female rats, anterior pituitary and uterus were collected for RT-PCR, and serum for steroid assays, at various times of the cycle. Temporal changes in PR A+B and PR B mRNA expression in anterior pituitary (A) and uterus (B), as detected by the two PCR protocols, and in serum E_2 and P_4 concentrations are shown in Figure 2. In this and the subsequent figures, mRNA data are presented as a fraction of the PR:internal control ratio at 0900 h on proestrus, which was arbitrarily defined as 1.00 for both PCR reactions. In the pituitary (Fig. 2A), mRNA levels for PR A+B and PR B, highest at 0900 h on proestrus, exhibited a progressive fall, which was already significant at 1830 h (not shown) and 2100 h on proestrus. Both declined further to trough values on the morning of metestrus (PR A+B) and estrus (PR B) and remained significantly lower than the 0900-h-proestrus level through most of the remainder of the cycle. Qualitatively, mRNA for PR B appeared to change more dramatically than for total receptor. In comparison, in the uterus (Fig. 2B), mRNA levels, which were also highest on the morning of proestrus and reached trough values on the morning of estrus, returned to proestrous values more rapidly than those in the pituitary. Values obtained at 1830 h on proestrus, the time of the primary gonadotropin surges, which did not differ significantly from those at 2100 h on proestrus, have been omitted from the figure for clarity. Not shown either are serum gonadotropin levels, which displayed the appropriate primary (LH, FSH) and secondary

TABLE 1. PR mRNA expression in anterior pituitary of male rats.

	PR signal/α-tubulin signal (fraction of 0900 h proestrous control)*	
Rat	PR A + B	PR B
Intact female (proestrous)	1.00 ± 0.08	1.00 ± 0.04
OVX female (48 h)	0.40 ± 0.02[a]	0.22 ± 0.02[a]
Intact male	0.21 ± 0.04	0.06 ± 0.01
Orchiectomized (48 h)	0.24 ± 0.02	0.06 ± 0.006

* Mean ± SE (n = 4).
[a] $P < 0.001$ vs. intact female.

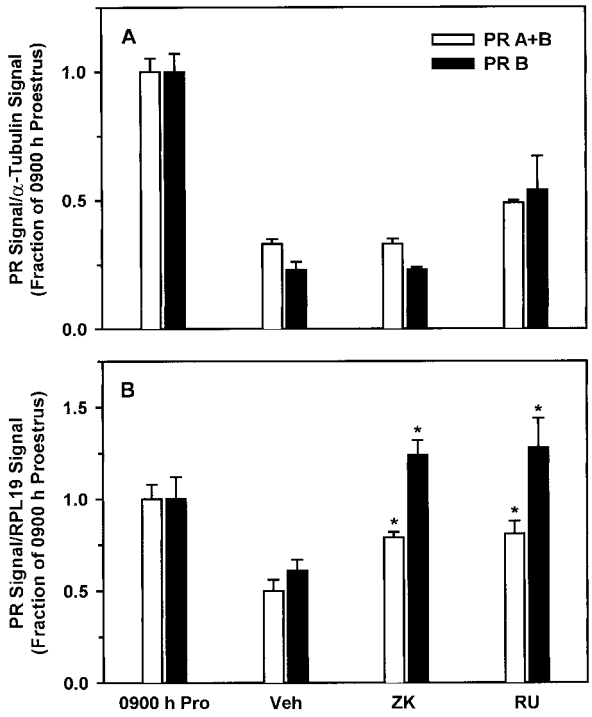

FIG. 4. Effects of s.c. administration of the antiprogestins ZK-98299 (ZK) and RU-486 (RU) at 1230 h on proestrus (Pro) on PR A+B and PR B mRNA levels in the anterior pituitary (**A**) and the uterus (**B**) at 0900 h on estrus. Data are the mean, and the error bar the SEM, of 4–5 observations. Messenger RNA levels were calculated as in the preceding figures. Neither antiprogestin had a significant effect on PR A+B or PR B mRNA levels in the pituitary. In the uterus, ZK and RU respectively induced a significant 1.6- and 2.0-fold increase in PR A+B and a 1.6- and 2.1-fold increase in PR B mRNA levels. *Significant difference ($P < 0.05$) compared to vehicle-treated controls.

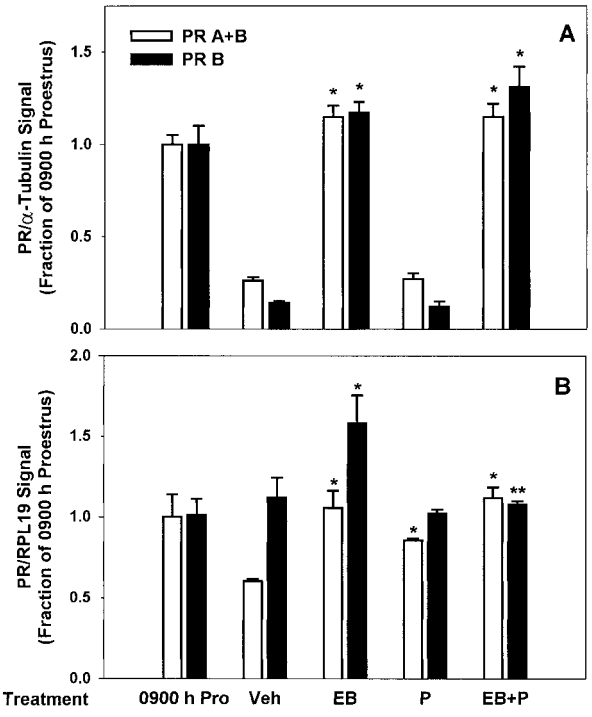

FIG. 5. Effects of ovariectomy and sequential E and P_4 replacement on Days 10 and 11 on PR A+B and PR B mRNA levels in anterior pituitary (**A**) and uterus (**B**) on Day 12. Data are the mean ± SEM of 4–5 observations. Messenger RNA levels were calculated as in the preceding figures. **A**) Ovariectomy significantly reduced mRNA levels for PR A+B and PR B ($P < 0.0001$), compared to intact, 0900-h-proestrus (Pro) rats. Fold induction of mRNA for PR A+B and PR B, compared to vehicle-treated controls, was, respectively, 4.4 and 8.4 (EB treatment) and 4.3 and 10.5 (EB + P_4 treatment). **B**) Ovariectomy significantly reduced mRNA levels for PR A+B only ($P = 0.01$), compared to intact, 0900-h-proestrus rats. Fold induction of mRNA for PR A+B and PR B, compared to vehicle-treated controls, was, respectively, 1.7 and 1.4 (EB treatment) and 1.85 and 1.0 (EB + P_4 treatment). *$P < 0.05$ compared to vehicle-treated control; **$P < 0.05$ compared to treatment with EB alone.

(FSH) surges on the evening of proestrus and the morning of estrus.

Experiment 2: Effect of Treatment with E on Proestrus on PR A+B and PR B mRNA Levels on Estrus and Metestrus

Administration of 50 μg EB to intact female rats at 1700 h on proestrus raised pituitary PR A+B and PR B mRNA expression 2.3- and 3.1-fold compared to vehicle-treated controls at 1230 h on estrus, and the effect persisted through the morning of metestrus, when induction by E was 2.3- and 2.8-fold, respectively (Fig. 3A). The effect of E treatment on the message for both total receptor and the B receptor was highly significant overall ($P < 0.0001$). In the uterus (Fig. 3B), treatment with E also had a highly significant effect on mRNA for PR A+B ($P < 0.0001$) and PR B ($P = 0.0002$): the fold increases of PR A+B and PR B mRNA were 4.6 and 3.1 on estrus and 3.5 and 1.8 on metestrus. The low gonadotropin levels at 1230 h on estrus and 0900 h on metestrus were not affected by administration of EB (data not shown). Serum E_2 levels of EB-treated animals were markedly elevated (> 500 pg/ml) on both estrus and metestrus, compared to the low levels present in vehicle-treated controls (15.4 ± 1.8 and 9.5 ± 1.6).

Experiment 3: Effects of Treatment with Antiprogestins on Proestrus on PR A+B and PR B mRNA Levels on Estrus

The effects of blockade of P_4 action by administration on late proestrus of ZK-98299 and RU-486 on PR A+B and PR B mRNA levels in the anterior pituitary (A) and the uterus (B) at 0900 h on estrus are shown in Figure 4. Blockade of P_4 action was confirmed by persistent uterine ballooning in all antiprogestin-treated animals. Treatment with antiprogestins did not have a significant effect on either PR A+B or PR B mRNA levels in the anterior pituitary. In contrast, in the uterus, both antiprogestins increased mRNA levels for PR A+B 1.6-fold ($P = 0.02$) and that for PR B 2-fold ($P = 0.0002$) at 0900 h on estrus. Consistent with our previously reported data [3], serum FSH was significantly suppressed by RU-486, but not by ZK-98299; serum LH was modestly elevated by both treatments.

Experiment 4: Effects of Ovariectomy and Replacement with E and P_4 on PR A+B and PR B mRNA Levels

The effects of ovariectomy and sequential E and P_4 replacement on Days 10 and 11 on PR mRNA levels in anterior pituitary (A) and uterus (B) on Day 12 are shown in Figure 5. In the anterior pituitary, ovariectomy significantly lowered mRNA for PR A+B and PR B to 26 ± 2 and 14 ± 1% of the proestrous control, respectively ($P < 0.0001$ for both). In the uterus, ovariectomy caused a smaller reduction in PR A+B, but not PR B, mRNA to 61 ± 1% of proestrous control ($P = 0.01$); it should be emphasized, however, that because of the marked (> 90%) decrease in the size of the uterus by 12 days postovariectomy, there was a significant overall reduction in total RNA and thus

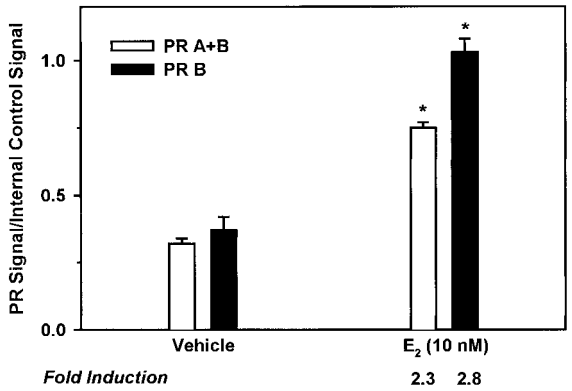

FIG. 6. Effects of exposure to E_2 (10 nM) for 96 h on PR A+B and PR B mRNA levels in primary monolayer culture of anterior pituitary cells from metestrous rats. Data are the mean ± SE of PR/α-tubulin ratios from four culture wells. Treatment with E_2 significantly increased the mRNA expression for both PR A+B and PR B. *Significant difference ($P \leq 0.0001$) compared to vehicle-treated controls.

mRNA for both isoforms. In the pituitary, treatment with E produced a robust 4.4 and 8.4 induction in the mRNA for PR A+B and PR B, respectively, compared to vehicle-treated controls; treatment with P, alone or in combination with E, on the other hand, had no effect on basal or E-stimulated PR mRNA. In the uterus, E alone caused a significant 1.7 ($P < 0.05$)- and 1.4 ($P < 0.01$)-fold increase in mRNA for PR A+B and PR B, respectively, compared to vehicle-treated controls. Again, it is important to note that the weight of the uterus more than doubled two days after E administration to rats OVX for ten days, making the increase in total receptor mRNA considerably greater than the fold induction based on a constant amount of input RNA in the RT-PCR. Treatment with P_4 alone, in contrast to the pituitary, caused a small but significant increase in PR A+B message ($P < 0.05$), without affecting the message for PR B; in combination with E, P_4 significantly reduced the PR B mRNA response to E from 1.85 to 1.0 times that of the vehicle-treated controls ($P = 0.02$). There was a marked increase in serum FSH after ovariectomy to levels that exceeded the preovulatory and secondary surges (29.8 ± 1.8 ng/ml); this elevated serum FSH was significantly suppressed by E. Treatment with P_4 alone did not affect serum FSH, but when given after E priming, reversed the E-induced suppression. Serum LH rose to a lesser degree in response to ovariectomy (7.3 ± 0.7); the elevated level of this gonadotropin was also significantly suppressed by E ($P < 0.0001$); P_4 replacement had no effect on basal serum LH, nor did it reverse the E-induced suppression, although there was a significant interaction between the two treatments ($P = 0.001$) (data not shown). Serum levels of E_2 and P_4 that resulted from administration of 50 μg EB or 5 mg P_4 on Days 10 and 11 were 535 ± 14 pg/ml and 4088 ± ng/ml, respectively.

Experiment 5: Effects of E Addition In Vitro on PR A+B and PR B mRNA Expression in Primary Anterior Pituitary Cell Cultures

Addition of 10 nM E_2 to primary cell cultures prepared from anterior pituitary of metestrous rats throughout the 96-h incubation raised mRNA levels for PR A+B and PR B 2.3- ($P < 0.0001$) and 2.8-fold ($P = 0.0001$) (Fig. 6). The extent of this induction was similar to that of the induction in the pituitary by EB administration to intact proestrous female rats in vivo.

DISCUSSION

Previous studies of PR mRNA regulation in primate anterior pituitary [10] and rat anterior pituitary cells [11] relied on in situ hybridization or Northern blot analysis with probes that did not distinguish between mRNA for the A and B isoforms. To investigate the possibility of differential regulation of mRNA for the two isoforms in rat anterior pituitary by gonadal steroids, in the present study we developed a new RT-PCR protocol with primers that anneal to the B-specific 5′ UTR of the rat PR; this protocol enabled us to analyze PR B mRNA expression in parallel with that for total (PR A+B) receptor. We demonstrate for the first time PR B mRNA expression in the anterior pituitary of the rat and its regulation by endogenous and exogenous E. An approach similar to that used in the present work was attempted previously by Kato and coworkers [23], who generated PR A+B- and PR B-specific hybridization probes by RT-PCR from RNA from rat uterus. However, because the sequence of the rat PR gene had not yet been reported at the time that their study was performed, their primers, based on the sequence of the human PR, yielded PCR products with only partial homology to rat PR. The PR-B-specific probe thus generated was used to examine PR mRNA levels only in the brain, not in the pituitary.

Because the promoter and coding sequence of PR A are fully contained in the coding sequence for B, it is not possible to detect mRNA for PR A selectively; changes in PR A mRNA can be inferred, however, from those in total receptor and PR B. The two region-specific PCR protocols have allowed us to differentiate between changes in PR B mRNA expression relative to that of total receptor as well as to draw certain conclusions about the relative abundance of the message for each isoform. First, a weak signal in the PR B-specific PCR reaction in the face of a strong signal in the PCR for total receptor would indicate that it is primarily the A isoform that accounts for the latter signal, while the B isoform is minimally expressed. Our data suggest that this may indeed be the case in the pituitary of the male rat, where the B-specific signal was barely detectable; this was not true, however, for the pituitary of female rats, which gave a strong PR B signal under all experimental conditions. Conversely, if the PR B signal were always to change in parallel with total message and its intensity were similar to that of total message under amplification conditions carefully equalized, then one could conclude that total message was accounted for entirely by that for the B isoform; this, too, was clearly not the case. Assuming comparable efficiencies of the two PCR reactions, the abundance of PR B mRNA was approximately one third that of total receptor in the anterior pituitary, compared to approximately one half in the uterus. Potential differences between the two reactions may, however, confound interpretation of these findings. Precise quantification of the ratio of the two isoforms in the rat pituitary will require immunoblot analysis, as has been performed in murine [24] and primate [25,26] target tissues and in the rat uterus [27]. When PR mRNA levels and PR protein were studied concurrently in the uterus across the ovine estrous cycle [28], parallel changes were consistently demonstrated, validating the use of steady-state mRNA levels as a sensitive index of PR gene expression.

A large body of evidence indicates opposite regulation of PR by the gonadal steroids E and P_4 in the uterus and certain breast cancer cell lines [15,16], with E increasing expression of the receptor and P_4 antagonizing this action

of E. The major finding of the present study was the marked up-regulation of the message for both isoforms of PR in anterior pituitary by E administration to both intact and OVX rats. The data further suggest rapid, sustained induction of PR mRNA as demonstrated in rat uterus [29] and in the Rat-1 cell line [30]. At the same time, we were unable to demonstrate down-regulation of mRNA expression for the two isoforms of PR by P, in conflict with earlier evidence that P_4 down-regulates PR protein and mRNA in the rat [7–9,11] and monkey [10,31] anterior pituitary. The explanation for this discrepancy may lie in the widely different timing of treatment as well as species difference. For example, the rapid and transient down-regulation of PR mRNA by P_4 in vitro described by Turgeon and coworkers [11] would not have been detected under our in vivo treatment schedule, in which blood and tissues were collected 17 h after administration of P. That our inability to demonstrate an effect of P_4 or antiprogestins in the pituitary was not due to an inherent flaw in methodology is ruled out by demonstration of appropriate changes in the uterus. Our finding that in the uterus the effect of E on PR B mRNA was antagonized by treatment with P_4 is consistent with that reported by Kraus and Katzenellenbogen [29], who demonstrated that P_4 blocked the effects of E on both PR mRNA and PR B protein in the uterus of immature rats and that RU-486 reversed this effect of P_4.

In the anterior pituitary, mRNA levels for both the PR A and PR B isoforms were highest at 0900 h on proestrus, a time when serum E_2 was highest and serum P_4 lowest. At 1830 h on proestrus, PR mRNA levels were already significantly lower than the 0900 h values; this was 3–4 h after the rapid fall in serum E_2 but before peak levels of serum P_4 had been reached [32]. Administration of E on the evening of proestrus prevented the dramatic fall in PR mRNA levels that occurred in vehicle-treated rats on the morning of estrus, coincident with low levels of serum E_2. On the basis of this finding, combined with our failure to demonstrate a significant effect of blockade of P_4 action on the evening of proestrus, we conclude that the observed changes in PR mRNA levels are more likely a consequence of changes in serum E_2 than in serum P_4 concentrations.

Our data reveal several instances of differential regulation of mRNA levels for PR B as compared to that for total receptor in the anterior pituitary. Although at most times across the estrous cycle PR B and total receptor changed in parallel, an exception to this was the morning of estrus when the mRNA levels for PR B reached an earlier and lower nadir than those for total receptor. Ovariectomy after two and ten days lowered PR B mRNA more than PR A+B mRNA, whereas treatment of OVX rats with E induced a greater fold induction of PR B than of PR A+B mRNA. Regulation of PR mRNA was sexually dimorphic: in the male rat, total receptor was expressed at a much lower level than in the female, and mRNA for the B isoform was virtually absent. Orchiectomy of short duration, contrary to ovariectomy, did not affect mRNA levels in this tissue, although long-term testosterone treatment was reported to up-regulate PR message in the lateral and dorsal lobes of the rat prostate [33]. The differential regulation of PR A and PR B that was demonstrated in the present study lends support to the existence in the rat of separate, E-inducible mRNA transcripts encoding the two isoforms, which to date has been established only for human PR [14]. Although in a recent study Bethea and Widmann [25] did not find evidence for differential regulation of the isoforms of PR, as determined by immunoblot analysis, this discrepancy may again be accounted for by a species difference.

Differential regulation of the two isoforms of PR does not explain our previous in vivo and in vitro findings of E-dependent suppression of FSH synthesis and secretion by the antiprogestin RU-486 [2,4]. Both isoforms of PR were expressed at low levels in the anterior pituitary at times when RU-486 was ineffective in lowering FSH secretion, i.e., on estrus or in primary pituitary cell cultures derived from metestrous rats. It is possible that in the absence of E this low level of PR message is not translated into functional receptor protein in sufficient quantity to bind the antagonist and inhibit FSH synthesis. Alternatively, E may induce, in addition to PR mRNA, another factor, such as a corepressor [34], whose recruitment is required for blockade of transcriptional activation of target genes by the antagonist-occupied PR.

In summary, our data demonstrate tissue-specific, E-regulated PR A and PR B gene expression in the rat anterior pituitary, which differs from that in the classical target organ, the uterus.

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