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## Research Article

# Progesterone receptors A and B differentially modulate corticotropin-releasing hormone gene expression through a cAMP regulatory element

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**Abstract.** Corticotrophin-releasing hormone (CRH) plays a major role in mechanisms controlling human pregnancy and parturition. Gene regulation by progesterone may be a key point in the control of placental CRH production. Studies in primary placental cells show that antagonism of progesterone activity or production by RU486 or trilostane leads to an increase in CRH promoter activity. This effect can be reversed by the addition of progesterone. Overexpression of progesterone receptor A (PR-A) or glucocorticoid receptor resulted in a decrease in CRH promoter activity following progesterone treatment, whereas an increase in promoter activity was observed with overexpressed PR-B. Studies including mutation of the cAMP regulatory element (CRE) confirm this site to be essential for the progesterone-mediated effects. In summary, our results demonstrate that progesterone regulates CRH gene transcription via a CRE in the CRH promoter and that PR-A and PR-B exhibit different actions in the regulation of CRH gene expression.

**Key words.** CRH; cAMP; steroid hormone; gene regulation; pregnancy.

Corticotrophin-releasing hormone (CRH), a 41-amino-acid neuropeptide secreted by the paraventricular nucleus of the hypothalamus, is the principal mediator of the hypothalamo-pituitary-adrenocortical response to stress [1]. CRH is also found in many tissues outside the central nervous system; during human pregnancy this peptide is produced in large amounts by the placenta [2–3]. Placental CRH is synthesized in syncytiotrophoblast cells and secreted into both maternal and fetal circulations. CRH derived from the placenta is identical to that produced in the hypothalamus in structure, immunoreactivity and bioactivity. Biosynthesis and secretion of placental CRH increase exponentially with advancing gestation and this increase is mirrored by exponential increases in CRH concentration in maternal plasma [4–6]. In the human, abnormally elevated maternal CRH levels during pregnancy are associated with preterm delivery, whereas unusually low levels correlate with longer than normal gestations [7–9]. Placental CRH production only occurs in humans and higher primates such as gorillas, chimpanzees and rhesus monkeys but not in lemurs or non-primates such as sheep [10, 11]. Consequently, placental CRH has been suggested to play a key role in the timing of birth [7, 12]. Thus, an understanding of how CRH production is controlled in placental syncytiotrophoblast cells is a prerequisite to gain an insight into this timing mechanism operated by CRH.

The control of CRH production by the placenta is multifactorial. A variety of endogenous factors are known to

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regulate placental CRH production, including norepinephrine, acetycholine, interleukin-1, vasopressin and steroid hormones [13–17]. In particular, glucocorticoid stimulates placental CRH production and gene expression, in contrast to its inhibitory effects in the hypothalamus [15, 16]. Moreover, we have shown recently that estrogen has a tonic inhibitory effect on CRH gene expression in placental cells [17]. Progesterone (P4) is also implicated in the regulation of CRH expression. Jones and coworkers [15] demonstrated that P4 decreased basal CRH release in primary cultures of syncytiotrophoblast. Furthermore, Karalis et al. [18] concluded that P4 down-regulation of CRH gene expression in placenta occurred through the glucocorticoid receptor (GR) because they did not detect P4 receptor (PR) expression in placental cells. However, several studies have shown that the PR is expressed by placental cells [19, 20]. Thus the mechanisms by which P4 could inhibit placental CRH production are unclear.

Therefore, the aim of this study was to examine the effects of P4 on CRH gene expression and to identify regulatory elements of the human CRH gene involved in the P4 response. We show that P4 down-regulates CRH gene promoter activity via a mechanism that is specifically directed through a cAMP response element (CRE) located in the CRH gene promoter region. In addition, GR, PR-A and PR-B have specific and distinct effects on CRH promoter activity.

## Materials and methods

### Plasmids

Luciferase reporter plasmids (Promega, Madison, Wis.) were used for transient transfections as described previously [21–23]. A human CRH genomic clone (CRH 1001+) containing 5.5 kb of the CRH gene 5′-flanking DNA was provided by J. Majzoub (Harvard University, Boston, Mass.) and deletions and mutants of the CRH promoter were made as described previously [21–23]. The expression vectors for PR-A and PR-B were provided by C. Clarke (University of Sydney, Australia) [24]. The GR expression vector was supplied by R. Evans (Salk Institute, San Diego, Calif.].

### Cell culture

Human term placentae were obtained from normal pregnant women after spontaneous vaginal delivery or elective cesarean section. Collection of placentae was performed with the approval of Changhai Hospital Ethics Committee (Shanghai, China). Cytotrophoblasts were cultured as described previously [25]. Briefly, cotyledons were removed from the maternal side and were dispersed with trypsin (Sigma-Aldrich) and deoxyribonuclease I (Sigma-Aldrich). A purified fraction of cytotrophoblasts was obtained following Percoll (Pharmacia, Uppsala, Sweden) gradient centrifugation. Cells were distributed into 12-well plates at a density of $1 \times 10^6$/well and grown in phenol-red-free DMEM (Life Technologies, Grand Island, N. Y.) containing 10% fetal calf serum (FCS).

### Transfections

Transient transfections were performed using a calcium phosphate method as described previously [21, 22, 26]. The transfection efficiency was routinely determined to be greater than 2% by in situ staining for $\beta$-galactosidase activity in primary placental cells transfected with an SV-galactosidase plasmid (Promega) at a cell density of $1 \times 10^6$ per well (12-well plate). Typically, 1 day before transfection, $1 \times 10^6$ cells/well were seeded and fed with phenol-red-free DMEM containing 10% charcoal-stripped FCS in 12-well plates, and incubated at 37 °C in a 5% $CO_2$ atmosphere. Each well was transfected with 6.5 µg (total) experimental DNA and 0.2 µg control DNA (PRL-TK vector; Promega) in 0.15 ml of HEPES buffered saline $CaCl_2$ solution, in a 5% $CO_2$ atmosphere at 37 °C. In cotransfections, 0.5 µg of steroid hormone receptor expression plasmid was used. Transfection medium was changed after 8 h to DMEM without FCS and treated with various agents as indicated. P4 and RU486 (RU) were purchased from Sigma-Aldrich; 8-bromo-adenosine-3′,5′-cyclic monophosphate [8-bromo cAMP] was purchased from Calbiochem (Biosicences. Inc. La Jolla, Calif.); Trilostane was provided by Sanofi Pharmaceuticals (Malvern, Pa.). P4, RU, trilostane and 8-bromo cAMP were added to the treatment medium from stock solutions in absolute ethanol. Control medium contained the same final solvent concentration (typically ≤ 0.01% v/v). The luciferase assay was carried out 3–48 h later with the dual luciferase assay kit (Promega). Relative luciferase activity is presented as firefly luciferase values normalized to *Renilla* luciferase activity.

### Western blot analysis

Cells were scraped off dishes in the presence of lysis buffer consisting of 60 mM Tris-HCl, 2% sodium dodecyl sulfate (SDS), 10% sucrose, 2 mM phenylmethylsulfonyl fluoride (Merck, Darmstadt, Germany), 1 mM sodium orthovanadate (Sigma-Aldrich), 10 µg/ml aprotinin (Bayer, Leverkusen, Germany). Lysates were quickly sonified, heated for 5 min at 95 °C, and stored at −80 °C until used. Protein concentrations were measured using a modified Bradford assay and samples were diluted in sample buffer [250 mM Tris-HCl (pH 6.8)], containing 4% SDS, 10% glycerol, 2% $\beta$-mercaptoethanol, and 0.002% bromophenol blue] and heated at 95 °C for a further 5 min. Aliquots of proteins were separated by SDS-PAGE (10%) and subsequently transferred to nitrocellulose membranes by electroblotting. Membranes

were blocked in 5% non-fat milk in TBS/Tween 20 (0.5%) for 30 min and incubated in the presence of the PR antibody (sc-538; Santa Cruz Biotechnology) or GR antibody (sc-1003; Santa Cruz Biotechnology) overnight at 4 °C. Proteins were detected using the enhanced chemiluminescence kit (Amersham-Pharmacia Biotech, Piscataway, N. J.) after incubation of blots for 2 h at room temperature with corresponding horseradish peroxidase-conjugated antibodies (Santa Cruz Biotechnology).

### Statistical analyses

Statistical analyses were carried out using Student's paired t tests. The values are expressed as the mean ± SE. Multiple comparisons were statistically compared by one-way ANOVA.

## Results

### P4 down-regulates CRH promoter activity

Because placental cells produce a high level of P4 endogenously, we used a common P4 antagonist, RU, to indirectly examine the effects of P4 on human CRH promoter activity in placental cells. Interestingly, a dose- and time-dependent increase in CRH promoter activity was observed after addition of RU (fig. 1A, B). To further confirm the inhibitory role of P4 in placental CRH expression, the endogenous production of P4 from placenta was inhibited by the addition of trilostane (a 3$\beta$-hydroxysteroid dehydrogenase inhibitor). A dose- and time-dependent inhibition of P4 production was observed with maximal inhibition achieved at 0.1 mM trilostane (data not shown) and, furthermore, this treatment with trilostane blocking P4 production resulted in a 30% increase ($p < 0.01$) in CRH promoter activity (fig. 1C, D). Maximal effects of both RU and trilostane was observed by 12 h. The induction of CRH promoter activity by RU and trilostane was reversed by the replacement of P4 (fig. 1E). These results strongly suggest an inhibitory role for P4 in regulating expression of the CRH gene in the placenta.

### Effects of cotransfection of PR and GR on CRH promoter activity

The expression of PR and GR in placental cells was examined by Western blot analysis (fig. 2). Placental cell proteins (50 µg) were separated in SDS-PAGE and detected with antibody against PR and GR (Fig. 2A). GR, PR-A and PR-B proteins were all detected in placental cells with PR-B levels being almost three-fold higher than PR-A. GR protein appears to be present at much higher levels than PR, an observation supported by transcript analysis [R. Nicholson, unpublished data]. Transfection of human GR, PR-A or PR-B expression vectors into placental cells resulted in an increase in the corresponding GR (fig. 2B), PR-A or PR-B (fig. 2C) protein levels.

Cotransfection of the CRH promoter reporter plasmid (CRH5500) with GR or PR expression vectors uncovered differential responses to P4 and RU treatment in cells overexpressing human GR, PR-A or PR-B proteins (fig. 2D). In cells overexpressing GR, P4 inhibited whereas RU and trilostane stimulated CRH promoter activity. Similarly, overexpressing PR-A resulted in a decrease in promoter activity after P4 treatment. In contrast, overexpression of PR-B resulted in a stimulatory effect on CRH gene transcription by P4 and an inhibitory effect by RU. The overexpression of PR-A resulted in a 28% reduction in basal CRH promoter activity, while overexpressing PR-B or GR had no noticeable effect on basal CRH promoter activity. These data suggest that PR-A is primarily responsible for the effect of P4 on CRH gene expression in placental cells, although the overall effect of P4 may be modulated by relative levels of PR-B and GR.

### Localization of P4 response region in the CRH 5′-flanking region

A series of 5′ deletion mutants from the 5.5-kb 5′-flanking region of the human CRH gene were analyzed in placental cells treated with 10 µM RU to localize the specific region that mediates the P4 effects (fig. 3). Progressive deletion to a construct containing only 248 bp of the promoter region did not affect RU-induced stimulation of human CRH promoter activity in placental cells. Further deletion of the sequence from −248 to −213 resulted in a loss of the RU-induced stimulation. This result indicates that the region between −248 to −213 (relative to the transcription start site) is involved in P4-mediated action. Further analysis by cotransfection of the cells with either a GR-, PR-A- or PR-B-expressing plasmid, together with the 5′ deletion mutant series reinforced the observation that the region between −248 to −213 is responsible for P4 action (fig. 3).

### P4 effects are through a CRE

There is a consensus CRE located within the region between −248 to −213 of the CRH promoter. To further explore whether this element is required for P4 regulation of the CRH gene, we used a CRH reporter fusion construct in which the CRE sequences were specifically mutated. P4 regulated transcriptional activity of the wild-type promoter, whereas mutation of the CRE abolished P4-mediated responses, independent of the absence or presence of overexpressed GR, PR-A or PR-B (fig. 4).

We further examined the role of the CRE by testing whether the CRE can transfer P4 regulation to a heterologous promoter (fig. 5). Linking the CRE DNA sequence, as found in the CRH promoter, in front of a minimal rabbit $\beta$-globin gene promoter resulted in a signifi-

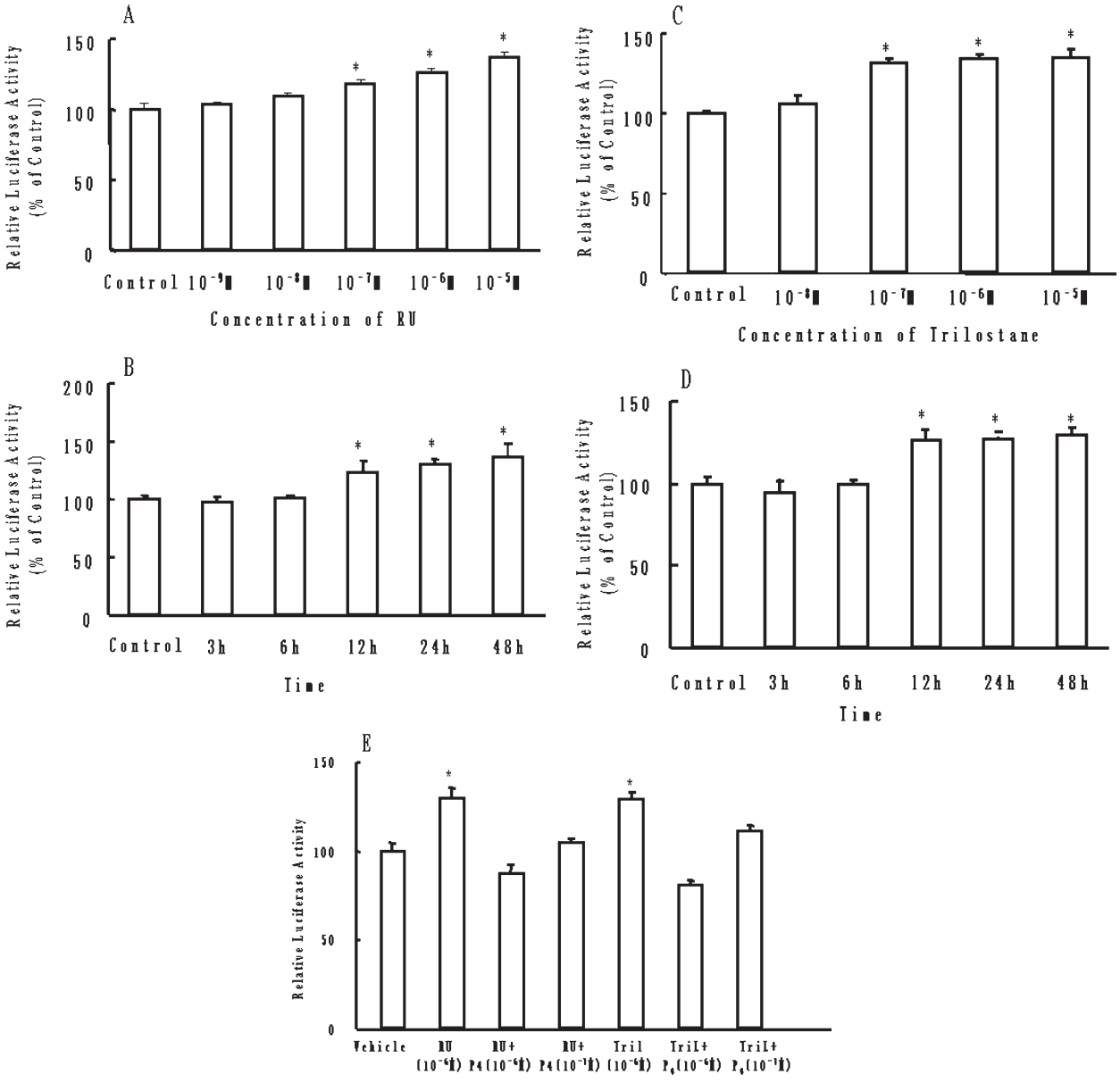


Figure 1. Time- and Dose-dependent regulation of CRH promoter activity in placental cells treated with RU (*A*, *B*), or trilostane (*C*, *D*), and reversal of the effect of P4 on RU and trilostane actions (*E*). (*A*) Primary placental cells were transfected with pCRH[5500]-GL3 plasmid DNA and exposed to either vehicle (0) or the indicated concentration of RU for another 24 h, then harvested for the luciferase assay. Relative promoter activity is shown as percentage of vehicle. Values represent the mean ± SE from four independent experiments. *$p < 0.01$ compared with vehicle controls. (*B*) Cells were transfected with pCRH[5500]-GL3 plasmid DNA and exposed to $10^{-6}$ mol/l RU for the indicated time before harvesting for the luciferase assay. Relative promoter activity is shown as percentage of vehicle. Values represent the mean±SEM from three independent experiments. *$p < 0.01$ compared with vehicle controls. (*C*) Primary placental cells were transfected with pCRH[5500]-GL3 plasmid DNA and exposed to either vehicle (0) or the indicated concentration of trilostane for 24 h, then harvested for the luciferase assay. Relative promoter activity is shown as percentage of vehicle. Values represent the mean ± SE from four independent experiments. *$p < 0.01$ compared with vehicle controls. (*D*) Cells were transfected with pCRH[5500]-GL3 plasmid DNA and exposed to $10^{-6}$ mol/l trilostane for the indicated time before harvesting for the luciferase assay. Relative promoter activity is shown as percentage of vehicle. Values represent the mean ± SE from three independent experiments. *$p < 0.01$ compared with vehicle controls. (*E*) Primary placental cells were transfected with pCRH [5500]-GL3 reporter gene. After transfection, the cells were incubated for an additional 24 h in the presence of RU and increasing concentrations of P4, or in the presence of trilostane and P4. Relative promoter activity is shown as percentage of vehicle. Values represent the mean ± SE from three independent experiments. *$p < 0.01$ compared with vehicle controls.

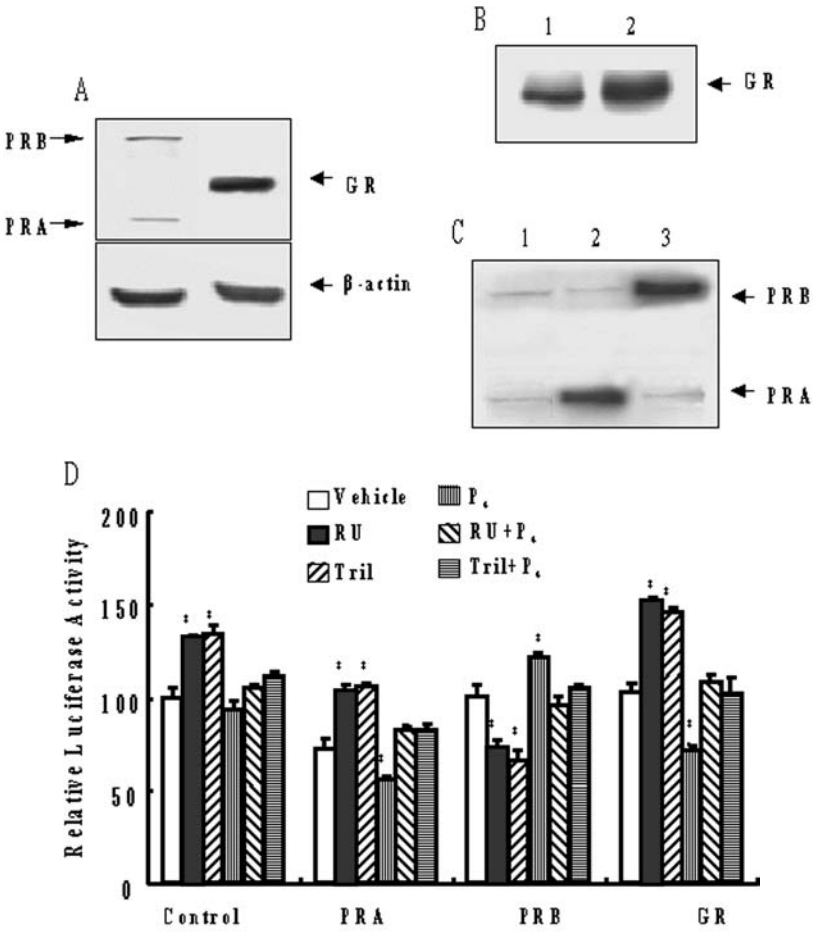


Figure 2. Western analysis of PR and GR. (*A*) Basal expression of GR and PR protein in primary placental trophoblasts. Total cellular protein isolated from placental cells was separated and immunoblotted with PR and GR antibodies as described in Materials and methods. (*B*) Overexpression of human GR. Total cellular protein was isolated from primary placental cells 8 h after transfection with 0.5 µg of GR-expressing plasmid, and then separated and immunoblotted with GR antibody as described in materials and methods. (*C*) Overexpression of PR-A (lane 2) and PR-B (lane 3). Total cellular protein was isolated from primary placental cells 8 h after transfection with 0.5 µg of the corresponding PR expression vector, and then separated and immunoblotted with PR antibody as described in Materials and methods. (*D*) Effect of overexpressing PR-A, PR-B and GR on CRH promoter activity in placental cells treated with P4 and RU. The pCRH[5500]-GL3 reporter construct-transfected cells were treated with $10^{-7}$ mol/l P4, $10^{-6}$ mol/l RU or $10^{-6}$ mol/l trilostane [Tril] for 24 h without (control) or with cotransfection of PR-A, PR-B and GR expression vector. Relative promoter activity is shown as a percentage of vehicle-treated, pCRH[5500]-GL3-transfected control cells. Values represent the mean ± SE from five independent experiments. *$p < 0.01$ compared with vehicle.

cant P4 regulatory effect on promoter activity, confirming that the CRE is necessary and sufficient to mediate P4 responses.

**P4 modulates cAMP-mediated CRH promoter activity**

To determine whether P4 modulates cAMP-stimulated CRH promoter activity, we examined the combined effects of P4 or RU and 8-bromo-cAMP on the expression of the CRH gene promoter in placental cells (fig. 6). Consistent with our previous results [21, 22], treatment with 8-bromo-cAMP increases CRH promoter activity. P4 inhibits whereas RU enhances cAMP-mediated promoter activity in the cells overexpressing GR or PR-A. In contrast, overexpression of PR-B in these cells results in RU-mediated inhibition of cAMP-stimulated promoter activity, and further stimulation of cAMP-stimulated promoter activity by P4.

## Discussion

The results show that the antiprogestin RU and the P4 synthesis inhibitor trilostane have stimulatory effects on CRH gene transcription in a dose-dependent manner, providing evidence that endogenous P4 has a tonic inhibitory effect on placental CRH synthesis. Due to the preexisiting high levels of endogenous P4, additional exogenous P4 has little or no effect on CRH gene expression, and this is consistent with the reduction in basal CRH gene expression in cells overexpressing PR-A.

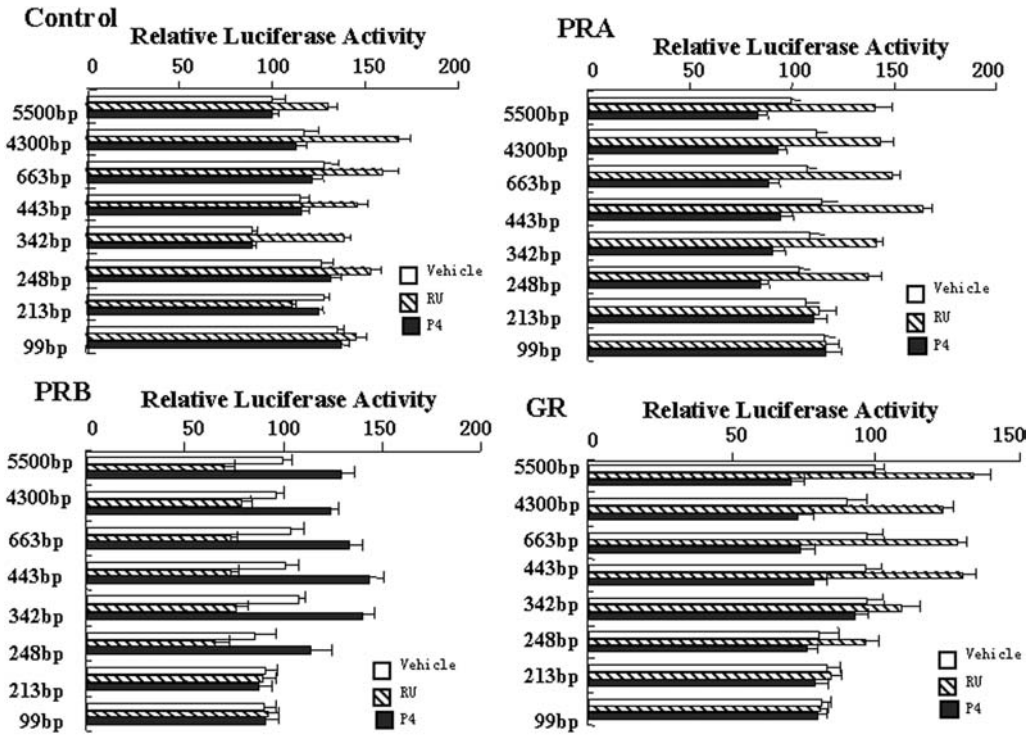


Figure 3. Localization of the P4 response region of the CRH promoter. Placental cells were transfected with constructs containing the indicated different lengths of the CRH gene promoter without (control panel) or with cotransfection of PR-A, PR-B and GR expression vector (as indicated), then treated with $10^{-7}$ mol/l P4, $10^{-6}$ mol/l RU or vehicle for 24 h. Relative promoter activities are shown as percentage of vehicle-treated, pCRH[5500]-GL3-transfected control cells. Values represent the mean ± SE from four independent experiments.

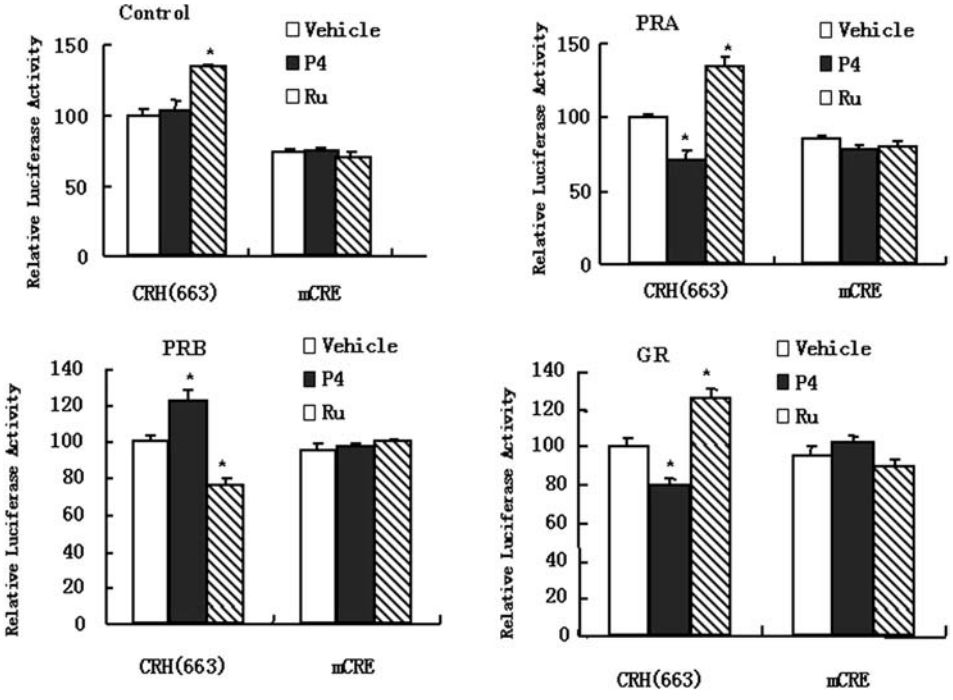


Figure 4. Mutation analysis of CRE sequences in the CRH promoter. Placental cells were transfected with the wild-type or mutant pCRH[663]-GL3 plasmids without (control panel) or with cotransfection of PR-A, PR-B and GR expression vector (as indicated), exposed to vehicle, $10^{-7}$ mol/l P4 or $10^{-6}$ mol/l RU for 24 h, and then harvested for the luciferase assay. Relative promoter activity is shown as percentage of vehicle-treated, pGLOB-GL3-transfected control cells. Values represent the mean ± SE from three independent experiments. *$p < 0.01$ compared with vehicle controls.

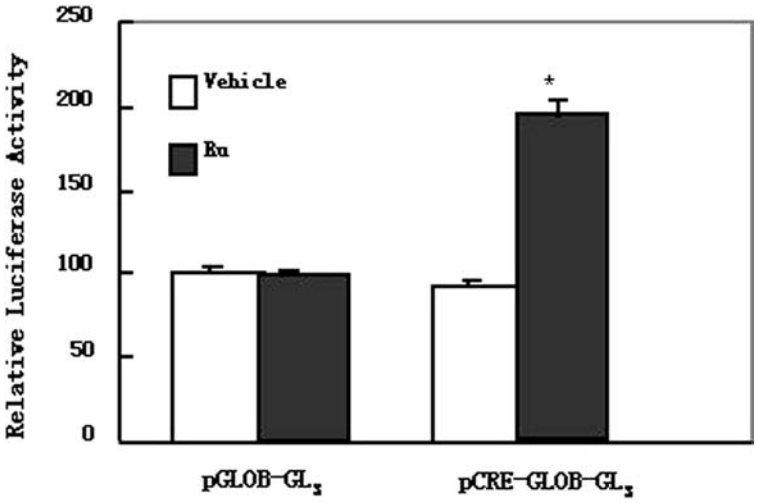


Figure 5. The CRH CRE confers progesterone responsiveness to the rabbit $\beta$-globin gene promoter in placental cells. Plasmids containing a luciferase reporter gene under the control of a basal rabbit $\beta$-globin promoter with [pCRE-GLOB-$GL_3$] or without (pGLOB-$GL_3$) a linked CRE sequence were transfected into placental cells and then exposed to vehicle, $10^{-7}$ mol/l P4 or $10^{-6}$ mol/l RU for 24 h. Values represent the mean ± SE from three independent experiments. *p < 0.01 compared with vehicle controls.

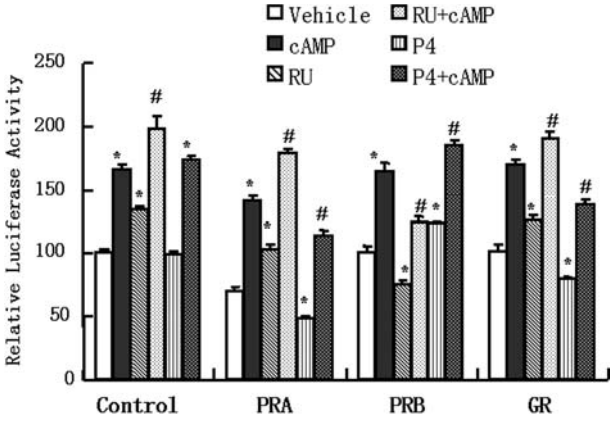


Figure 6. The effect of progesterone on cAMP-induced CRH promoter activity. Placental cells were transfected with pCRH[663]-GL3 without (control) or with cotransfection of PR-A, PR-B or GR expression vector (as indicated). Cells were then exposed to 0.5 mmol/L 8-bromo-cAMP, $10^{-7}$ mol/l P4, $10^{-6}$ mol/l RU or vehicle for 24 h before harvesting for the luciferase assay. Relative promoter activity is shown as percentage of vehicle-treated, pCRH[663]-GL3-transfected control cells. Values represent the mean ± SE from three independent experiments. *p < 0.01 compared with vehicle controls; #p < 0.01 compared with cAMP.

Reversal of P4-mediated repression by RU and trilostane confirms the negative effect of P4 in placental cells. Our results are consistent with the report by Karalis et al. [18] of CRH negative regulation by endogenous P4 in primary placental cells.

P4 mediates its biological activity by interacting with the PR within the target cells. However, there is some controversy as to whether the PR is expressed in placental trophoblasts. Several studies have not been able to detect PR in the placenta [18, 27, 28], whereas a number of other studies have [19, 20]. PR exists in human as two proteins, PR-A and PR-B, encoded by a single gene. The two forms result from alternate initiation of transcription from two distinct promoters [29]. Our Western blot analysis demonstrates that the PR-A and PR-B proteins are both present in human placental cells. To further study the role of the two PR proteins in mediating P4-induced effects in placenta, expression vectors for human PR-A and PR-B were cotransfected into primary placental cells. Overexpression of PR-B resulted in an increase in CRH promoter activity in the presence of exogenous P4 whereas, in contrast, overexpressed PR-A decreased CRH promoter activity in the presence of P4. This shows that PR-A and PR-B have opposing functions in regulating the expression of the CRH gene upon P4 treatment in placental cells. Interestingly, Cheng and coworkers [30] have also shown that PR-A down-regulates whereas PR-B up-regulates gonadotropin-releasing hormone receptor gene transcription in placenta [30]. Many other reports have also demonstrated that PR-A and PR-B mediate opposing transcriptional actions on various genes [31–35]. In most contexts, PR-B typically functions as an activator of progesterone-response genes, while PR-A is transcriptionally inactive [31, 32]. A recent report has indicated that the mechanism underlying the differential activities of the two human PR proteins is associated with differential cofactor binding [36].

Within the steroid receptor superfamily, PR and GR share regions of high homology, including the ligand-binding and DNA-binding domains [37]. P4 can not only bind to PRs but can also bind, with one-quarter the affinity of cortisol, to GR [38]. We have observed that there is a relatively large amount of GR, compared with PR, expressed in placental cells. Furthermore, overexpression

of GR resulted in decreased CRH promoter activity with P4 treatment, supporting a P4-mediated inhibitory role on CRH gene expression in placenta. These results are consistent with the findings of Patel et al. [39] where P4 stimulation of prostaglandin dehydrogenase is mediated not only by PR but also by GR. A number of other reports have also demonstrated physiological action of P4 via GR in both the absence and presence of PR [40–44].

The progressive 5′ deletion of the human CRH flanking region, mutation of the CRE within the CRH promoter context, and insertion of a CRE to drive a basal rabbit $\beta$-globin promoter conferred RU responsiveness similar to that seen with the intact CRH promoter. These results clearly indicate that a functional CRE is necessary and adequate for P4 repression of CRH gene expression in placental syncytiotrophoblast cells. These results are also consistent with the findings of Yamamoto et al. [45] where P4-mediated repression of hCG$\alpha$ gene transcription was shown to require a CRE sequence motif.

Furthermore, cotransfection analyses of various CRH promoter constructs with GR, PR-A or PR-B expression vectors revealed that mutation of the CRE within the CRH promoter context caused a loss of responsiveness to P4 involving GR, PR-A or PR-B. Indeed, several earlier reports have shown that PR and GR can regulate gene expression via the same regulatory element [46].

The amount of placental CRH production is thought to be crucial in determining human pregnancy length [4–9, 12]. When they did not detect PR in placenta, Karalis et al. [18] proposed that a blockage by glucocorticoid of an inhibitory effect of P4 on CRH gene expression accounts for the rise in CRH levels in late gestational ages. However, our data show that PR is present in placenta and, by using overexpressed GR, we showed that GR-mediated P4 inhibition of CRH gene expression occurs even in the presence of PR. Glucocorticoid, estrogen and a number of endogenous factors have already been shown to regulate CRH production and secretion [13–18, 21, 22]. Thus, the balance between up-regulation of CRH production and down-regulation of CRH synthesis may be crucial for determination of gestational length. This study and others [21–23, 47] combine to indicate that the CRE is a critical element involved in the regulation of the CRH gene.

The physiological outcome will depend on the relative concentrations of PR-B, PR-A and GR, with the former leading to stimulation of transcription and the latter two to inhibition. This fine modulation may be important in determining the exponential increase of CRH over the 9 months of human pregnancy.

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