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Parallel Regulation of Prolactin and c-*fos* Gene Expression by 17β-estradiol and Stress in the Mouse Pituitary

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The aim of this study was to investigate expression patterns of the prolactin (PRL) and c-*fos* genes by 17β-estradiol (17β-E) and stress in the mouse pituitary. In the pituitary, the levels of PRL mRNA were found high with some fluctuation at 30, 60, and 90 min whereas the levels of PRL mRNA were low at 120 min when ovariectomized female mice were injected with 17β-E or vehicle. PRL mRNA levels began to increase again at 4 h and remained high up to 24 h only in the 17β-E-treated mice. The overall changes in c-*fos* mRNA by 17β-E were very similar to those in PRL mRNA in the pituitary. Subsequent study revealed that these high initial levels of PRL and c-*fos* mRNAs were caused by stress during injection, not by 17β-E, since vehicle injection alone into the ovariectomized mice could increase the levels of PRL and c-*fos* mRNAs. The stress-induced elevations of PRL and c-*fos* mRNAs were inhibited by bromocriptin, a dopamine agonist, suggesting that the dopaminergic system is involved in the action route of injection stress. In addition, the induced levels of c-*fos* mRNA by 17β-E and stress in the pituitary were very low compared with those in the uterus. The time course changes in c-*fos* mRNA level were different between the pituitary and uterus. Taken together, these data indicate that PRL and c-*fos* gene expression in the pituitary are regulated by 17β-E and stress in a parallel manner, supporting the notion that c-Fos plays a role in regulation of PRL gene expression.

Prolactin (PRL) is a reproductive hormone which plays an important role in maintenance of lactation, survival of most mammalian young after birth (Leong et al., 1983; Neill, 1988), and stimulation of immune responsiveness (Walker et al., 1993). The synthesis and secretion of PRL are under the complex regulation of various neurotransmitters and gonadal steroids (Maurer, 1982; Leong et al., 1983; Neill, 1988; Cho et al., 1993).

c-Fos, a member of the Fos family, has been implicated in cell growth (Holt et al., 1986), differentiation (Distel et al., 1987), and development (Ruther et al., 1987). All of the members of the Fos family are able to form heterodimers with the Jun protein family members (Halazonetis et al., 1988; Hai and Curran, 1991). c-Fos / c-Jun heterodimers bind to DNA at AP-1 sites and influence a large number of genes, including collagenase and adipocyte P2 (Rausher et al., 1988; Schonthal et al., 1988).

During PRL gene expression, involvement of the c-*fos* gene has been anticipated since estrogen affects c-*fos* gene expression in the pituitary (Allen et al., 1997) as well as in the uterus (Loose-Mitchell et al., 1988). Moreover, human and rat PRL genes have AP-1 or AP-1 like site on their 5′ flanking regions (Pernasetti et al., 1997; Caccavelli et al., 1998). Thus, this study was undertaken to gain an insight into the possible involvement of c-Fos in PRL gene expression by estrogen and stress.

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Materials and Methods

Animals

Adult female ICR mice purchased from the Daehan Animal Center were maintained under light- and temperature-controlled conditions with food and water available *ad libitum*. Animals were then used according to the experimental designs.

Experimental designs

To investigate the time course changes in PRL and c-*fos* gene expression in the pituitary, adult female

mice were ovariectomized under ether anesthesia and treated with 17β-E (Sigma). 17β-E was dissolved in a small amount of 100% ethanol before dilution in saline for S.C. injection to give a final concentration of 1.2 μg 17β-E/200 μl saline. These mice were sacrificed at 30, 60, 90, and 120 min in one set of experiment and at 4 and 24 h in another set of experiment after steroid treatment. To test the responsiveness of the PRL gene to 17β-E at 120 min in the pituitary, ovariectomized mice were treated with 17β-E at doses of 0, 0.24, 1.2, 6, and 30 μg per mouse and sacrificed 120 min after injection.

To verify the effects of stress on PRL and c-*fos* gene expression, three sets of experiments were designed. In the first set, ovariectomized female mice were maintained in one cage without separation and then assigned into three groups: mice were maintained intact in group1, sham-injected in group2, and injected with saline in group3, respectively. In the second set, ovariectomized female mice were separated into two groups in each cage one day before injection. Mouse was uninjected in group1 and injected with saline in group2. In order to exclude the interaction between mice, only one mouse was maintained in each cage. In the third set, ovariectomized female mice were divided into two separate cages in order to test the possible involvement of the dopaminergic system during injection stress. In the first group, mouse was injected with saline for four consecutive days and sacrificed 30 min after the last injection. The second group was injected with bromocriptin (3 mg/kg), a dopamine agonist, instead of saline.

To compare the level of c-*fos* mRNA between the pituitary and uterus, ovariectomized adult female mice were sacrificed at 30, 60, 90, and 120 min after 17β-E injection. All experiments were repeated at least three times and representative results are presented.

Northern blot analysis

RNA extraction: RNA was extracted from three pituitaries by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987) and analyzed by RT-PCR or Northern blot as previously described (Cho et al., 1993; 1994). Briefly, one or three pituitaries were homogenized with denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% N-lauryl sarcosine, 0.1 M 2-mercaptoethanol). The homogenate was phenol/chloroform extracted and RNA was precipitated. RNA was then quantitated by a U.V. spectrophotometer (U.V. 2000, Pharmacia). Ratios of the A_{260}/A_{280} ranged from 1.8 to 2.0.

Electrophoresis: For size separation, electrophoresis on a 1.2% agarose gel was performed at 70V for 1.5 h. After RNA transfer onto Nytran filters (0.45 μm, Schleicher & Schuell) by diffusion blotting, the filters were hybridized with radio-labeled probes in a plastic bag (Kopak). cDNAs for the rat PRL (a kind gift from Dr. RA Maurer, University of Iowa) and for the mouse c-*fos* were labeled with ^{32}P-dCTP by the random primer labeling method (Feinberg and Vogelstein, 1983). After hybridization, the filters were washed, dried, and autoradiographed with X-ray film (Konica AX film) at -70℃ for 1 d for PRL and for 5 d for c-*fos*, respectively.

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR: Five micrograms of total RNA was used after quantifying in duplicate or triplicate to reduce the difference in the starting quantity. RNA was then treated with DNase (5U, Promega) at 37℃ for 10 min in order to exclude the contamination of genomic DNA, and reverse transcribed at 42℃ using random hexamer primers and AMV reverse transcriptase (Promega) in a 20 μl reaction. A mix including the oligonucleotide primers (500 ng each), dNTP, and *Taq* DNA polymerase (2.5U) was added to each reaction, the total volume was brought to 100 μl with 1 X PCR buffer [10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01% gelatin], and the sample was overlaid with light mineral oil. Amplification was performed for 30 cycles using an annealing temperature of 65℃ on an Omn-E thermal cycler (Hybaid Limited, UK). For the PRL gene, the primers were designed to generate a 651 bp PCR product. 5′ primer sequence is "GGTCAGCCCAGAAAGC AGG" and 3′ primer sequence is "GGGCAATTTGGCA CCTCAGGA". For the c-*fos* gene, the primers were designed to generate a 337 bp product. 5′ primer sequence is "CGGGTTTCAACGC CGACTACG" and 3′ primer sequence is "GACACGGTCTTCACCATTCCC". After amplification, the samples were chloroform-extracted, dried, resuspended in a 10 μl TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA), and electrophoresed on a 1.2% agarose gel. The gel was photographed after ethidium bromide staining.

RT-PCR with Biotin: During RT-PCR, dUTP-biotin was added to the reaction buffer. After amplification, PCR products were chloroform-treated, dried, and resuspended in TE buffer. After electrophoresis, PCR products were transferred to Nytran by diffusion blotting. The filters were then incubated with streptavidin alkaline phosphatase (Promega) at room temperature for 1 h. After washing, the bands were visualized with stabilized substrate (Promega) at room temperature for 5 h.

Results and Discussion

When ovariectomized female mice were injected with 17β-E or vehicle and sacrificed at 30, 60, 90, and 120 min, the levels of PRL mRNA were found high with some fluctuation at 30, 60, and 90 min whereas the levels of PRL mRNA were consistently reduced at 120

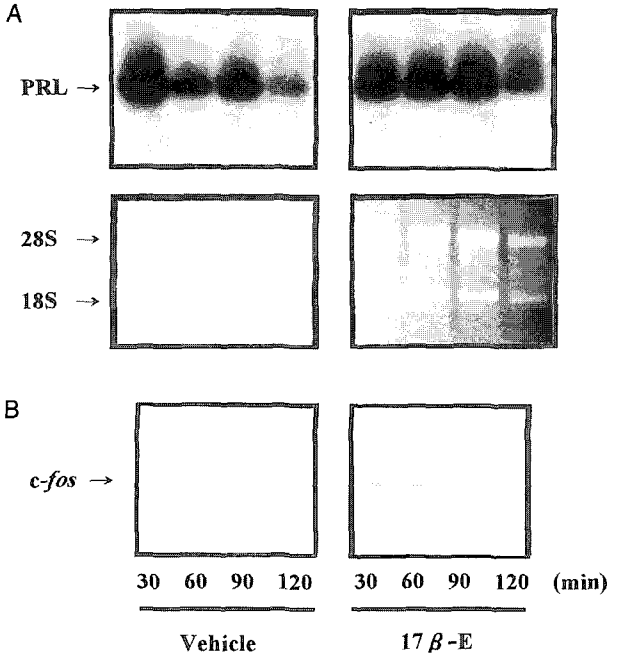

Fig. 1. Changes of PRL and c-*fos* gene expression by 17β-E. A, Ovariectomized female mice were injected with 17β-E or vehicle at the indicated times. RNAs were purified, quantitated, and electrophoresed on 1.2% agarose gels. After transfer into the Nytran, the filters were hybridized with ^{32}P-labeled cDNA. B, RNAs were reverse transcribed and subjected to PCR with dUTP-biotin and primers designed for detection of c-*fos* mRNA. After electrophoresis and transfer into the Nytran, the bands were visualized by streptavidin conjugated alkaline phosphatase.

min in both groups (Fig. 1A). Nevertheless, the PRL mRNA level of estrogen-treated mouse at 120 min was slightly higher than that of the vehicle-treated one (Fig. 1A). The overall changes in c-*fos* mRNA were very similar to those in PRL mRNA in the pituitary (Fig. 1B). However, the levels of c-*fos* mRNA were found to be very low since it could be detected only by RT-PCR utilizing biotin, not by Northern blot hybridization analysis. When the effects of 17β-E were investigated in extended time periods, the level of PRL mRNA began to increase at 4 h and remained high until 24 h (Fig. 2A). At these time points, c-*fos* mRNA were also induced so that the c-*fos* mRNA were able to be detected (Fig. 2B). The pituitary seemed to have potential to respond to 17β-E at 120 min because the level of PRL mRNA could be increased in proportion to doses of 17β- E (Fig. 3). Our results revealed that the initial high levels of PRL and c-*fos* mRNAs in both vehicle and 17β- E treated mice were caused not by 17β-E and true effects of 17β-E was observed beginning from 120 min after the treatment.

To dissect the initial elevation of PRL and c-*fos* mRNAs in detail, we performed three sets of experiments as follows. First, mice were housed in a single cage and given by injection. The results showed that the level of PRL mRNA was not changed among the intact, sham-injection, and saline-injected mice (Fig. 4B, a). Second, when the mouse was separated one day

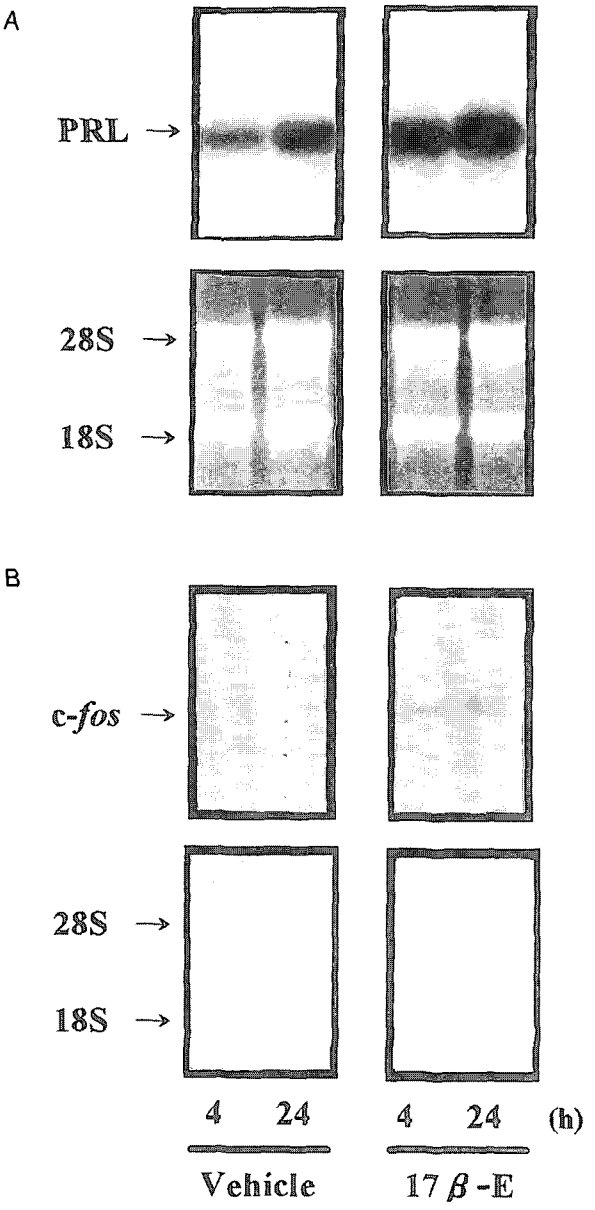

Fig. 2. Changes of PRL and c-*fos* gene expression by 17β-E. Ovariectomized female mice were injected with 17β-E or vehicle at 4 h or 24 h before sacrifice. Northern blots were performed as described in Fig.1.

before in order to exclude mutual influence during saline injection, a more enhanced level of PRL mRNA was found in the saline-injected than in uninjected mouse (Fig. 4B, b left). The level of c-*fos* mRNA was also higher in saline-injected than uninjected mouse (Fig. 4B, b right). Thus, the initial levels of PRL and c-*fos* mRNAs up to 90 min can be attributed to the injection stress and 17β-E appears to stimulate PRL mRNA 120 min after injection. Third, when bromocriptin, a dopamine agonist, was used for four consecutive days before saline injection, the levels of PRL and c-*fos* mRNAs were found lower in bromocriptin-treated mouse than in control (Fig. 4C). The dopami-

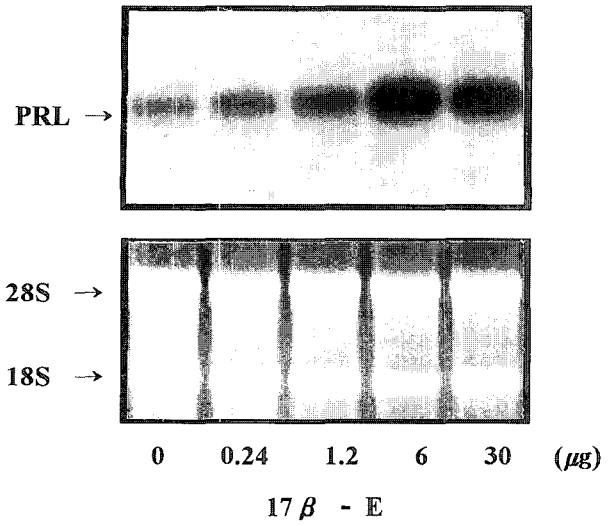

Fig. 3. Responsiveness of PRL gene to 17β-E. Ovariectomized female mice were injected with various doses of 17β-E at 120 min before sacrifice. Northern blot was performed as described in Fig.1.

nergic neurons (Leong et al., 1983; Neill, 1988) and stress (Kehoe et al., 1992) have been reported to be involved in PRL release. Blockade of stress-induced elevation of PRL and c-*fos* mRNA levels by bromocriptin in this study suggests that the dopaminergic system might be an action route of injection stress.

To compare the expression pattern of the c-*fos* gene in response to 17β-E in the pituitary and uterus, we determined the c-*fos* mRNA level in both tissues from the same mice. As revealed in Fig. 5, c-*fos* mRNA level in the uterus was increased, reached peak, and then returned to basal level within 120 min in 17β-E-treated mice whereas c-*fos* mRNA level of control animals remained undetectable with an exception of the 90 min time period (Fig. 5A). The temporal pattern of the c-*fos* mRNA level in the mouse uterus was very similar to the previous result in the rat uterus (Loose-Mitchell et al., 1988). In contrast to the pituitary, the level of c-*fos* mRNA at 4 h and 24 h after 17β-E injection was undetectable in the uterus (data not shown). The level of c-*fos* mRNA could be enhanced at 120 min by 17β-E over 0.24 μg/mouse (Fig. 5B). The equal loading of RNA in each lane was ensured as revealed by ethidium bromide staining of 28S and 18S rRNA in all figures. In addition, the absolute level of c-*fos* mRNA was much higher in the uterus than in the pituitary as revealed in the results of Northern blot hybridization analysis (Fig. 5C, left) and RT-PCR (Fig. 5C, right).

We suggested that c-Fos mediates PRL gene expression by estrogen and stress based on the following reasons. First, as already reported by several groups, 5′ flanking regions of the human (Pernasetti et al., 1997; Caccavelli et al., 1998) and rat PRL gene (Gourdji and Laverriere, 1994) contain an AP-1 site or an AP-1-like site, which are believed to be functional.

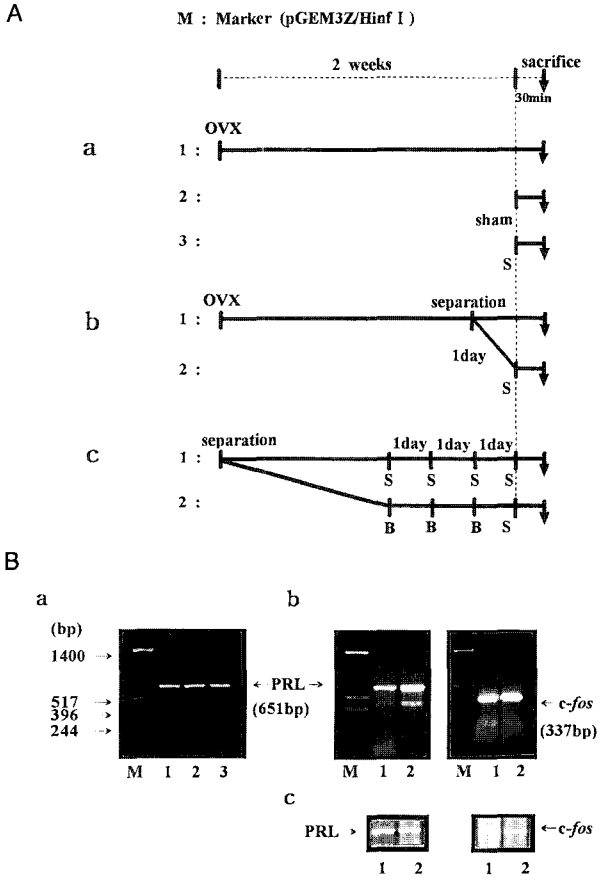

Fig. 4. PRL and c-*fos* mRNAs induced by stress. A, Experimental designs are schematically represented. B, (a) Ovariectomized female mice were maintained in one cage without separation. The first group was not injected (lane 1), the second was sham-injected (lane 2), and the third was injected with saline (lane 3) 30 min before sacrifice. (b) In contrast, ovariectomized female mice were separated into two groups in each cage one day before injection. The first group was maintained uninjected (lane 1) and the second was injected with saline (lane 2). (c) Ovariectomized female mice were separated into two groups in each cage. The first group were injected with saline for four consecutive days and sacrificed 30 min after the last injection (lane 1). The second were injected with bromocriptin instead of saline (lane 2). Five micrograms of RNA from one pituitary was reverse transcribed and subjected to PCR. After electrophoresis, the bands were visualized with ethidium bromide. S, saline; B, bromocriptin.

Second, a temporal coincidence was observed between the levels of PRL and c-*fos* mRNAs during the action of estrogen and stress in our *in vivo* study. Similarly, a parallel increase of PRL and c-*fos* mRNA levels was reported during the action of calcium (Bandyopadhyay and Bancroft, 1989) and thyrotropin-releasing hormone in cells cultured *in vitro* (Passegue et al., 1995). Finally, a low abundance of c-*fos* mRNA in the pituitary which is detectable only by RT-PCR, in addition to the differential pattern of c-*fos* mRNA between the pituitary and uterus, implies other roles of c-Fos in the pituitary such as gene regulation not related to cell proliferation (Holt et al., 1986; Ruther et al., 1987). Another possibility, though very unlikely, is that newly synthesized PRL acts, in turn, on the c-*fos* gene by feedback mechanism, resulting in a rapid

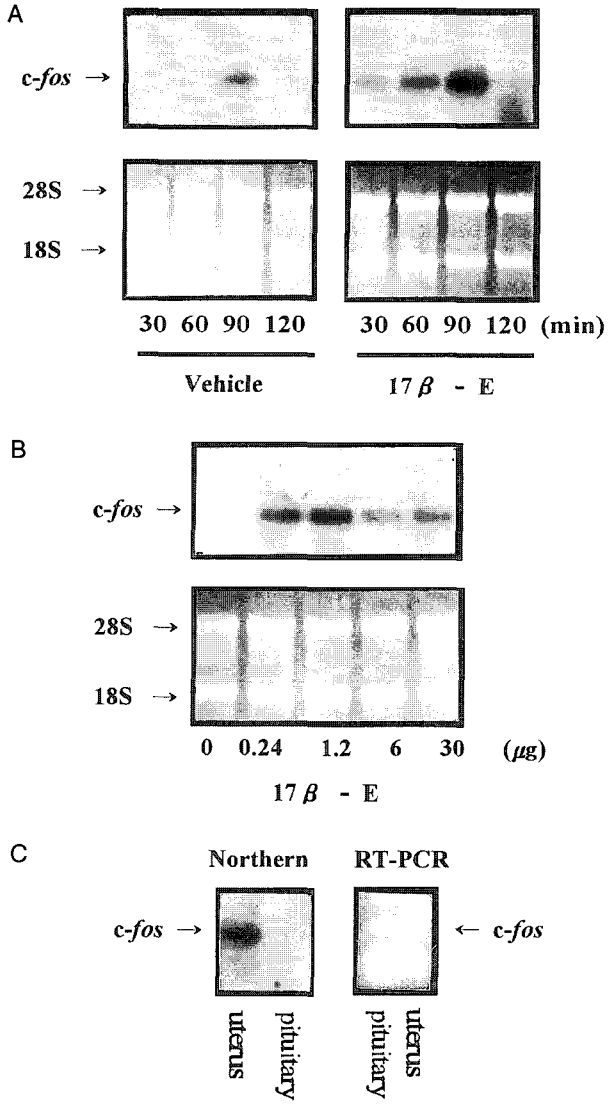

Fig. 5. c-*fos* gene expression in the uterus and pituitary. A, Ovariectomized female mice were injected with 17β-E at the indicated times before sacrifice. B, Ovariectomized female mice were injected with various concentrations of 17β-E at 120 min before sacrifice. RNAs were extracted from the uteri, quantitated, and electrophoresed on 1.2% agarose gels. After transfer into the Nytran, the filters were hybridized with ^{32}P-labeled c-*fos* cDNA. C, Relative levels of c-*fos* mRNA in the uterus and pituitary. Left and right figures reveal the results of Northern blot and RT-PCR, respectively.

c-*fos* mRNA increase, as reported in rat hepatocytes (Berlanga et al., 1995).

Recently, a new estrogen receptor, ERβ (Kuiper et al., 1996; Tremblay et al., 1997) was reported to act via the AP-1 site (Paech et al., 1997), suggesting that another pathway exists during estrogen action in addition to the already known ERα pathway via the estrogen responsive element, termed ERE (Maurer and Notides, 1987). In the case of humans, 5′ flanking region of the PRL gene contains both ERE and AP-1, so that estrogen may act through either ERE or AP-1 or both. Until recently the genomic structure of the murine PRL gene has not been reported, though it is likely that structural similarity exists between the murine and human PRL genes. Thus, pathway adopted by estrogen to regulate the PRL gene in mouse remains to be elucidated.

Taken together, these data indicate that 17β-E and stress stimulate the PRL and c-*fos* gene expression in a parallel manner, supporting the notion that c-Fos may mediate 17β-E and stress signals in regulation of PRL gene expression.

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