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NOTE

Increase in Pit-1 mRNA Is Not Required for the Estrogen-Induced Expression of Prolactin Gene and Lactotroph Proliferation

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Abstract. Estrogen has been shown to stimulate lactotroph proliferation and expression of the prolactin (PRL) gene. Recently it has been established that Pit-1, a pituitary-specific transcription factor, is required for lactotroph proliferation. Furthermore, *in vitro* studies showed that an increase in the PRL promoter activity caused by estrogen was dependent of the amount of cotransfected Pit-1-expressing plasmid. These findings led us to examine whether the induction of Pit-1 mRNA is required for the estrogen-increased PRL gene expression in the rat anterior pituitary *in vivo*. Short term estrogen treatment was achieved by means of a single intramuscular injection of estradiol dipropionate. DNA synthesis, the levels of PRL and Pit-1 messenger RNAs in the anterior pituitary were determined. Estradiol dipropionate resulted in a significant increase in DNA synthesis 24 h after administration and in PRL mRNA after 48 h. In contrast, the Pit-1 mRNA level was not altered. Since Pit-1 is expressed not only in lactotroph but also in somatotroph and thyrotroph, and the lactotroph cell population has been reported to be less than 10% in the pituitary, the change in the Pit-1 mRNA level in lactotrophs was not seen following only short term estrogen treatment. An increase in the lactotroph cell population was therefore achieved by chronic estrogen treatment (subcutaneous implantation of a silastic tube containing 17β-estradiol powder for 30 days). This treatment resulted in the marked proliferation of lactotrophs and a 3-fold increase in PRL mRNA. However, no alteration in Pit-1 mRNA was observed. These results suggest that the increase in Pit-1 mRNA is not required for the estrogen-induced lactotroph proliferation or PRL gene expression.

Key words: Pit-1, Prolactin, Estrogen, Rat

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RECENTLY it has been established that Pit-1, a pituitary-specific transcription factor, is necessary for the transcription of prolactin (PRL) and growth hormone (GH) genes [1–4]. In addition, Pit-1 plays a critical role in the normal development of the lactotroph, somatotroph and thyrotroph, since it was shown that the mutation or rearrangement of the Pit-1 gene was responsible for hypoplasia or the absence of these cells in the pituitaries of the Snell and Jackson dwarf mice [5]. In a human cretinism patient, Tatsumi *et al.* [6] demonstrated that a mutation in the Pit-1 gene caused combined deficiency of PRL, GH and thyrotropin. Together with these reports, Pit-1 is essential not only for the establishment and maintenance of the differentiated phenotype of three pituitary cells but for their proliferation as well.

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It is well known that estrogen administration to the rat increases the production and secretion of PRL with hypertrophy of the pituitary [7–9]. The PRL gene activity is regulated through protein interactions within at least two distinct regions of the 5′ flanking sequence, a proximal promoter region and a distal enhancer region [1]. Both of these regulatory sequences contain several recognition sites of Pit-1 [1]. While the two regions appear to be responsible for PRL gene expression, the distal enhancer region differs from the proximal region in that it contains a sequence which confers responsiveness on estrogen (an estrogen response element = ERE) [1]. Recently Day *et al.* [10] demonstrated that both Pit-1 binding sites and ERE were required for distal enhancer activation by estradiol. Furthermore, when the plasmids expressing Pit-1 and estrogen receptor were cotransfected with the distal enhancer reporter gene into Cos-1 cells, activation of the reporter gene by estrogen was dependent upon the amount of the cotransfected Pit-1 expressing plasmid [10]. Castrillo *et al.* [11] reported that specific inhibition of Pit-1 synthesis by introducing its antisense oligonucleotide into GC cells (a pituitary tumor cell line) led to a marked decrease in GH and PRL gene expression and in proliferation of the cells. These *in vitro* studies suggest that, through an increase in Pit-1 gene expression, estrogen may induce the proliferation of lactotrophs and expression of the PRL gene. The present investigation was undertaken to examine this possibility *in vivo* by administering estrogen to male rats.

Materials and Methods

Animals and hormone treatment

Male Wistar rats were obtained from Nakashima Experimental Animal Research Laboratory, Nagoya, Japan. Each animal weighed approximately 200 g at the start of estrogen treatment. They were maintained on a constant light (0700 h–1900 h) and dark cycle at a temperature of 24°C. Food and water were given *ad libitum*.

Intra-muscular injection of estradiol dipropionate (Teikokuzoki Pharmaceutical Co., Tokyo, Japan) was performed once at a dose of 100 μg/100 g body weight. Six rats were assigned to each of 5 groups corresponding to the time intervals after estrogen administration (0, 12, 24, 48, and 72 h). Rats were sacrificed by cardiac exsanguination under ether anesthesia between 0900 h and 1000 h to avoid variation related to the diurnal changes in PRL secretion. The anterior pituitary glands were removed, immediately placed in ice-chilled phosphate buffered saline (PBS), and washed with the same buffer 4 times. After blotting excess moisture, the anterior pituitaries were weighed and three pituitaries were immediately used for the determination of DNA synthesis independently. The remained three pituitaries were frozen on a block of dry ice and kept at −80°C until the determination of PRL and Pit-1 mRNAs.

Three other rats received subcutaneous implantation of a 5 cm-long silastic tube (Dow Corning, No. 602–305, 0.078 in i.d., New York, U.S.A.) filled with 40 mg of 17-β estradiol into their back as previously reported [7–9]. The rats were killed 30 days after the implantation and anterior pituitaries were obtained. Anterior pituitaries from three control rats to which silastic tubes were not implanted were also prepared.

Determination of DNA synthesis

DNA synthesis was determined by incorporation of ^{3}H-thymidine as previously described [12]. In brief, each anterior pituitary was cut into 4 pieces and pre-incubated for 1 h at 37°C in 1 m*l* of Krebs Ringer bicarbonate buffer containing 2.56 mg/m*l* D-glucose (KRBG). The medium was then replaced with 1 m*l* KRBG containing 10 μCi of ^{3}H-thymidine (New England Nuclear, 20 Ci/mM, Boston, U.S.A.). After incubation for 1 h under the same conditions, the tissue was rinsed in ice chilled PBS and homogenized in 0.5 m*l* of the same buffer. A 200 μ*l* aliquot was precipitated with trichloroacetic acid (TCA) and the precipitate was solubilized with 80 μ*l* NCS tissue solubilizer (Amersham International plc, Buckinghamshire, England). The radioactivity of ^{3}H-thymidine incorporated into TCA-insoluble material was determined after the addition of 1.4 m*l* of xylene-based scintillation liquid. An aliquot of each homogenate was analyzed for DNA content by diphenylamine reaction.

Cloning of rat Pit-1 cDNA

The cDNA encoding full length of Pit-1 was am-

plified from rat pituitary mRNA by the reverse transcription-polymerase chain reaction (RT-PCR) method. Total RNA was extracted according to the method described by Chomczynski and Sacchi [13]. After cDNA was synthesized from total RNA by reverse transcriptase and with oligo $d(T)_{15}$ as a primer, the PCR was carried out with the sense primer (5'-GTTCTCTACTCTCTTGTGGGAATG-3') and the antisense primer (5'-CATGGCTA-CCACAGGCAAGTCTTA-3') as described previously [14]. Thirty five cycles were performed; each cycle was 30 sec at 94°C, 2 min at 54°C and 1 min at 74°C. The PCR product was ligated to pCR1000 plasmid according to the supplier's protocol (TA cloning system, Invitrogen Co. San Diego, U.S.A.). Sequence analysis confirmed that this PCR product contained the entire coding sequence of rat Pit-1.

Determination of PRL and Pit-1 mRNAs

The amounts of PRL and PRL and Pit-1 mRNAs were determined by the Northern blot hybridization method. The procedure was described previously [15, 16]. In brief, after the fractionation of 20 μg total RNA on 0.8% agarose gel, the gel was stained with ethidium bromide. The RNA was then transferred onto Gene Screen Plus membrane (New England Nuclear, Boston, U.S.A.). The amounts of 28S and 18S ribosomal RNAs in each lane were estimated from the photograph of the membrane taken under ultraviolet waves (254 nm). The membrane was hybridized with PRL or Pit-1 cDNA probe labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; New England Nuclear, Boston, U.S.A.) by means of a random primed labeling kit (Boehringer Mannheim, GmbH, Mannheim, Germany). Rat PRL cDNA was kindly provided by Dr. J. A. Martial. The full length Pit-1 cDNA was used as a probe. After hybridization, the membrane was washed and exposed to XAR-5 film (Kodak, New York, U.S.A.) at −80°C with an intensifying screen for two weeks (Pit-1) or at room temperature for 3 h (PRL). The relative amounts of the respective mRNAs were determined by densitometry and expressed as a percentage of the control.

Results

Effect of short term estrogen treatment on DNA synthesis in the anterior pituitary is shown in Table 1. A two fold increase in DNA synthesis was observed 24 h after an intramuscular injection of estradiol dipropionate when expressed as either radioactivity incorporated per pituitary or per μg of DNA. This significant increase continued until 48 h after estrogen administration, whereas the DNA synthesis slightly declined at 72 h.

Northern blot analyses of PRL and Pit-1 mRNAs in the pituitaries obtained after short term estrogen treatment are shown in Fig. 1A. PRL mRNA was detected as a 1.0 kb single band. Pit-1 mRNA was detected as three bands, one major band and two minor bands. The major band was 2.5 kb and the minor bands were 4.1 and 1.6 kb. These minor bands may represent a nuclear precursor and a transcript in which a proximal polyadenylation site is utilized, respectively [1]. Since the densities of the three bands varied in parallel, the amount of Pit-1 mRNA was determined by the densitometry of the major 2.5 kb band. Fig. 1B depicts the changes in Pit-1 and PRL mRNA levels after a bolus injection of estradiol dipropionate. No change in the Pit-1 mRNA level was observed, but a significant increase in PRL mRNA was observed 48 h after the estrogen administration.

The effect of continuous estrogen stimulation on

Table 1. Effect of estrogen on pituitary DNA synthesis

Hours after estrogen	Pituitary weight (mg)	DNA content (μg/pit)	DNA synthesis (cpm/pit)	DNA synthesis (cpm/μg pit DNA)
0	6.8 ± 0.1	46.8 ± 5.2	9,092 ± 996	194 ± 20.7
12	6.4 ± 0.2	56.7 ± 3.6	10,064 ± 592	178 ± 8.1
24	6.2 ± 0.7	44.2 ± 8.3	18,415 ± 2,832[b]	417 ± 3.9[a]
48	7.0 ± 0.1	52.5 ± 9.9	20,725 ± 1,152[a]	395 ± 17.9[a]
72	8.1 ± 0.3[a]	49.2 ± 9.0	16,362 ± 1,532[b]	333 ± 11.0[a]

The data were expressed as the mean ± SEM. Statistical significance of difference from the basal level at 0 h was analyzed by Student's *t*-test (a, $P<0.001$; b, $P<0.005$).

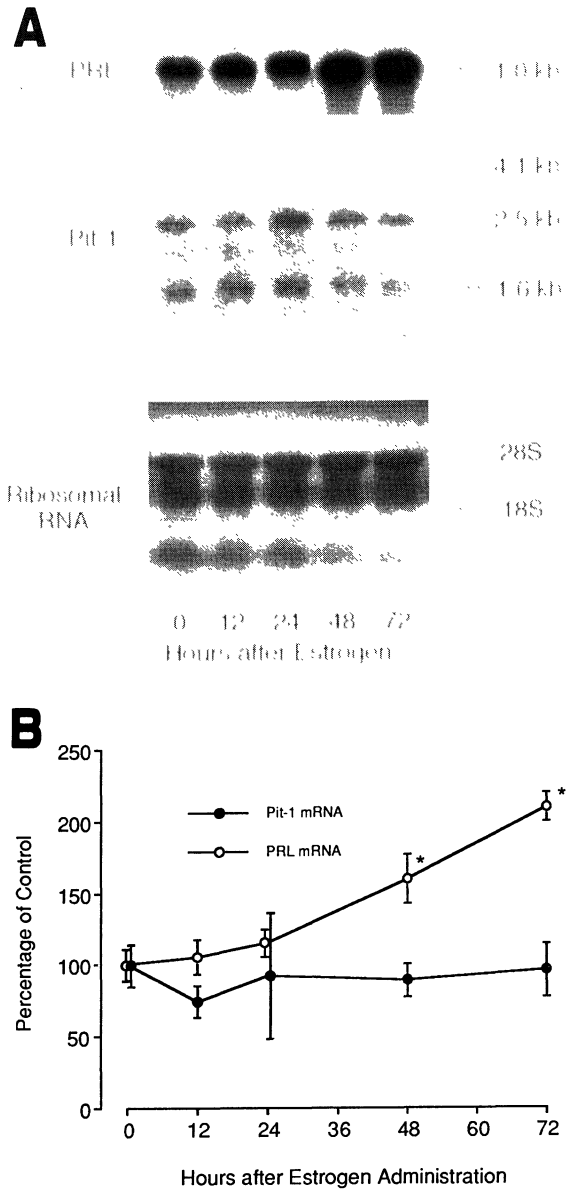

Fig. 1. Northern blot analyses of PRL and Pit-1 mRNAs in the anterior pituitaries obtained after short term estrogen treatment. A: Photograph of the autoradiography and staining of ribosomal RNA. Twenty micrograms of total RNA was used. The sizes of the respective mRNAs are indicated. As reported previously [1], the Pit-1 mRNA was detected as three different sizes. Application of equal amounts to each lane was evident from the staining of ribosomal RNAs. B: The changes in the amounts of PRL and Pit-1 mRNAs determined by densitometry of the autoradiograph. The changes in PRL mRNA are shown in an open circle and that of Pit-1 mRNA in a closed circle. The results are plotted as percentages of control levels (mean ± SEM, n=3; *, $P<0.005$ *vs.* 0 h).

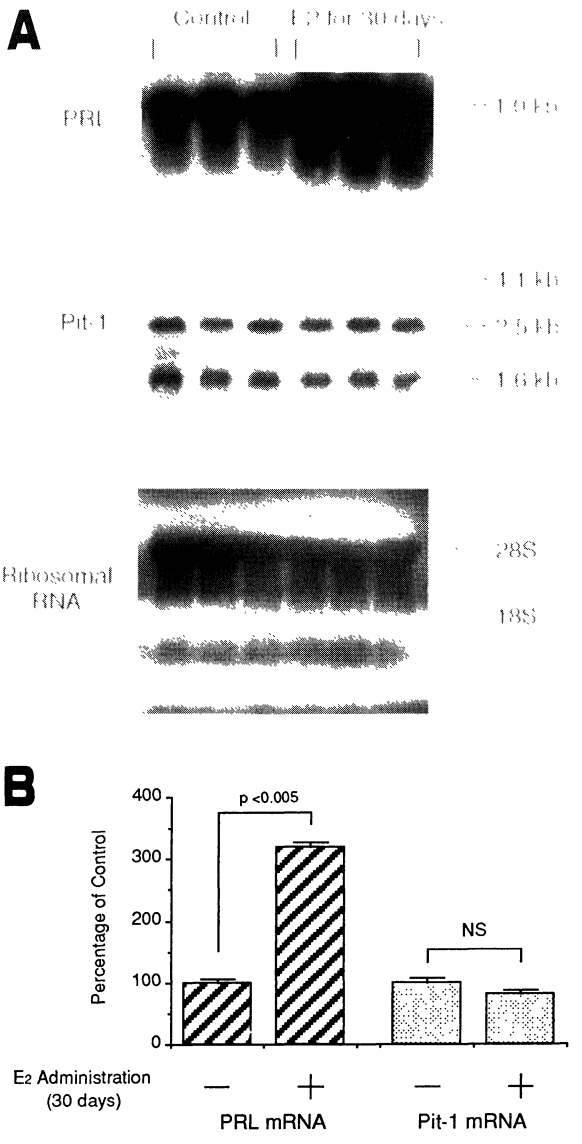

Fig. 2. Effect of continuous estrogen stimulation by implanting a silastic tube containing 17β-estradiol on PRL and Pit-1 mRNAs in the rat anterior pituitary. A: Photograph of the autoradiography and staining of ribosomal RNA. B: The changes in the amounts of PRL and Pit-1 mRNAs determined by the densitometry of the autoradiograph. The results are expressed as a percentage of control levels (mean ± SEM, n=3; NS, not significant).

Pit-1 and PRL mRNAs in the anterior pituitary was studied by implanting a silastic tube containing 17β-estradiol (Fig. 2A and B). A significant increase in PRL mRNA (about 320% of the non-stimulated control level) was observed 30 days after the implantation. In contrast, no increase in Pit-1 mRNA was observed.

Discussion

Pit-1 is expressed in mature lactotrophs, somatotrophs and thyrotrophs which arise sequentially during development of the anterior pituitary [17]. The *in vitro* studies suggest that the increased expression of Pit-1 may be associated with an estrogen-dependent increase in the expression of the PRL gene [10, 11]. But it remains to be determined whether the expression of the Pit-1 gene is altered when the lactotroph cell is stimulated by estrogen *in vivo*.

Short term estrogen treatment achieved by means of a single intramuscular injection of estradiol dipropionate resulted in a significant increase in DNA synthesis and PRL mRNA but no change in Pit-1 mRNA in the anterior pituitary. Since it has been shown that most of the increased DNA synthesis after estrogen administration is attributable to the mitosis of lactotrophs [18, 19], the results suggest that lactotroph proliferation induced by estrogen may not be accompanied by an increase in Pit-1 gene expression.

It is possible, however, that a change in the Pit-1 mRNA level in lactotrophs could not be observed following short term estrogen treatment, because Pit-1 is expressed not only in lactotroph cells but also in other types of hormone producing cells in the anterior pituitary, and the lactotroph cell population is less than 10% in the pituitary of male rats [20]. An increase in lactotroph cell population was therefore achieved by the chronic estrogen treatment. It was reported that subcutaneous implantation of a silastic tube containing 17β-estradiol powder for 30 days increased the lactotroph cell population to 40% of the total number of cells in the anterior pituitary [8]. Other studies reported that chronic estrogen administration stimulates not only lactotroph cell proliferation but also the PRL mRNA level [18, 19]. In agreement with the previous results, a significant increase in PRL mRNA was observed 30 days after the implantation, but no increase in Pit-1 mRNA was observed.

To examine another possibility, i.e. that Pit-1 may increase only transiently as reported in c-*fos* gene induction by estrogen [21, 22, 23], 17β-estradiol was intravenously administered at a dose of 100 μg/100 g body weight. No alteration was observed 1 h after estrogen administration (data not shown).

Recently an alternative splicing variant of Pit-1 called Pit-1a, Pit-1β, or GHF-2 has been reported [24–26]. This splicing variant has 26 amino acid insertions in the transactivation domain of the original Pit-1. Since the Northern blot analysis employed in the present study could not separate the two species, the amounts of Pit-1 mRNA estimated were the sum of mRNAs for Pit-1 and the variant. As mentioned already, Pit-1 transactivates both GH and PRL promoters [2–4]. However, the variant has been shown to transactivate GH promoter as efficiently as Pit-1 [25, 26] whereas it activates PRL promoter much less efficiently [24] or not at all [25, 26]. If estrogen could increase the ratio of Pit-1 to the variant by affecting the alternative splicing, it could enhance the expression of the PRL gene without changing the total amount of Pit-1 mRNAs. However, it is unlikely that estrogen affects the alternative splicing since Theill *et al.* [26] reported that the ratio of Pit-1 to the variant mRNA was approximately 7:1 in both young male and pregnant female rat pituitaries.

The present study indicates that no increase in the Pit-1 mRNA is required for the estrogen-induced activation of the PRL gene or proliferation of the lactotroph *in vivo*. In agreement with our results, Zhang *et al.* [27] demonstrated that estrogen did not change the level of Pit-1 mRNA in GH_3 cells cultured in a defined medium but it increased PRL mRNA. Our results also suggest that basal Pit-1 expression could be sufficient to cause an estrogen-dependent increase in lactotroph proliferation and PRL gene expression. Since it has been shown that phosphorylation of Pit-1 modulates its transcriptional activity [28], it should be determined whether estrogen affects the phosphorylation status of the Pit-1 molecule.

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