Estrogen enhancement of androgen-responsive gene expression in hormone-induced hyperplasia in the ventral prostate of F344 rats

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It has been postulated that, in addition to the crucial role of androgens, estrogens may be involved in development of prostate hyperplasia and cancer. In rats, combined administration of estrogen and androgen synergistically increases ventral prostate weight, and continued treatment results in the development of glandular hyperplasia. Prostate adenocarcinoma can be induced by chemical carcinogens in rats, and estrogen given together with an androgen generally shortens the latent period or increases the incidence and/or multiplicity of carcinomas. However, the mechanisms responsible for these synergistic effects of estrogen and androgen are poorly understood. In the present study, we examined the combined effects of 17 -estradiol (E2) and testosterone (T) on gene expression in an early stage of prostate hyperplasia in an F344 rat model. ER expression, which has been suggested to contribute to development of prostatic hyperplasia, was increased by the combined treatment with T and E2, while it was suppressed by T alone. Expression levels of two androgen-responsive genes, probasin and kallikrein S3, were increased in the ventral prostate of rats treated with T plus E2 for 4 weeks in a dose-dependent manner, while short-term treatment did not alter the expression. These results suggested that enhancing effects of E2 on transcription of androgen-responsive genes, as well as an increased level of ER may play roles in the synergistic effects of E2 on T-induced prostate hyperplasia. (Cancer Sci 2004; 95: 711–715)

Androgen plays a crucial role in development of benign prostatic hyperplasia (BPH) and prostate cancer (PC), but it has been postulated that estrogens may also be involved. In humans, BPH is known to be linked with both serum estrogen levels and urinary estrogen content.[1,2] Canine BPH is inducible by simultaneous administration of androstanediol and estradiol,[3] and in rats, administration of estrogen concomitantly with androgen promotes prostate growth.[4] Furthermore, combined estrogen and androgen treatment enhances prostate carcinogenesis in Noble rats and in chemical carcinogen-treated F344 rats.[5,6] Radioligand-binding assays and immunohistochemical studies with ER antibodies have demonstrated the presence of ER in both BPH and prostate carcinomas, but with no apparent correlation between receptor levels and pathologic features.[7] The discovery of ER has given rise to a new understanding of the physiological roles of ER.[8,9] In ER knockout mice, multiple hyperplastic foci are observed in the ventral prostate[10] and immunohistochemical studies in human prostate cancer have provided evidence of decreased ER expression in human BPH and PC.[11,12] These findings suggest that estrogen receptors are involved in regulating cell proliferation and in carcinogenesis in the prostate gland.

In the present study, we examined the combined effects of 17 -estradiol (E2) and testosterone (T) on gene expression in an early stage of prostate hyperplasia in an F344 rat model. RNAs isolated from the ventral prostate tissue were subjected to quantitative real-time RT-PCR to analyze expression of ER, the androgen receptor (AR) and several androgen-responsive genes, including probasin, kallikrein S3 and cystatin-related protein-1 (CRP1).

Materials and Methods

Hormones. 17 -Estradiol benzoate was purchased from Sigma Chemicals, St. Louis, MO, and testosterone propionate (T) from Wako Junyaku KK, Osaka, Japan.

Animals. Animal experiments were conducted in accordance with 'A Guide for the Care and Use of Laboratory Animals of Hiroshima University.' Male F344 rats were purchased at 4 weeks of age from Charles River Japan Co. (Kanagawa, Japan) and maintained with free access to basal diet and tap water. For 2- and 4-week experiments, the animals were divided into six groups: intact controls, castrated, T 50 mg and T plus E2, 0.1, 0.5 and 2.5 mg. For 24-h treatment, they were divided into five groups: intact controls, castrated, T and T plus E2, 0.5 and 2.5 mg. For hormone administration, 25 mm silastic tubes containing 50 mg of testosterone were subcutaneously implanted. Similarly, 10 mm silastic tubes were used for the various doses of E2. After treatment, animals were sacrificed under ether anesthesia and the ventral prostate lobe was dissected under a microscope, weighed and immediately frozen in liquid nitrogen and stored at −80°C.

Quantification of mRNAs by real-time RT-PCR. RNA preparation was carried out with an SV-total RNA isolation kit (Promega Co., Madison, WI). Total RNA (1 μg) was reverse-transcribed with 200 U of MMLV-RT (Invitrogen Corp., Carlsbad, CA) and 2.5 pmol of oligo-dT primer (Invitrogen) in 25 μl of buffer containing 1 m*M* dNTP, 100 m*M* Tris HCl (pH 8.3), 150 m*M* KCl, 6 m*M* $MgCl_2$, 60 m*M* dithiothreitol and 5 U/μl RNasin with incubation at 37°C for 60 min.

The real-time PCR method with a QuantiTect Sybr Green PCR kit (Qiagen) and an ABI Prism 7700 (PerkinElmer Life Sciences, Boston, MA) was employed for quantitative measurement following the supplied protocol.[13] Specific primer sets with a T_m of about 59°C were designed for each mRNA. The PCR conditions were 15 min of initial activation followed by 45 cycles of 15 s at 94°C, 30 s at 50°C and 60 s at 72°C. Prior to quantitative analysis, PCR products were prepared separately and purified by gel-electrophoresis. Extracted fragments were used as standards for quantification. The DNA sequences were confirmed with a capillary DNA sequencer, ABI 310 (Perkin-Elmer Life Sciences). All mRNA contents were normalized with reference to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA.

PCR primers. The following forward and reverse PCR primers

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Table 1. Body, pituitary and ventral prostate weights for F344 rats treated with T and/or E2

	Number of animals	Body w. (g)	Ventral prostate w. (mg/100 g B.W.)	Pituitary w. (mg)	Serum T (ng/ml)	Serum E2 (pg/ml)
2 weeks						
Control	5	158±4.1	40.2±5.6	11.2±0.3	0.9±0.20	33.7±5.30
Castration	4	141±6.6	6.5±0.4##	10.6±0.6	Not detected	31.1±3.75
T	5	165±4.5	104.8±9.7	10.9±0.2	2.8±0.15##	44.5±2.29
T+E2 0.1 mg	5	138±1.9	112.5±6.2	10.6±0.5	3.6±0.34##	35.0±2.44
T+E2 0.5 mg	5	154±2.7	123.8±8.5	11.4±1.1	3.4±0.05##	127.6±4.62
T+E2 2.5 mg	5	143±5.4	111.9±9.2	10.6±0.7	3.3±0.20##	164.2±18.75
4 weeks						
Control	5	254±6.0	107±2.0	6.6±0.6	1.6±0.07	17.8±1.95
Castration	5	178±4.9	8.5±1.2##	6.0±0.4	Not detected	17.0±1.80
T	7	245±3.7	202±3.5	7.1±0.4	2.3±0.03##	17.0±1.63
T+E2 0.1 mg	7	224±6.1	217±4.2	10.2±0.7	2.1±0.15#	18.8±2.61
T+E2 0.5 mg	7	212±4.3	247±10.1	10.6±0.8	2.6±0.03##	25.7±5.64
T+E2 2.5 mg	7	189±3.3	218±6.9	12.5±0.8	2.6±0.16##	79.3±22.3

##, significant difference from the control value at $P<0.01$. and , significant difference from the T-alone value at $P<0.05$ and $P<0.01$, respectively.

Table 2. Ki-67 mRNA expression in the ventral prostate of F344 rats treated with T and/or E2

	Ki67 mRNA level (fmol/pmol G3PDH)	Fold increase
2 weeks		
Control	20.8±3.3	1.00
Castration	4.1±1.5##	0.20
T	29.0±2.8	1.39
T+E2 0.1 mg	77.7±28.2	3.74
T+E2 0.5 mg	269.6±59.6	13.0
T+E2 2.5 mg	141.7±27.8	6.81
4 weeks		
Control	11.9±2.3	1.00
Castration	2.1±0.2##	0.18
T	7.3±0.8	0.61
T+E2 0.1 mg	27.7±13.5	2.33
T+E2 0.5 mg	47.0±13.2	3.95
T+E2 2.5 mg	95.8±20.6	7.00

##, significant difference from the control value at $P<0.01$. and , significant difference from the T-alone value at $P<0.05$ and $P<0.01$, respectively.

were used for quantification by the real-time PCR method: 5 GTATCGGACGCCTGGTTACC and 5 TTCCAAGTGACTTTCTCGGGA for rat G3PDH (+32–+190, 161 bps); 5 CCAGAATGGCCGAGAGAGAC and 5 CCAGACCC-CATAATGGTAGCC for rat ER (+419–+614, 196 bps); 5 TGCCAATCATCGCTCCTCTAT and 5 GGCACAACTGCTCCCACTAAG for rat ER (+142–+267, 126 bps); 5 CACCATGCAACTTCTTCAGCA and 5 CGAATTGCCCCCTAGGTAACT for rat AR (+504–678, 175 bps); 5 CCG-CCAATCCAACTCAAGTAA and 5 TTCCAAGTGACTTT-CTCGGGA for rat Ki-67 homologue (+194–+349, 156 bps, GenBank Accession: XM225460); 5 CCTCCTGCTCACACTGGATGT and 5 GCGACGGAAGTAGGTCCTCA for rat probasin (+15–+171, 157 bps). 5 TGCTCCTACTGGCCATCTTTG and 5 TGTCAGCACTGTGCGTGTTG for rCRP1 (+26–+190, 165 bps); and 5 AATTCCCAACCCTGGCA-AGT and 5 GCTGAGCAAAGGGTTCATC for rat kallikrein S3 (+100–+260, 161 bps).

Serum T and E2 levels. Serum T and E2 levels were measured with an ELISA kit, purchased from Neogen Corp. (Lexington, KY).

Statistical analysis. Statistical comparisons were made by using ANOVA followed by Scheffe's test.

Results

Body, ventral prostate and pituitary gland weights and serum T levels. Body weights were decreased in the castrated groups, while T administration did not cause any change (Table 1). Co-administration with E2 also resulted in decrease after 2- and 4-week treatments. T-alone treatment increased the prostate weights to almost twice the intact control weight and co-administration of E2 resulted in significant additional increases by 8, 20 and 8% (0.1, 0.5 and 2.5 mg of E2, respectively) over 4 weeks of treatment. There were no differences in pituitary weights after 2 weeks of treatment, but a significant increase was seen after 4 weeks of E2 co-administration. T administration increased the serum T level while co-administration of E2 did not alter the serum T level at any dose.

Cell proliferation in tissue. mRNA expression for a proliferative antigen, Ki-67, was significantly increased by co-administration of E2 in a dose-dependent manner with both 2- and 4-week treatment, except in the case of 2.5 mg E2 plus T administration for 2 weeks (Table 2). On the other hand, castration strongly suppressed Ki-67 expression.

ER , ER and AR mRNA expression. ER mRNA expression was significantly decreased by castration, while ER mRNA levels were increased dramatically with both 2- and 4-week treatments (Table 3). T administration reduced ER mRNA expression and co-administration increased the ER mRNA level over the control in a dose-dependent manner, especially after 4 weeks of treatment. In contrast, no significant change in ER mRNA was noted with T-alone or T plus E2 treatments. Castration significantly reduced AR mRNA levels, while T administration had no effect, with or without E2.

Expression of androgen-responsive genes: probasin, kallikrein S3 and CRP1. Expression levels of all three genes were not altered by T administration alone, while they were decreased by castration. Interestingly, co-administration of E2 increased the expression over that of T alone in a dose-dependent manner. Effects were most pronounced for the probasin gene, for which a 4.5-fold increase was noted (Fig. 1; Table 4). On the other hand, no significant influence was apparent on T-induced CPR1 expression. However, 24-h treatment with T or T plus E2 did not alter the mRNA expression of either of the genes (Table 5).

Discussion

Although androgen is essential for prostate development and growth, a number of experimental reports have suggested direct

Table 3. mRNA levels of ERs and AR in the ventral prostate of F344 rats treated with T and/or E2

	ER (fmol/pmol G3PDH)	ER (fmol/pmol G3PDH)	AR (fmol/pmol G3PDH)
2 weeks			
Control	1.30±0.11	83.0±7.5	224±25
Castration	4.51±0.61##	4.1±0.4##	67±13##
T	0.29±0.06##	91.4±9.3	172±26
T+E2 0.1 mg	0.42±0.09	85.1±5.4	108±13
T+E2 0.5 mg	0.42±0.07	90.3±12.0	143±18
T+E2 2.5 mg	0.46±0.08	79.5±7.6	108±7
4 weeks			
Control	0.21±0.06	37.3±4.4	131±8
Castration	1.40±0.30##	34.8±2.2##	61±4##
T	0.17±0.04	32.6±4.0	165±35
T+E2 0.1 mg	0.33±0.03	43.9±7.7	176±27
T+E2 0.5 mg	0.40±0.07	41.5±7.5	145±18
T+E2 2.5 mg	0.35±0.12	59.7±6.7	142±22

##, significant difference from the control value at $P<0.01$. and , significant difference from the T-alone value at $P<0.05$ and $P<0.01$, respectively.

Table 4. mRNA levels of testosterone-responsive genes in the ventral prostate of F344 rats treated with T and/or E2

	Probasin (nmol/pmol G3PDH)	Kallikrein S3 (nmol/pmol G3PDH)	CRP1 (nmol/pmol G3PDH)
2 weeks			
Control	9.8±3.0	20.5±4.5	1195±207
Castration	0.8±0.1#	0.04±0.01	1±0.4
T	13.4±1.2	34.4±9.6	1314±225
T+E2 0.1 mg	16.0±3.8	24.9±6.8	1081±192
T+E2 0.5 mg	30.6±5.2	46.5±3.0	1587±322
T+E2 2.5 mg	48.9±13.6	31.7±6.3	1793±333
4 weeks			
Control	7.8±2.2	113±12.9	1698±285
Castration	0.5±0.2#	0.02±0.01	0.12±0.02
T	14.9±3.5	141±13.5	1347±182
T+E2 0.1 mg	28.1±4.5	220±21.3	1097±213
T+E2 0.5 mg	23.8±6.8	249±31.6	1833±234
T+E2 2.5 mg	40.4±5.8	219±35.7	1753±202

#, significant difference from the control value at $P<0.05$. and , significant difference from the T-alone value at $P<0.05$ and $P<0.01$, respectively.

effects of estrogen on processes in this gland, including imprinting during the neonatal period and prostate hyperplasia and dysplasia.[14–16] A recent study with the hypogonadal mouse model also pointed to direct action in the prostate gland.[17] In rats, combined administration of E2 and T synergistically increases ventral prostate weight and continuous treatment results in the development of glandular hyperplasia.[4] However, the mechanisms responsible for such synergistic effects of estrogen and androgen are poorly understood. The present study provided strong evidence that estrogen can enhance transcription of androgen-responsive genes. The results also indicated that combined treatment with T and E2 increases ER expression, which was suppressed by T administration alone. Since involvement of ER in the development of prostate hyperplasia and cancer has been suggested,[10–12] increase in ER itself could contribute to the synergistic effect.

In the present study, when T and E2 were administered to intact male rats in combination, ventral prostate weight was increased over that of the T-alone group. Expression of the proliferation marker, Ki-67, known to approximate the growth fraction in prostate cancers,[18] also clearly demonstrated an enhancing effect of E2, although the maximal increase was noted

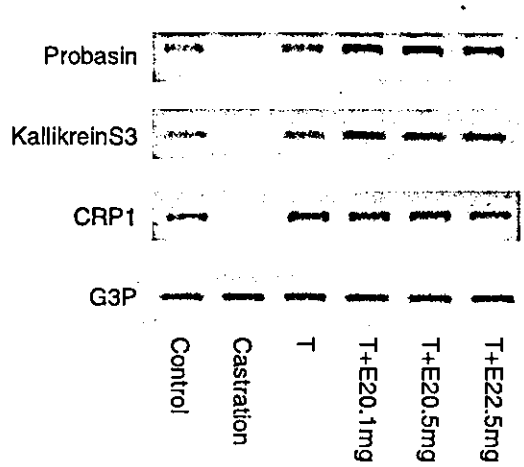

Fig. 1. Representative results of RT-PCR detection of probasin, kallikrein S3 and CRP1 in the ventral prostate in F344 rats treated with T and/or E2.

Table 5. mRNA levels of testosterone-responsive genes in the ventral prostate of F344 rats after 24-h treatment with T and/or E2

	Number of animal	Probasin (nmol/pmol G3P)	Kallikrein S3 (nmol/pmol G3P)	CRP1 (nmol/pmol G3P)
Control	4	36.0±5.1	26.0±0.3	580±53
Castration	4	2.2±0.8##	0.0±0.01##	10±0.5##
T	4	36.2±3.3	23.8±2.6	621±59
T+E2 0.5 mg	4	36.3±4.4	31.0±4.8	664±15
T+E2 2.5 mg	4	34.9±7.6	25.9±4.7	637±50

##, significant difference from the control value at $P<0.01$.

with 0.5 mg of E2 for both prostate weights and Ki-67 mRNA levels after the 2-week administration. The expression level of Ki-67 seems to be E2-dose-dependent after 4 weeks of treatment. Measurement of serum T levels confirmed that the treatment with T in silastic tubes boosted the hormone level and co-administration of E2 did not alter the level. However, an effect of E2 on the pituitary weight was evident. Administration of estrogen to adult males suppresses pituitary gonadotropins and testicular androgen production is diminished, which may mask direct effects of estrogen.[19,20] Thus, treatment with estrogen alone was not conducted.

Prostate cancer is the most frequently diagnosed malignancy in Western countries and it is androgen-dependent in its early stages.[21] To study the mechanisms of prostate carcinogenesis, several animal models have been developed. In Noble rats, long-term treatment with a high dose of testosterone induces prostate adenocarcinomas.[5,6] Lesions can also be induced by chemical carcinogens such as MNU (*N*-methyl-*N*-nitrosourea), *N*-nitrobis(2-oxopropyl)amine and DMAB (3,2 -dimethyl-4-aminobiphenyl) and are similarly androgen-dependent.[16,20–23] In experimental models, estrogen given together with androgen generally shortens the latent period or increases the incidence and/or multiplicity of carcinomas. Interestingly, when testosterone is given either alone or with estrogen, invasive adenocarcinomas have been found in the dorso-lateral and anterior prostate, regardless of the strain.[24] Chemical carcinogen-induced carcinomas, on the other hand, tend to be non-invasive and found in the ventral region of the prostate.

Although early studies demonstrated the presence of ER in normal prostate and prostate cancer tissues in both human and rodents, no correlation between their levels and any clinicopathological parameters has been reported.[7,25,26] Estrogen's effects on the prostate have been postulated to be indirect, possibly through the hypothalamic-pituitary-testis axis, until recently. Interest in ER and its roles in the prostate gland were recently revived by the cloning and characterization of ER .[8,9] Subsequent studies have revealed that ER is localized in epithelial cells, in contrast to the presence of ER in the stromal tissue in both primates and rodents.[27,28] Since treatment with T enhances glandular hyperplasia, a decrease in ER expression may be due to an increase in epithelial cell population, and an increase in ER by combined treatment with T plus E2 may suggest a potential impact of estrogen on stromal cells. Expression of ER , on the other hand, seems to be directly regulated by testosterone in epithelial cells.[10,29] It was recently revealed that expression of ER is frequently lost in human prostatic dysplasia and primary carcinomas[11,12] and in ER -knockout mice (BERKO), multiple hyperplastic foci develop in the ventral prostate. These findings suggest an anti-proliferative role of ER , in contrast to the stimulation of proliferation thought to occur with ER . In the present study, 4-week treatment with E2 plus T resulted in an increase in the ER mRNA level without any change in ER mRNA expression.

Expression levels of several androgen-responsive genes were measured to examine the effects of co-administration of estrogen. Kallikrein S3 is a serine protease that is abundantly expressed in rat ventral prostate and is strictly regulated by androgen.[30] This protein is probably a rat homologue of human prostate-specific antigen (PSA), a useful, albeit non-specific, marker for human prostate cancer.[31] The upstream region of the PSA gene contains consensus and non-consensus androgen-responsive elements (AREs) which account for hormone-dependent transcription, although the promoter structure of rat kallikrein S3 has not been determined.[32] CRP1 (cystatin-related protein-1), originally described as an androgen-regulated 20- or 22-kDa protein, is secreted in the rat ventral prostate[33,34] and probasin, belonging to the lipocalin superfamily, is both secreted and accumulated in the nuclei of prostate cells. Two distinct AR-binding sites have been identified as responsible for its androgen-responsive transcription.[35] Probasin is expressed in the ventral prostate, but it is abundant in the dorso-lateral lobes. Levels of all three mRNAs were reduced by castration, which confirmed the androgen-dependent regulation of their transcription. Among the three, the amount of CRP-1 mRNA in control prostate is the highest at 100 times that of G3PDH, a house-keeping gene used as an internal control in the present experiments. Kallikrein S3 mRNA expression was also high at a level equivalent to that of G3PDH. Although epithelial cells expressing these genes may expand during the development of hyperplasia, T administration alone did not change mRNA expression over the intact control levels, except for kallikrein S3 at 2 weeks. However, co-administration of E2 caused an increase in their mRNAs in a dose-dependent manner. On the other hand, 24-h treatment with E2 plus T did not change the mRNA levels, which may suggest that the synergistic effect of E2 was not due to direct interaction of ER and AR at promoter sites in these genes. Castration reduced AR mRNA level by one-third, which is consistent with a previous report.[36] However, administration of T with or without E2 did not change AR mRNA expression, in agreement with previous findings in Noble rats.[37] In the present experiment, only the ventral prostate was subjected to study. Since there are large variations in basal levels and hormonal regulation of gene expression among the different lobes of the rodent prostate, examination of the other lobes should be conducted in the future.

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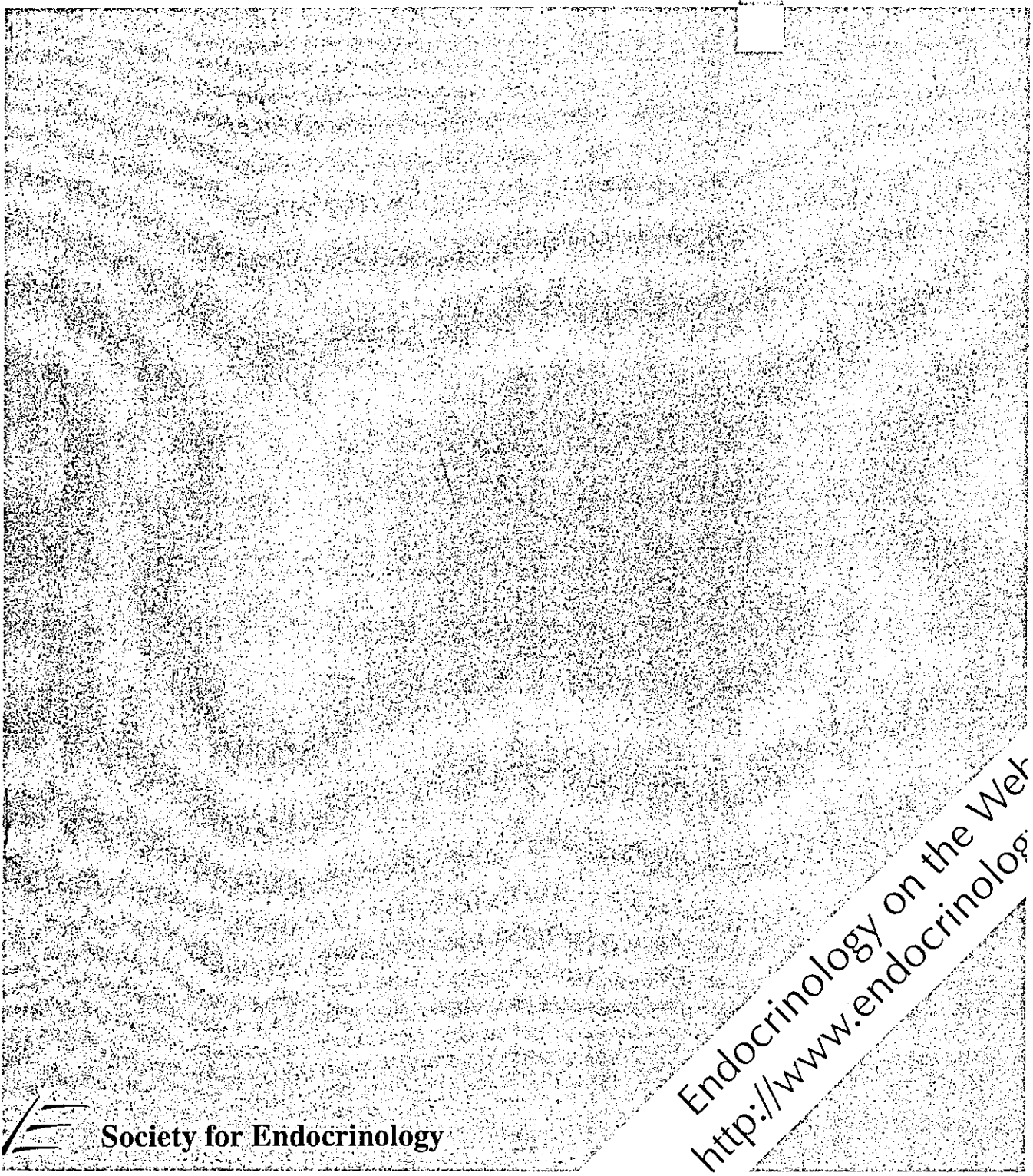

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Activation of estrogen response element dependent transcription by thyroid hormone with increase in estrogen receptor levels in a rat pituitary cell line, GH3

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Abstract

Interrelationships between thyroid hormone and estrogen actions have been documented with regard to a variety of physiological functions. Both hormones stimulate transcription of target genes by binding to their nuclear receptors that interact with specific responsive elements (estrogen and thyroid hormone response elements, i.e ERE and TRE, respectively) in the regulatory regions of the gene. *In vitro* studies have suggested that interplay between the two hormones might be due to cross-talk at hormone responsive elements, with the respective hormone receptors and ligands able to interact, although physiological relevance has yet to be proved. We have proposed a simpler mechanism for thyroid hormone effects on estrogen responses via increase in estrogen receptor α (ERα) with resultant increase in progesterone receptors, prolactin production and tumor growth. A pituitary cell line, GH3, has been widely used to investigate the function of mammo-somatotropic cells, especially regarding regulation of GH and prolactin production. In the present study, an ERE-luc reporter was transfected into GH3 cells and the responses to endogenous ERα were examined. We demonstrated that: (1) L-3,5,3′-triiodothyronine (T3) induces mRNA expression of ERα; (2) T3 alone is able to induce ERE-luc activity and this is inhibited by OH-tamoxifen; (3) T3 synergistically acts on estradiol (E2)-induced ERE responses; and (4) ERE-luc activity is enchanted by co-transfection of an ERα expression vector. These results support the hypothesis that estrogen responses are potentiated by T3 through up-regulation of ERα levels.

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Introduction

Interrelationships between thyroid hormones and estrogen actions have been documented with regard to a variety of physiological functions (Vasudevan *et al.* 2002) and elevation in thyroid hormone levels has been shown to have adverse effects on reproduction in rodents (Dellovade *et al.* 1996). Stimulation of mitosis in the uterine epithelium by estrogen is reduced in hypothyroid rats (Franklyn *et al.* 1994), while hyperthyroidism is known to decrease bone density by depressing estrogenic action. Regulation of GH by thyroid hormones depends on the thyroid hormone response element (TRE) in the promoter region of the growth hormone (GH) gene and the estrogen receptor (ER) also binds to this TRE and promotes transcription *in vitro* (Graupner *et al.* 1991). Although the thyroid hormone receptor (TR) has a higher affinity for TRE than the ER, estrogen could result in interference with thyroid hormone dependent transcription. Conversely, TR is also able to interact with estrogen repsonse elements (EREs) of both the progesterone receptor and the vitellogenin A2 promoters which influence transcription (Scott *et al.* 1997). However, no evidence has yet been provided regarding any physiological significance for such cross-talk demonstrated in *in vitro* experiments. We have proposed a simple enhancing mechanism of thyroid hormones on estrogenic actions via an increase in ERα levels; we previously found this is to be the case in a transplantable pituitary tumor line, MtT/F84, with potentiation of estrogen responses such as prolactin production and the induction of progesterone receptors (Fujimoto *et al.* 1988, 1991, 1996, Ito *et al.* 1985).

GH1 and GH3 are widely used rat pituitary cell lines, originally isolated from the MtT/W5 pituitary tumor, whose growth and prolactin/GH synthesis are stimulated by both thyroid hormone and estrogen (Sorrentino *et al.* 1976). They express high amounts of ERα and also TR (Haug *et al.* 1978). In the literature, there is a large

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variation in the reported estrogen-responsive growth of this cell line, from insensitive to very sensitive (Haug & Gautvik 1976, Kiino & Dannies 1981, Lieberman *et al.* 1981, Scammell *et al.* 1986, Amara *et al.* 1987, Rhode & Gorski 1991). The Health Science Research Resources Bank in Osaka, Japan, which carries a collection of cell lines available to the research community, have two strains of GH3 cells in stock, both of which are very sensitive to estrogen as well as thyroid hormone with reference to promotion of cell growth.

In the present study, we further examined, in GH3 cells, our hypothesis of enhancement of estrogenic action by thyroid hormones via an increase in ERα levels. For this purpose, ERα mRNA expression and ERα-mediated responses were examined *in vitro* by mRNA quantification using the real-time PCR method and an estrogen-dependent transcription assay with an ERE-luciferase reporter.

Materials and Methods

Chemicals

17β-estradiol (E2), L-3,5,3′-triiodothyronine (T3) and an estrogen antagonist, 4-hydroxytamoxifen (OH-tamoxifen), were purchased from Sigma. Each was dissolved in ethanol to give stock solutions.

Cell culture

The pituitary cell line, GH3, was obtained from the Health Science Research Resources Bank (Osaka, Japan) and maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 mixed medium (Sigma) containing penicillin and streptomycin with 10% horse serum (HS, Gibco/Invitrogen) and 2·5% fetal bovine serum (FBS, Gibco/Invitrogen). The MCF-7 cell line was maintained in DMEM containing penicillin and streptomycin with 5% FBS. Before transfection experiments and cell growth assays, cells were maintained for a week in phenol- red-free medium (Sigma) containing the same antibiotics along with dextran-charcoal-treated serum. For cell growth assays, GH3 cells were seeded in 24-well plates at 1×10^4 cells/well, and hormones were added the next day. Growth was measured after 5 days by means of a modified MTT assay with WST-1 (Dojindo Chemicals, Kumamoto, Japan).

RNA isolation and reverse transcription

GH3 cells were seeded in 60 mm dishes at 1×10^6 per dish, treated with hormones the next day and harvested after 24 h. RNA preparation was carried out with an SV-total RNA isolation kit from Promega, following the supplied protocol. One microgram of total RNA was reverse transcribed with 200 U Moloney murine leukemia virus reverse transcriptase (Invitrogen) 2·5 pmol oligo-dT primer (Invitrogen) in 25 µl b containing 1 mM dNTP, 100 mM TrisHCl (pH 150 mM KCl, 6 mM $MgCl_2$, 60 mM dithiothreitol 5 U/µl RNasin with incubation at 37 °C for 60 min

Measurement of ERα mRNA by quantitative real-time P

The real-time PCR method with a QuantiTect Green PCR kit (Qiagen) and an ABI Prism (PerkinElmer Life Sciences, Boston, MA. USA) employed for quantitative measurement of rERα glyceraldehyde-3-phosphate dehydrogenase (G3 P mRNAs following the supplied protocol (Woo *e* 1998). The primers for rERα were 5′-CCAATTCTC AATCGACGC (+470/+489, located in exon 2) 5′-TCTTATCGATGGTGCATTGGTT (+688/+ located in exon 3) giving a PCR product of 241 bp. primer pairs for G3 PDH were GGGTGATGCTGC CTGAGT (+255/+274) and TGGCATGGACTC GTCATG (+516/+515) resulting in a 262 bp proc The PCR conditions were a 15 min initial activation followed by 45 cycles of 15 s at 94 °C, and 30 50 °C and 60 s at 72 °C. Prior to the quantitative ana PCR products were prepared separately and purifie gel-electrophoresis. Fragments extracted from the were used as standards for quantification. ER mR contents were normalized with reference to the G3 P mRNA level.

ERE-luc reporter gene assay

The estrogen-responsive reporter plasmid, $(ERE)_3$-SV luc, contains three consensus ERE motifs from the *Xen* vitellogenin A2 gene (Kudoh *et al.* 1996). A rat E expression plasmid was constructed by inserting the Ec fragment of pRcER6 (Koike *et al.* 1987) into an Ec site of the pSG5 vector. phRL-CMV was a re luciferase expression plasmid from Promega.

GH3 cells were plated at 4×10^4 cells/well in 48-plates and transiently transfected with 0·4 µg (ER SV40-luc along with 0·01 µg phRL-CMV (Prom with TransFast transfection reagent containing a syntl cationic lipid (Promega), following the supplier's prot The weight ratio of TransFast reagent to DNA was After 24 h incubation with hormones, cells were harve with 30 µl cell lysis buffer (Promega) and the firefly renilla luciferase activities were determined wit dual luciferase assay kit (Promega) by measuring lu nescence with a Wallac Micro-Beta scintillation cou (PerkinElmer Life Sciences). Firefly luciferase rep activity was normalized to the renilla luciferase act from phRL-CMV.

Statistical analysis

Statistical comparisons were made using Student's *t*-t

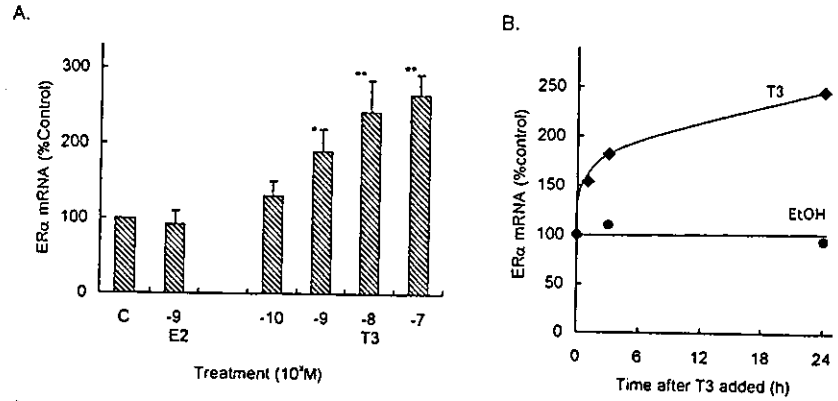

Figure 1 Dose- and time-dependent changes in ERα mRNA levels in GH3 cells. (A) Cells were treated with T3 at 10^{-10} to 10^{-7} M for 24 h. (B) Cells were exposed to 10^{-7} M T3 for 0, 1, 3 and 24 h. Total RNA was subjected to quantitative real-time RT-PCR. Each point represents a mean ± S.E.M. ($n=4$). Significant differences from the control values are: $^{*}P<0.05$, $^{**}P<0.01$. ERα was the major type of ER found expressed in GH3 cells. The absolute value for ERα mRNA was 3·3 ± 0·5 fmol/pmol G3 PDH in the control, 110-fold the amount of ERβ mRNA (0·03 fmol/pmol G3 PDH).

Results

Induction of ERα mRNA expression by T3

Treatment with T3 for 24 h significantly increased the ERα mRNA level in GH3 cells (Fig. 1). The response was dose dependent between 10^{-9} and 10^{-7} M. A time-course study revealed that the mRNA level was already significantly increased after 1 h of treatment with T3 at 10^{-7} M.

Activation of ERE-luc transcription by E2 and T3

The ERE-luc reporter plasmid transfected into GH3 cells responded well to E2 at concentrations between 10^{-11} and 10^{-9} M. Interestingly, T3 was also able to induce ERE-directed luciferase activity at 10^{-9} M, albeit with lower activity than E2 (Fig. 2A). An anti-estrogen, OH-tamoxifen (TAM), inhibited both T3- and E2-induced ERE-luc responses. When the same reporter was transfected into MCF-7 cells, ERE-luc activity was again dependent on the concentration of E2. T3, on the other hand, did not induce any ERE-luc activity in this cell line (Fig. 2B).

Enhancement of E2-induced ERE-luc responses by T3 in GH3 cells

E2-induced ERE-luc responses were enhanced from 4- to 6-fold with E2 alone to 6- to 11- fold with E2 plus

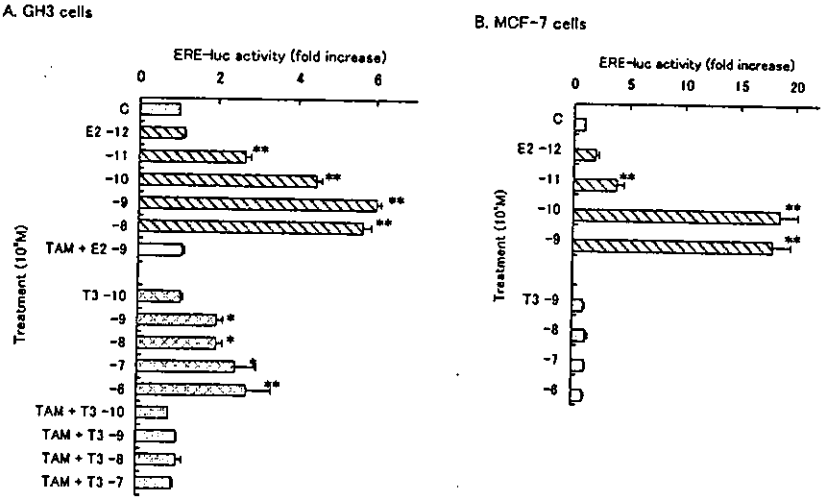

Figure 2 Effects of E2, T3 and OH-tamoxifen on ERE-luc activity in GH3 and MCF-7 cells. Cells were transiently transfected with the $(ERE)_3$-SV40-luc reporter plasmid and/or treated with E2, T3 and OH-tamoxifen (TAM) for 24 h. Each bar represents a mean ± S.E.M. ($n=5$ or 6). Significant differences from the control values are: $^{*}P<0.05$, $^{**}P<0.01$.

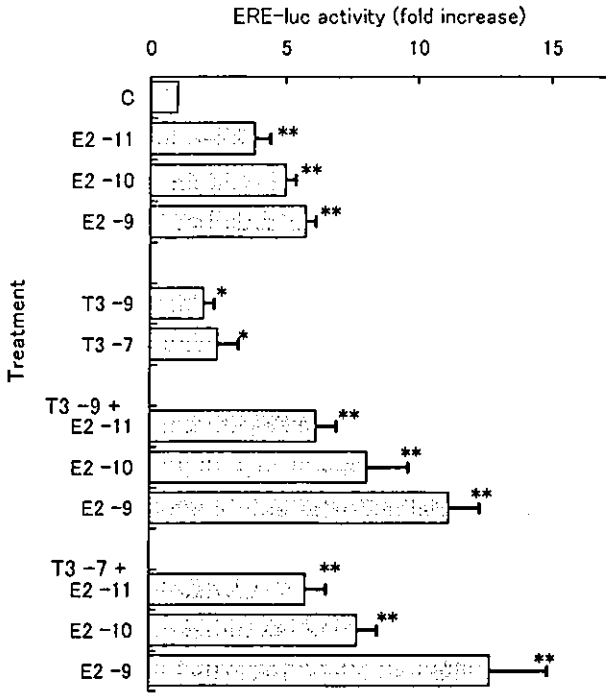

Figure 3 Synergistic effects of T3 on ERE-luc activation by E2 in GH3 cells. Cells were transiently transfected with the $(ERE)_3$-SV40-luc reporter and treated with different combinations of E2 and T3. Concentrations are given as 10^x M (e.g. 'E2 − 11' represents 10^{-11} M E2).

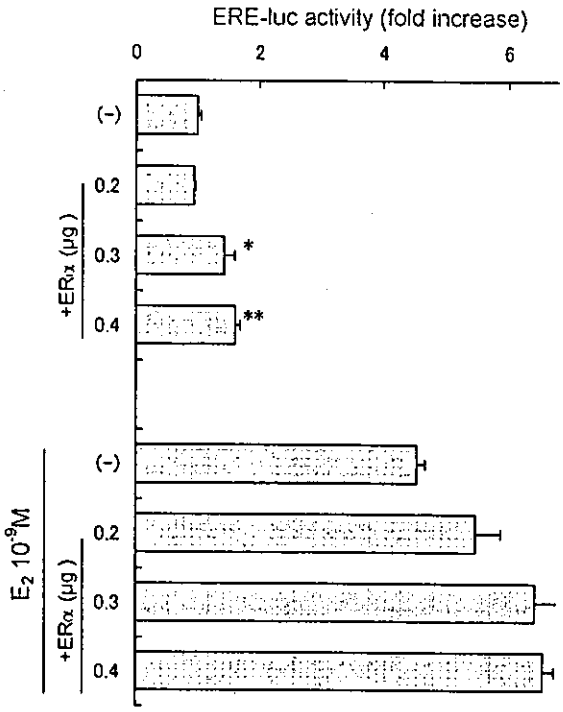

Figure 4 Effects of co-transfection of rERα on ERE-luc activity. Cells were transiently transfected with the $(ERE)_3$-SV40-luc reporter and pSG5-rERα expression plasmids at 0·2, 0·3 and 0·4 μg with or without E2. Each bar represents a mean ± S.E.M. ($n=5$). Significant differences from the control values are: $^*P<0.05$, $^{**}P<0.01$.

10^{-9}M T3, while the elevation due to T3 alone was only 2- to 2·5-fold (Fig. 3). Similar findings were obtained with T3 at 10^{-7} M.

Effects of rERα expression vector transfection on ERE responses

Co-transfection of the rERα expression vector, pSG5-rERα, significantly increased the basal level of ERE-luc activity 1·6-fold (Fig. 4). When 10^{-9} M E2 was administered, significantly higher luciferase activities were noted in ERα-transfected cells.

Effects of E2 and T3 on GH3 cell proliferation

Data for the effects of E2 and T3 on cell proliferation of GH3 are summarized in Fig. 5. The growth of the cells almost stopped when the medium was replaced with phenol-red-free DMEM with dextran-charcoal-stripped serum. The cell growth was very sensitive to estrogen, with significant growth noted at 10^{-12} M and reaching the maximum at 10^{-9} M. With T3, significant growth was noted at 10^{-10} M and was maximal at 10^{-8} M. OH-tamoxifen at 10^{-7} M inhibited both E2- T3-induced cell proliferation, while not exerting influence when given alone.

Discussion

MtT/F84 is a transplantable mammo-somatotropic tu which was originally induced by chronic E2 adi istration and subsequent transplantation in estroge F344 rats. It is characterized by the production of GH prolactin and by the presence of ER and TR. We previously described that growth of MtT/F84 corre well with the administered dose of estrogen (Fuji *et al.* 1991). Under hypothyroidal conditions, interesti estrogen responses (including tumor growth and gesterone receptor induction) are suppressed, accompa by a decrease in ER levels. On isolation of a seri pituitary cell lines from MtT/F84, we proved regulati ER expression by thyroid hormones *in vitro* (Fuji *et al.* 1997), suggesting that this is the mechanism ur lying the modification of estrogen responses. Long b our cell lines were established, GH1 and GH3 cell were isolated from the rat pituitary tumor MtT/W5.

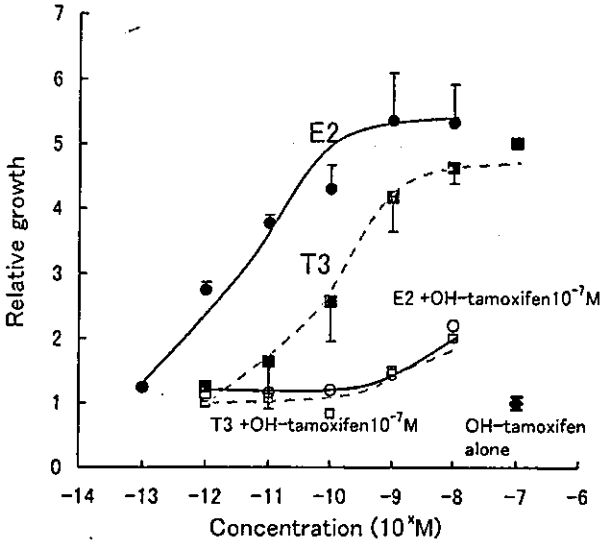

Figure 5 Effects of E2 and T3 on GH3 cell growth. Cells were treated with T3, E2 and/or OH-tamoxifen. After 5 days of exposure, cells were subjected to a modified MTT assay with WST-1.

have been widely used to investigate the function of mammo-somatotropic cells, since the regulation of GH and prolactin production in this cell line appears to be physiologically relevant with dependence on hormones such as T3, E2 and dexamethasone. In the present study of GH3 cells, we further tested and confirmed our hypothesis that T3 stimulates estrogenic actions by increasing the ER level using an ERE-reporter gene assay and ER mRNA measurement by real-time PCR.

We have demonstrated that the mRNA of ERα is up-regulated by T3 in GH3 cells. Since ERα is the dominant type of the receptor in GH3 (ERβ mRNA level was only about 1/60 of ERα according to measurements by real-time PCR, data not shown), only ERα was investigated in the present study. In the pituitary gland, it has been reported that the ER concentration is decreased in hypothyroid and increased in hyperthyroid rats (Andre *et al.* 1982). T3 has been shown to increase the ER level in rat liver (Freyschuss *et al.* 1994) and in *Xenopus* liver cells, playing a crucial role in preparing the latter to respond to estrogen by switching-on ER expression (Ulisse & Tata 1994). The cells thus become able to produce E2-dependent vitellogenin protein only after T3 treatment. In the mammary gland, one of the primary targets for estrogen and which generally expresses high levels of ERα, there are no reports indicating an effect of thyroid hormones on ER expression either *in vivo* or *in vitro*. Indeed T3 did not influence ER mRNA expression in MCF-7 cells in the present study (data not shown). Promoter regions of the ERα gene have been reported and well characterized. In humans, transcription is from three different promoters, while only two promoters are involved in the rat (Grandien *et al.* 1995, 1997, Freyschuss & Grandien 1996). However, there seem to be no thyroid hormone responsive transcriptional motifs in the known promoter structures. Although the induction of ERα mRNA by T3 occurred within 1 h, the transcription may not be regulated directly by T3 through the TR–TRE mechanism.

It is evident that thyroid hormones and estrogen actions are interrelated in a variety of physiological functions involving both hormones, such as growth of the uterine epithelium, change in bone density and determination of sexual behavior (Franklyn *et al.* 1994, Dellovade *et al.* 1996). It has been speculated that the interrelations might be the result of the competition and/or co-operation of their receptors in promoter regions, since over-expressed ER and TR can interfere with each other *in vitro* at TRE and ERE sites. In the present study, the ERE-luc reporter was transfected into GH3 cells endogenously expressing ER and TR, which makes our model more realistic. In the ERE-luc-transfected GH3 cells, we showed that: (1) T3 is able to induce ERE-luc activity; (2) this T3-dependent activity can be inhibited by an anti-estrogen; (3) T3 synergistically stimulates E2-induced ERE responses; and finally (4) ERE-luc activity could be enhanced by co-transfection of an ERα expression vector. The results support our previously proposed hypothesis that estrogen responses are potentiated by T3 through up-regulation of ER levels, rather than involving TR and ERE. T3 alone activated ERE-luc expression in the present study, possibly due to responses to endogenous estrogen in the culture serum remaining after the dextran-charcoal treatment.

Effects of T3 and E2 on pituitary cell growth have long drawn attention. Early work at Sirbasku's laboratory indicated that *in vivo* growth of GH3 indeed depends on both estrogen and thyroid hormones (Kirkland *et al.* 1976, Sorrentino *et al.* 1976). However, only thyroid hormones are required for growth in cell culture. While findings for T3-responsive growth are consistent, reported sensitivity to estrogen has varied in the literature (Kiino & Dannies 1981, Scammell *et al.* 1986, Amara *et al.* 1987, Chun *et al.* 1998, Rhode & Gorski 1991). This inter-laboratory variation might be due to differences in strain, since GH3 has a rather old origin and has been widely used. However, technical problems with the charcoal treatment of serum to remove estrogenic substances could have had an impact (Riss & Sirbasku 1989). The estrogenic activity of phenol red or related contaminants in common culture medium was not recognized until Katzenellenbogen's group provided convincing evidence (Berthois *et al.* 1986). With updated culture conditions, Sirbasku's group successfully reconstructed estrogen-responsive growth in GH4 cells. Our previous studies with pituitary cell lines have repeatedly shown that as little as 10^{-12} M E2 is effective in inducing estrogen-dependent reporter gene activity as well as E2-responsive cell growth. The data thus suggest

that GH3 cells respond to both E2 and T3 in the culture condition with careful removal of estrogenic compounds.

The growth of GH3 cells responsive to both E2 and T3 has been found to be inhibited by OH-tamoxifen; this is consistent with a report concerning F4Z2 cells, another estrogen-dependent pituitary cell line (Zhou-Li *et al.* 1992). The fact that the required concentration of T3 for growth stimulation (10^{-11} to 10^{-8} M) did not coincidence with that for ER induction (10^{-9} to 10^{-7} M) suggests direct effects of T3 on pituitary cell proliferation in this case.

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Identification of estrogen-responsive genes in the GH3 cell line by cDNA microarray analysis

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Abstract

To identify estrogen-responsive genes in somatolactotrophic cells of the pituitary gland, a rat pituitary cell line GH3 was subjected to cDNA microarray analysis. GH3 cells respond to estrogen by growth as well as prolactin synthesis. RNAs extracted from GH3 cells treated with 17β-estradiol (E2) at 10^{-9} M for 24 h were compared with the control samples. The effect of an antiestrogen ICI182780 was also examined. The array analysis indicated 26 genes to be up-regulated and only seven genes down-regulated by E2. Fourteen genes were further examined by real-time RT-PCR quantification and 10 were confirmed to be regulated by the hormone in a dose-dependent manner. Expression and regulation of these genes were then examined in the anterior pituitary glands of female F344 rats ovariectomized and/or treated with E2 and 8 out of 10 were again found to be up-regulated. Interestingly, two of the most estrogen-responsive genes in GH3 cells were strongly dependent on E2 in vivo. #1 was identified as calbindin-D9k mRNA, with 80- and 118-fold induction over the ovariectomized controls at 3 and 24 h, respectively, after E2 administration. #2 was found to be parvalbumin mRNA, with 30-fold increase at 24 h. Third was c-*myc* mRNA, with 4.5 times induction at 24 h. The levels were maintained after one month of chronic E2 treatment. Identification of these estrogen-responsive genes should contribute to understating of estrogen actions in the pituitary gland.

Keywords: Estrogen-responsive genes; cDNA microarray; Pituitary; GH3; Rats

1. Introduction

Estrogen regulates multiple functions in different cell types in the anterior pituitary gland [1–3]. In the somatolactotrophs (GH/prolactin cells), it is well documented that estrogen activates prolactin mRNA transcription through the estrogen-responsive element (ERE) located in the 5′-upstream regulatory region [4,5]. The storage and release of prolactin are also regulated by estrogen [6]. In addition to hormone production, estrogen promotes cell proliferation in somatolactotrophs, which is prominent in the rat case [7–9]. Although estrogen-responsive expression of a series of genes must be involved in these biological functions of the pituitary cells, only a few have so far been reported to be regulated by estrogen [2].

GH3 is a widely used rat pituitary somatolactotrophic cell line, originally isolated from the MtT/W5 pituitary tumor, whose growth and prolactin synthesis are stimulated by estrogen [10,11]. There is a variation in the estrogen-responsiveness of this cell line reported in the literature [5,12–15], but the cells obtained from the Health Science Research Resources Bank in Osaka, Japan, display high sensitivity with regard to induction of cell proliferation. In the present study, we performed a gene expression analysis of estrogen action in GH3 cells using the cDNA microarray technique and found many of the identified estrogen-responsive genes to also be similarly regulated in vivo in the anterior pituitary in F344 rats.

2. Materials and methods

2.1. Chemicals

17β-estradiol (E2) was purchased from Sigma Chemicals, St. Louis, MO, USA and ICI182780 was obtained from Tocris Cookson Ltd., Bristol, UK. Each was dissolved in

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ethanol to give stock solutions. Actinomycin D and cycloheximide were purchased from Wakojunyaku KK, Osaka, Japan.

2.2. Cell culture

The pituitary cell line GH3 was obtained from the Health Science Research Resources Bank (Osaka, Japan) and maintained in DME/F12 mixed medium (Sigma Chemical Co.) containing penicillin and streptomycin with 10% horse serum (HS, Gibco/Invitrogen Corp., Carlsbad, CA, USA) and 2.5% fetal bovine serum (FBS, Gibco/Invitrogen). Before estrogen treatment, cells were maintained for a week in phenol red-free medium (Sigma Chemicals) containing the same antibiotics along with dextran-charcoal-treated serum. For cell growth assays, GH3 cells were seeded in 24-well plates at 1×10^4 cells/well, and hormones were added the next day. Growth was measured after five days by means of a modified MTT assay with WST-1 (Dojindo Chemicals, Kumamoto, Japan). For microarray analysis, 3×10^6 GH3 cells were seeded in 90 mm dishes and treated with E2 at 10^{-9} M and/or ICI at 10^{-7} M and harvested after 24 h treatment. Cells were harvested after addition of Isogen (Wakojunyaku). For mRNA quantification, cells were treated with E2 at 10^{-12} to 10^{-9} M and/or ICI182780 at 10^{-7} M. After the indicated period of time, cells were harvested with cell lysis buffer supplied with an SV-total RNA isolation kit (Promega Co., Madison, WI, USA).

2.3. Animals

Animal experiments were conducted under the guidelines of the 'A Guide for the Care and Use of Laboratory Animals of Hiroshima University'. Female F344 rats were purchased at four weeks of age from Charles River Japan Co. (Kanagawa, Japan). They were maintained with free access to basal diet and tap water. All animals except the intact control underwent surgical ovariectomy upon receipt and implanted with pellets containing 10 mg of E2 subcutaneously as described previously [16]. Animals were sacrificed under ether anesthesia after 3, 8, 24 and 48 h in the short-term experiment. Treatment was extended between 7 and 30 days for the long-term experiment. The pituitary gland and the uterus of each rat were weighed and immediately frozen in liquid nitrogen and stored at −80 °C.

2.4. The GeneChip analysis

Total RNAs were extracted with Isogen, a premixed RNA isolation reagent, based on the acid guanidium thiocyanate–phenol–chloroform extraction method. The supplied protocol was followed.

First-strand cDNA was synthesized by incubating 5 μg of total RNAs with 200 U SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA), 100 pmol T7-(dT)24 primer [5′-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-(dT)24-3′], 1 × first-strand buffer (50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM MgCl2, 10 mM DTT) and 0.5 mM dNTPs at 42 °C for 1 h. Second-strand synthesis was performed by incubating the first-strand cDNAs with 10 U *E. coli* ligase (Invitrogen), 40 U DNA polymerase I (Invitrogen), 2 U RNase H (Invitrogen), 1× reaction buffer (18.8 mM Tris–HCl pH 8.3, 90.6 mM KCl, 4.6 mM $MgCl_2$, 3.8 mM DTT, 0.15 mM NAD, 10 mM $(NH_4)_2SO_4$) and 0.2 mM dNTPs at 16 °C for 2 h. Ten units of T4 DNA polymerase (Invitrogen) were then added, and the reaction was allowed to continue for another 5 min at 16 °C. After phenol–chloroform extraction and ethanol precipitation, the double-stranded cDNA was resuspended in 12 μl DEPC-treated dH_2O. Labeling of the dsDNA was achieved by in vitro transcription using a BioArray HighYield RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, NY). Briefly, the dsDNA was mixed with 1× HY reaction buffer, 1× biotin labeled ribonucleotides (NTPs with Bio-UTP and Bio-CTP), 1× DTT, 1× RNase inhibitor mix and 1× T7 RNA polymerase. The mixture was incubated at 37 °C for 4 h. The labeled cRNA was then purified using a RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The purified cRNA was fragmented in 1 × fragmentation buffer (40 mM acetate, 100 mM KOAc, 30 mM MgOAc) at 94 °C for 35 min. For hybridization with the GeneChip Rat Genome U34A (Affymetrix), 15 μg fragmented cRNA probe was incubated with 50 pM control oligonucleotide B2, 1× eukaryotic hybridization control (1.5 pM BioB, 5 pM BioC, 25 pM BioD and 100 pM Cre), 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated BSA and 1× manufacturer-recommended hybridization buffer in a 45 °C rotisserie oven for 16 h. Washing and staining were performed with a GeneChip Fluidic Station (Affymetrix) using the appropriate antibody amplification washing and staining protocol. The phycoerythrin-stained arrays were scanned as digital image files and scanned data were analyzed with GeneChip software (Affymetrix) [17].

2.5. Quantification of mRNAs by real-time RT-PCR

RNA preparation was carried out with an SV-total RNA isolation kit. One microgram of total RNA was reverse-transcribed with 200 U of MMLV-RT (Invitrogen) and 2.5 pmol of oligo-dT primer (Invitrogen) in 25 μl buffer containing 1 mM dNTP, 100 mM Tris–HCl (pH 8.3), 150 mM KCl, 6 mM $MgCl_2$, 60 mM dithiothreitol and 5 U/μl RNasin with incubation at 37 °C for 60 min.

The real-time PCR method with a QuantiTect Sybr Green PCR kit (Qiagen) and an ABI Prism 7700 (The Perkin–Elmer Co) was employed for quantitative measurement for following the supplied protocol [18]. Specific primer sets with a T_m of about 59 °C were designed for each mRNA selected from the microarray analysis (Table 1). The PCR conditions were a 15 min of initial activation step followed by 45 cycles of 15 s at 94 °C, 30 s at 50 °C and 60 s at

Table 1
Nucleotide sequences of primers for quantitative real-time PCR

Gene	GenBank accession#	Forward	Reverse
#1	K00994	AACCAGCTGTCCAAGGAGGA	CTTCTCCATCATCGTTCTTATCCA
#2	AI175539	TTTCTTCAGGCCCACCATCT	TTGCAGGATGTCGATGACAGA
#3	AI014135	GAACCAATTCTCCTAGCACAAGTG	CACGCCTGTGTTGGGCTAA
#4	AI178971	GGTGTGAAATCCCCAGGGT	CCCTGTCCACTCTGAGCGAC
#5	S81478	GATCAACGTCTCGGCCAATT	GCACAAACACCCTTCCTCCA
#6	D26393	GATTTCTAGGCGGTTCCGGA	ACTCGGAGCACACGGAAGTT
#7	AI230712	TGGCAGAAAAATCAATCCAGC	AAAGCCAGCCCCAAATCAC
#8	AF081366	CATCTGGACAACTGTGCTGGA	GGCACCACACATGAAGGAATT
#9	Y00396	CCGAGCTACTTGGAGGAGACA	AGGCCAGCTTCTCGGAGAC
#10	U02553	GATCAACGTCTCGGCCAATT	GCACAAACACCCTTCCTCCA
#14	U24175	CAGTGGATCGAGAGCCAGC	TGCCCCAGCTTGATCTTCAG
#15	D13623	ACCAAGACCGGTAGCAAGGG	GAAATCCGACGGAAGAGTGC
#21	AA892522	CCTTCGACTCAGCCACAAAAA	ACAGGGTCTTACCCTGCCTTC
#22	L16922	AGCCAGAGCCCACAATATGG	GCAATCATTTCTTCCGGCAC
G3PDH	AB017801	TGAAGGTCGGTGTGAACGGATTTG	TGATGGCATGGACTGTGGTCATGA

72 °C. Prior to the quantitative analysis, PCR products were prepared separately and purified by gel electrophoresis. The fragments extracted from the gel were used as standards for quantification. The DNA sequences were confirmed with a capillary DNA sequencer, ABI 310 (The Perkin–Elmer Co.). All mRNA contents were normalized with reference to G3PDH mRNA.

2.6. Statistical analysis

Multiple comparison was made by ANOVA followed by Scheffe's test. Otherwise, Student's *t*-test was applied.

3. Results

3.1. Estrogen-dependent cell proliferation of GH3

The relative cell numbers were measured at day 5 of treatment with E2 at concentrations from 10^{-13} to 10^{-9} M (Fig. 1). Significant stimulation of cell proliferation was observed at 10^{-12} M and the response appeared to reach a maximum at 10^{-11} M. The sizes of individual cells treated with E2 appeared to be larger than without hormone.

3.2. Estrogen-responsive genes identified by cDNA microarray

Differentially expressed genes based on the ratio of the measured hybridization intensities on GeneChip Rat Genome U34A between control and E2-treated cells are listed in Table 1. A minimal change of two-fold was applied to select up- and down-regulated genes. Two independent experiments were carried out and the genes showing reliable hybridization for both experiments were counted. The genes are listed according to average values of E2 induction. The results of ICI182780 treatment alone or with E2 are also given in Table 2. The genes regulated by E2 but not showing inhibition by ICI182780, which only accounted for four in total, are not included in the table. Interestingly, only 26 genes were categorized as up-regulated and seven as the down-regulated, out of approximately 8000 genes on the chip.

3.3. Confirmation of mRNA changes

From Table 2, the top ten genes and four others (#14, #15, #21 and #22) were selected and subjected to quantification of mRNA levels to confirm the results of cDNA microarray analysis. cDNAs from GH3 cells treated with E2 at 10^{-12} to 10^{-9} M and/or ICI at 10^{-7} M are examined and

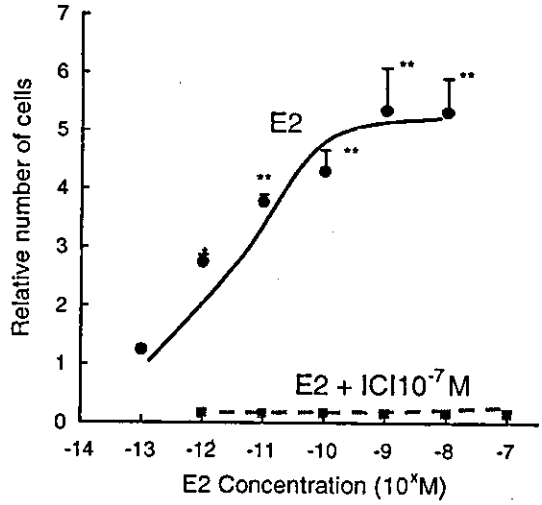

Fig. 1. Effects of 17β-estradiol (E2) and ICI182780 (ICI) on GH3 cell proliferation. Cells were seeded in 24-well plates at 1×10^4 cells per well. After five days treatment with E2 at 10^{-13} to 10^{-9} M alone or with ICI at 10^{-7} M, cell proliferation was measured by a modified MTT assay. Each point represents a mean ± S.E.M. ($n = 4$). *,** Indicates significant differences from the control value at 0.05 and 0.01, respectively.

Table 2
Genes up- and down-regulated by estrogen two or more fold in the microarray study

Genbank accession#		Gene name/blast match	Fold change in expression			
			E2(Exp1)	E2(Exp2)	E2+ICI	ICI
Genes up-regulated						
#1	K00994	Calbindin-D9k*	8.12	6.20	0.70	0.33
#2	AI175539	Parvalbumin*	7.58	4.54	0.81	0.28
#3	AI014135	Ribosomal RNA*	6.23	4.93	1.17	0.94
#4	M17083	Alpha globin*	5.23	4.99	0.39	0.59
#5	S81478	3CH134/CL1 ATPase	4.77	4.12	0.97	1.11
#6	D26393	Type II hexokinase	2.75	3.15	0.14	0.49
#7	AI230712	PACE4*	2.98	2.73	0.44	0.15
#8	AF081366	K + channel ROMK2.1 isoform	3.21	2.44	0.88	0.20
#9	Y00396	c-*myc* protein	2.99	2.59	0.76	0.35
#10	U02553	Protein tyrosine phosphatase	3.32	2.23	0.67	0.44
#11	AF036548	RGC-32	3.47	2.05	1.12	0.37
#12	U53505	Type II iodothyronine deiodinase	2.26	2.87	0.77	0.34
#13	Y09507	Hypoxia-inducible factor 1	2.60	2.38	1.13	0.69
#14	U24175	Regulator of transcription 5a1	2.77	2.01	0.61	0.46
#15	D13623	p34 protein	2.43	2.32	1.05	1.02
#16	M58040	Transferrin receptor	2.37	2.38	0.73	0.30
#17	AA819776	EST (similar to HSP86)	1.93	2.76	1.82	1.97
#18	AA875126	EST (unknown)	2.33	2.27	0.58	0.70
#19	M14656	Osteopontin	1.89	2.69	1.37	1.22
#20	X67788	Ezrin, p81	2.28	2.23	0.47	0.50
#21	AA892522	EST (unknown)	2.19	2.23	0.60	0.82
#22	L16922	Progesterone receptor	2.30	2.04	0.89	0.67
#23	U57097	APEG-1 protein	2.36	1.97	1.43	1.51
#24	M24852	Neuron-specific protein	1.87	2.45	1.73	1.57
#25	AA817846	EST (similar to D-β-hydroxy butyrate dehydrogenase)	1.86	2.37	0.97	0.96
#26	AI169417	Phosphoglycerate mutase type B subunit mRNA*	1.98	2.23	0.97	0.92
Genes down-regulated						
	U67080	Zinc finger protein r-MyT3	0.49	0.47	1.38	1.06
	AA799964	EST (unknown)	0.49	0.41	0.51	0.71
	AI639263	EST (unknown)	0.46	0.41	0.68	0.32
	M27925	Synapsin 2a	0.47	0.35	1.31	1.31
	E03229	JP 1991272688-A/2	0.47	0.30	1.31	0.95
	AI237654	Vdup1*	0.40	0.35	0.81	0.84
	AA893280	EST (similar to adipose differenti-ation-related protein)	0.47	0.21	0.91	0.98

Gene are listed in order of average E2 fold change in Experiments 1 and 2. **Four E2 up-regulated genes were not inhibited by ICI, which are not included in this table (The GenBank accession numbers of these are AI138070, AA866485, D84480 and X74293).

* Indicates genes originally listed as ESTs but found to have perfect match by BLAST.

the results were summarized in Fig. 2. Although the fold increases of E2 induced gene expression were slightly lower than in the microarray analysis, up-regulation and inhibition by ICI182780 were confirmed except with three genes, #3, #6 and #15, which showed no responses. Time dependence of gene expression induced by E2 was also examined and the results are summarized in Fig. 3. As expected, some of the genes were expressed early after E2 administration and others increased gradually. Since the microarray analysis was carried out at only one time point, 24 h after E2 treatment, early responding and quickly muting genes would not be expected to be identified.

To determine E2 in inducing the transcription of genes #1 and #2, GH3 cells were treated with E2 in the presence of 0.5 μg/ml of actinomycin D (a transcription inhibitor) and 10 μg/ml cycloheximide (a translation inhibitor) for 3 and 24 h (Table 3). Increase in mRNA levels by E2 was blocked

Table 3
Effects of cycloheximide and actinomycin D on E2-induced mRNA change of calbindin D9k and parvalbumin in GH3 cells

	3 h	24 h
Gene#1: calbindin D9k		
Control	5.45 ± 0.70**	4.02 ± 0.33**
CHX	4.03 ± 0.11**	3.74 ± 0.27**
ActD	1.01 ± 0.21	1.13 ± 0.23
Gene#2: parvalbumin		
Control	1.81 ± 0.41	4.52 ± 0.94*
CHX	2.51 ± 0.19**	8.34 ± 0.37**
ActD	0.93 ± 0.09	1.58 ± 0.31

Cell were treated with E2 at 10^{-9} M for 3 and 24 h with or without cycloheximide (CHX) at 10 μg/ml or actinomycin D (ActD) at 0.5 μg/ml. The inductions by E2 were calculated for each treatment (mean ± S.E.M., $n = 4$).

* Indicates significant induction at 0.05 and 0.01, respectively.

** Indicates significant induction at 0.05 and 0.01, respectively.

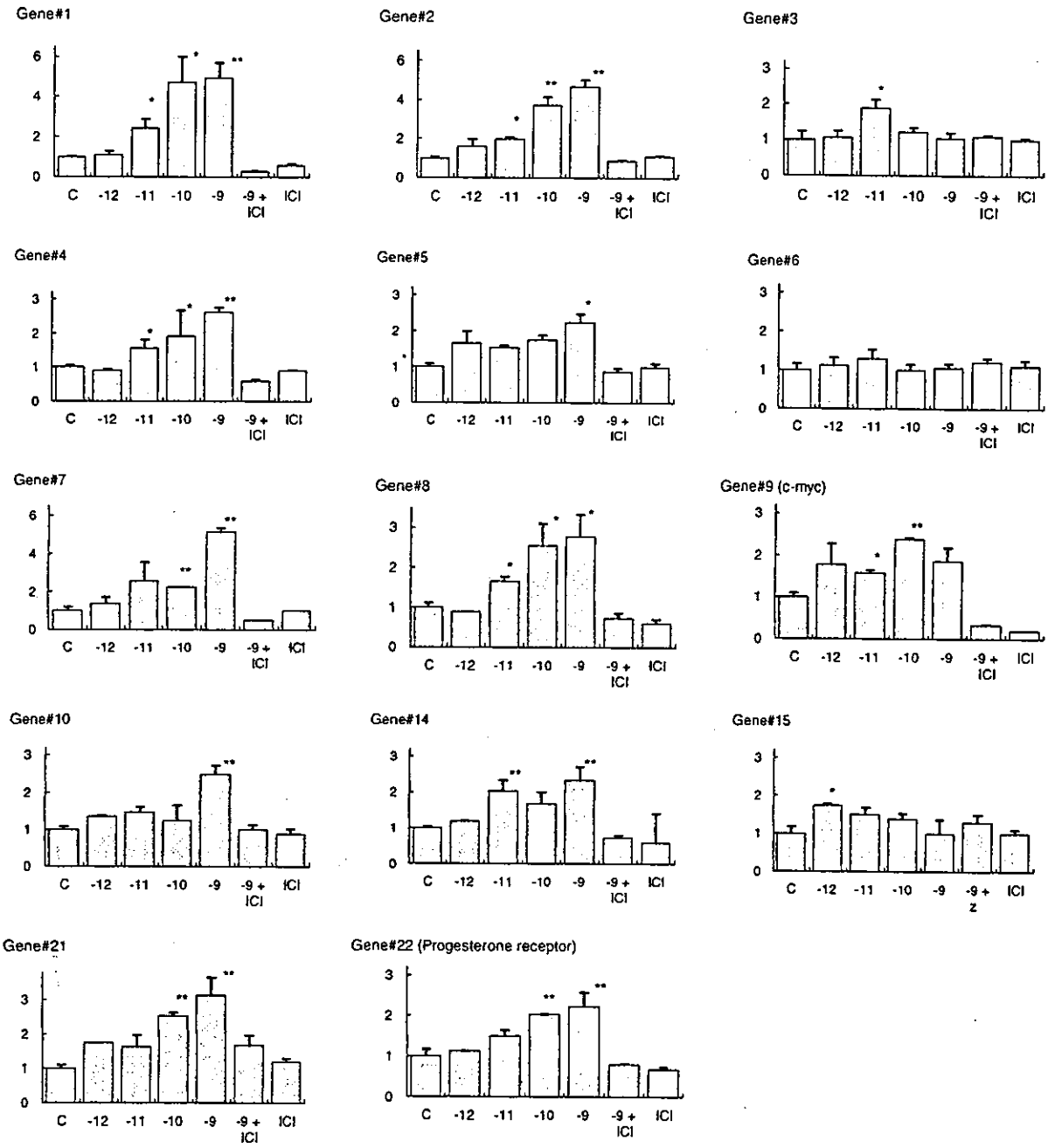

Fig. 2. Dose-dependent changes in gene expression levels measured by quantitative real-time RT-PCR. Cells were treated with different concentrations of E2 at 10^{-12} to 10^{-9} M and/or a single dose of ICI 182780 (ICI) at 10^{-7} M for 24 h. All mRNA contents were normalized with reference to G3PDH mRNA. The fold changes were calculated based on the gene expression in the cells treated with vehicle. Each point is an average of two independent experiments.

by actinomycin D but not by cycloheximide, which indicates that E2 regulates these genes at the transcriptional level.

3.4. Expression of genes in the pituitary gland

Expression of estrogen regulated genes in GH3 cells was further investigated in the anterior pituitary gland. First, mRNA expression of eleven-responsive genes was examined in short-term (24 h) and long-term (30 days) E2-treated ovariectomized F344 rats. Findings for estrogen-dependent increase for each gene are summarized in Table 4 as fold change of mRNA in E2-treated animals over that in the ovariectomized controls. All the genes except #4 were up-regulated in pituitary tissue by the short-term and long-term treatment of E2. Estrogen dependence of expression of gene #1 (calbindin-D9k) and gene #2 (parvalbumin) was extremely strong, over 100-fold induction being noted. For these and gene #9 (c-*myc*), more detailed time-dependent analysis was carried out. In Fig. 4, each mRNA level was calculated based on the level in ovariectomized rats at day

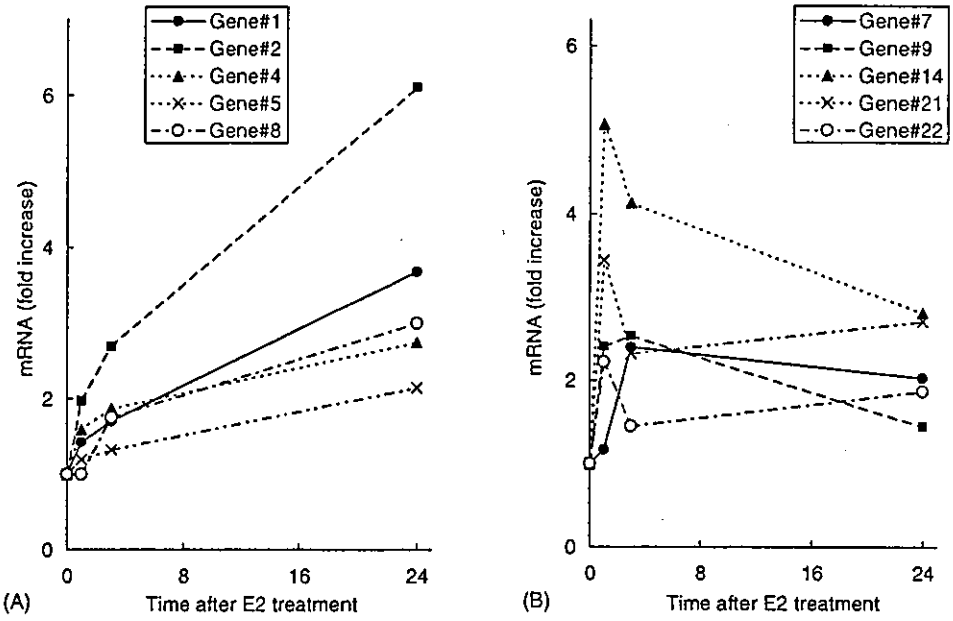

Fig. 3. Time-dependent change in gene expression levels measured by quantitative real-time RT-PCR. All mRNA contents were normalized with reference to G3PDH mRNA. Cells were treated with E2 at 10^{-9} M for 0, 1, 3 and 24 h. Each point represents a mean ± S.E.M. ($n = 4$). *,** Indicates significant differences from the control values at 0.05 and 0.01, respectively.

Table 4
Estrogen-responsive genes identified by the microarray study in the pituitary tissues in ovariectomized F344 rats

Gene	GenBank accession#	Fold change in expression	
		24 h	1 month
#1	K00994	118	95.0
#2	AI175539	28.9	70.0
#4	M17083	1.1	0.6
#5	S81478	2.3	2.0
#7	AI230712	2.9	4.7
#8	AF081366	9.9	2.0
#9	Y00396	4.5	17.7
#10	U02553	3.1	1.6
#14	U24175	2.4	4.0
#21	AA892522	2.0	5.1
#22	L16922	4.2	9.4

Ovariectomized F344 rats were treated subcutaneously with pellets containing E2 for 1 and 30 days. The gene expression was measured by quantitative real-time RT-PCR in pituitary tissue and the fold changes were calculated based on the mRNA level in ovariectomized controls at time 0 ($n = 5$).

0. All the three mRNAs, for calbindin-D9k, parvalbumin and c-*myc*, were induced significantly within 3 h of subcutaneous E2 administration, although the increase was most prominent for calbindin-D9k, with a 72-fold elevation. Higher levels were still maintained after a month of chronic E2 treatment.

4. Discussion

The GH3 cell line has been widely used to investigate the functions of somatolactotrophic cells, since regulation of its GH and prolactin production appears to be physiologically relevant with dependence on thyroid hormones, estrogen and glucocorticoid [11,12,19]. In the present study, we applied microarray analysis and identified a number of estrogen-responsive genes.

In terms of GH3 estrogen-responsiveness, there are two distinct parameters, prolactin synthesis and cell proliferation. However, reported sensitivity to estrogen has varied in the literature [4,13–15,20]. The inter-laboratory variation may be due partly to differences in strain, since GH3 has a rather old origin and has been widely used. Technical problems with charcoal treatment of serum for removing estrogenic substances may have had an impact in some cases [21]. The estrogenic activity of phenol red or related contaminants in common culture media was not recognized until Katzenellenbogen's group provided a convincing evidence [22]. Prior to the present microarray analysis, GH3 cells were examined in our culture conditions and found to be very sensitive to estrogen, exhibiting induction of cell proliferation in response to E2 at a concentration as low as 10^{-12} M. The high sensitivity on cell proliferation appears typical for pituitary cell lines, like the MtT/E-2 cell line we have established and another lactotrophic cell line, PR1 [4,23]. ERα is the major type of ER expressed in GH3 cells with a ratio to ERβ of 380:1 according to quantitative PCR (data not shown).

Recently, estrogen-responsive genes have been investigated by cDNA microarray in human breast cancers and the normal uterus [24,25]. However, the pituitary gland has not been explored for estrogen-responsive genes by this approach, to our knowledge. In the present microarray analysis, a relatively small number of genes were found to be