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Mitogen-Activated Protein Kinase Pathway Is Involved in Androgen-Independent *PSA* Gene Expression in LNCaP Cells

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BACKGROUND. Prostate specific antigen (PSA) is regulated by growth factors and hormones through functional androgen responsive elements in the promoter region of the *PSA* gene. However, the molecular basis for androgen independent PSA elevation in hormone refractory prostate cancer is unknown. The purpose of this study was to investigate the role of MAP kinase activation in androgen independent regulation of PSA expression.

METHODS. LNCaP cells transfected with MEK1 expression vector with or without the MAP kinase inhibitor U0126 under low androgen conditions were analyzed by luciferase assay and electrophoretic mobility shift assay (EMSA).

RESULTS. Transfection experiments of the proximal PSA promoter linked to Luc-reporter identified one region designated as "B" motif centered at −60 bp to be essential for basal activation. Co-transfection with the MEK1 activated vector enhanced PSA expression, while mutation of the "B" motif totally abrogated this induction. EMSA showed a specific DNA–protein complex, but Sp1 family members and AR do not interact with the "B" region by supershift analysis.

CONCLUSIONS. Our data suggest that enhanced androgen-independent *PSA* gene expression in MAP kinase-induced LNCaP cells is mediated, at least in part, by the "B" motif of the PSA promoter. *Prostate 56: 319–325, 2003.* © 2003 Wiley-Liss, Inc.

KEY WORDS: **PSA; androgen-independence; mitogen-activated protein kinase; prostate cancer**

INTRODUCTION

Prostate specific antigen (PSA) is a chymotrypsin-like protein protease synthesized by normal, hyperplastic, and malignant prostatic epithelia [1,2] and its expression is regulated by androgens through the androgen receptor (AR) [3–5]. The AR modulates transcription through its interaction with its consensus DNA binding site, GGTACAnnnTGTT/CCT, termed the androgen response element (ARE). The proximal promoter has been delineated to an −630 fragment containing a core TATA box and two AREs, AR I and AR II [6,7]. An enhancer element, centered at approximately −4.2 kb is located within a 6-kb region [8]. The enhancer was originally identified as a 1.6-kb fragment containing sites recognized by the androgen receptor, Ap1 cAMP-responsive element binding protein and Fos [9].

PSA is a useful biomarker for prostate cancer. It has been shown that serum PSA level is proportional to tumor volume and correlates positively with the clinical stage of the disease [10]. Progression of prostate cancer to androgen independent state is commonly associated with increase in serum PSA [11]. When the tumor becomes androgen-independent, PSA mRNA is up-regulated through an as yet unknown mechanism that presumably involves the promoter and enhancer regions of the *PSA* gene. These regions have been

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sequenced and the following DNA response elements have been characterized: (1) TATA box, −28 to −23; (2) androgen response elements, −170 to −156 and −4148 to −4134; and (3) androgen response region, −395 to −376 [7,9,12]. The fact that PSA production increases in an androgen-deprived environment suggests that other factors are involved in the androgen-independent induction of *PSA* gene expression. So far, little is known about the androgen-independent regulation of PSA in hormone-refractory prostate cancer cells.

In the present study, to clarify the mechanism of androgen-independent regulation of PSA, first, we idenified the cis-elements in the proximal promoter essential for basal activity. Then we showed that MAP kinase pathway is involved, without AR interaction, in the regulation of PSA expression, using LNCaP prostate cancer cells in an extremely low androgen environment.

MATERIALS AND METHODS

Cell Culture and Reagents

The human cancer cell line, LNCaP was purchased from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in RPMI-1640 supplemented with 10% FBS. For all experiments, cells were cultured in phenol red free RPMI-1640 medium supplemented with 2% charcoal-stripped FBS. To inhibit MAP kinase kinases 1 and 2, cells were treated with either 10 μM 1,4-diamino-2,3-dicyano-1,4 bis [2-aminophenylthio] butadiene (U0126: Promega, Madison, WI) or dimethyl sulfoxide (DMSO). The anti-androgen Casodex was a gift from Astra-Zeneca Pharmaceuticals (Macclesfield, UK).

Determination of PSA Secretion

The level of PSA secreted into the medium was determined using a Tandem-PSA radiometric assay kit (Beckman Coulter, Inc., CA) according to the instructions provided by the manufacturer and normalized to protein concentration.

Plasmid Construction

5′-Deletion constructs (−761Luc, RI-Luc [13], and −100Luc) within the PSA promoter were generated by PCR using oligonucleotides primers that contained convenient restriction sites for cloning into the promoterless pGL3 basic vector (Promega). The forward primers were: 5′-ACGTGAGCTCTATTTGTTGGAGAAGGGGCATTGG-3′, 5′-TTAAGAGCTCCTCCCCTCCCCTTCCACAG-3′, and 5′-TTAAGAGCTCGCAGCATGGGGAGGGCCTTGGTCA-3′corresponding to positions −761/−738, −153/−134, and −100/−77 relative to the transcriptional start site and a common reverse primer 5′-GAGGGGCTGGGGGTATGGGCTTGG-3′ for the +224/+247 site into the first intron. PCR products were cloned into the pCR®II (Invitrogen, Leek, The Netherlands), verified by sequencing, digested with SacI and HindIII, and subcloned into the pGL3 Basic vector (Promega). Several point mutations were introduced into the −100Luc and RILuc constructs using the U.S.E. Mutagenesis kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) following manufacturer's protocol. The −100 mLuc represents mutation of the possible Sp1 consensus sequence and the RImLuc for the RILuc construct. Lowercase boldface letters represent base substitution mutations on the PSA promoter. The primers used for mutagenesis were as follows: 5′-AGCAGGGCAG**ttt**-CGGAGTCCTG-3′, 5′-CTGGGTGCCA**cggaGtact**GG-GCGGAGTCC-3′ for the −100mLuc and RImLuc, respectively. Mutant clones with the correct digestion pattern were further confirmed by sequencing and then subcloned into the pGL3 basic vector.

Transient Transfection and Reporter Gene Activity Assay

Briefly, LNCaP cells (2×10^5) were plated on 24-well plates and incubated in RPMI 1640 with 10% fetal bovine serum for 24 hr before transfection. Cells were transfected with designed plasmid constructs and 1 μg of reporter plasmid of pRL-TK vector containing cDNA encoding Renilla luciferase (Promega) for normalization, using the Tfx transfection reagent (Promega) in phenol red free RPMI 1640 containing 2% charcoal-treated serum. For MAP kinase treatment, cells were cotransfected with 500 ng of the MEK1 cDNA activated in pUSEamp vector (Upstate Biotechnology, NY). Following transfection, serum free media containing the 10-nM testosterone analog R1881 with or without 100 μM anti-androgen Casodex or Casodex alone were added. For experiments involving the MEK inhibitor U0126, cells were pre-incubated with 10 μM of U0126 for 1 hr before transfection assays. Twenty-four hours after transfection, cells were washed and lysed. Luciferase activity in cells lysates was assayed using the Dual-Luciferase Reporter assay system (Promega). All values in transfection experiments were normalized for transfection efficiency by co-transfection with the pRL-TK vector.

All transfections were carried out in the above-described charcoal-free conditions, unless otherwise specified.

Nuclear Extract Preparation

Nuclear extracts were prepared as described earlier [14]. All the following steps were performed at 4°C. Briefly, cells were resuspended in sucrose buffer

[0.32 M sucrose, 3 mM $CaCl_2$, 2 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol (DTT), 0.5 mM PMSF, and 0.5% NP-40], and the lysate was then centrifuged at 500*g* for 5 min to obtain the nuclei pellet, which were washed with sucrose buffer without NP-40. The nuclei were resuspended in low salt buffer [20 mM HEPES (pH 7.9), 25% glycerol, 0.02 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1% NP-40, 0.5 mM DTT, and 0.5 mM PMSF] followed by addition of high salt buffer [20 mM HEPES (pH 7.9), 25% glycerol, 0.8 M KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1% NP-40, 0.5 mM DTT, and 0.5 mM PMSF] with incubation for 20 min on a rotatory platform. Samples were diluted in "diluent buffer" [2.5 volumes of HEPES (pH 7.6), 25% glycerol, 0.1 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF] and then centrifuged at 13,700*g* for 20 min. Five-µg aliquots of supernatant (nuclear extract) were stored at −70°C.

Electrophoretic Mobility Shift Assay (EMSA)

To use as probes, double-stranded oligonucleotides spanning the region between −64/−37 of the human PSA promoter were prepared: wild-type ("B"WT) 5′-CCAGCAGGGCAGGGGCGGAGTCCTGGGGA-3′ (Sp1 consensus sequence underlined) and mutated ("B"mut) 5′-CCAGCAGGGCAGGtttGGAGTCCTGGGGA-3′ (substituted bases represented by lowercase boldface letters). Binding reactions were performed by incubating ^{32}P-labeled double-stranded oligonucleotides with nuclear extract in binding buffer (10 mM Tris-HCl (pH 7.6), 5% glycerol, 1 mM EDTA, 50 mM NaCl, 1 mM DTT, and 0.1 µg/ml poly (dI–dC). For oligonucleotide competition assays, 25- or 100-fold molar excess of unlabeled oligonucleotides were also added to the binding reaction. After incubation at 25°C for 30 min, the reaction mixtures were analyzed on 4% nondenaturing polyacrylamide gel. For antibody supershift assays, 1 µl of anti Sp1, Sp2, −Sp3 or −Sp4 and AR antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the reaction mixture prior to the addition of the probes and incubated at 4°C for 1 hr. Protein–DNA complexes were fractionated on 4% nondenaturing polyacrylamide gels in 0.5× Tris-Borate-EDTA buffer at 4°C and visualized by autoradiography.

Northern Blot

Total RNA was isolated from U0126, MEK, or vehicle treated cells using the Ultraspect RNA isolation system (Biotecx Laboratories, Houston, TX). RNA was quantitated electrophotometrically, and 5 µg of each sample were fractionated on 1.2% agarose formaldehyde gel and transfer to Zeta-Probe Blotting Membrane (Bio-Rad). A 300-bp PSA cDNA fragment was prepared by PCR using the primers 5′-TGGACAGGGGGCAAAAGCAC-3′ and 5′-AGGACACAGAGAGGACAAAA-3′ end labeled and used for hybridization as described previously. Autoradiographs were analyzed using the NIH Image 1.62 (National Institute of Health) program and normalized to 28S RNA.

RESULTS

Identification of the Minimal PSA Promoter Region and the cis-Regulatory Element

To discern the minimal region necessary for PSA promoter activity under androgen-deprived conditions, a series of deletions were generated by PCR (Fig. 1). Transfection of LNCaP cells with the construct −761, RI, and −100 in the absence of androgens revealed notable basal promoter activity. The −37Luc construct showed a low promoter activity. These results indicated that the region −37 to −100 is essential for basal PSA promoter activity (Fig. 1). The sequence −100 to −37 of the PSA promoter contains a possible Sp1 transcription factor consensus sequence centered at −60 bp upstream from the transcription start site. To investigate the role of Sp1, three points mutation were introduced in the −100 construct (see "Materials and Methods" for sequence of mutated −100 construct). Transient transfection of LNCaP cells with the mutated −100Luc construct showed that −100mLuc resulted in complete loss of promoter activity (Fig. 1). Because this

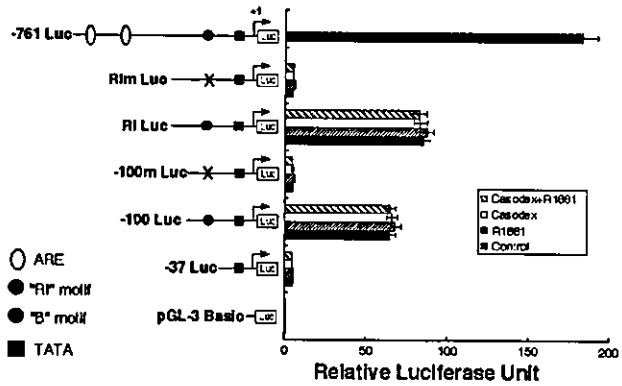

Fig. 1. 5′-Deletion and mutation analysis of the human *PSA* gene promoter. The nucleotide sequence −761 to +5 bp of the human *PSA* gene promoter was cloned into the pGL3 basic vector and prepared for transfection. Deletion constructs were transiently transfected in LNCaP cells in the presence of 10 nM R1881, 100 µM Casodex, or both R1881 and Casodex. The diagram on the left is a map of a series of 5′deletions of PSA promoter in Luc-plasmid. Potential consensus binding sites for ARE (open circles), "B" motif (closed circles) "RI" region (hatched circles), and TATA (closed boxes) are indicated. Base substitution mutations in the "B" and "RI" sequences are represented by a cross. Normalized luciferase activities are depicted on the right. The data are mean ± SEM values (n = 3).

site overlaps with a previous reported ("RI") region [13], transfection of cells with the RILuc construct also was associated with a substantial promoter activity consistent with the mentioned report. The RILuc activity was slightly higher than that of the −100 construct, but both mutants showed a significant decrease of promoter activity. To achieve a complete hormone free environment and to further characterize the effect of androgens on the "B" region, we used the anti-androgen Casodex for blocking the AR transcriptional activity. As shown in Figure 1, addition of Casodex did not change −100Luc and RILuc transcriptional activities. Further, treatment with the synthetic androgen R1881 exerts no effect on both constructs. These findings suggest that the "B" region located at −60 from the start codon, is the key element in the −100 bp of the PSA promoter sequence, and essential for basal activity under androgen-independent conditions.

Sp1 Transcription Factor and AR Are Not Involved in the Proximal PSA Promoter Transcriptional Activity

A database consensus site search showed that the "B" region shares high homology with the Sp1 transcription family binding site. Thus, we evaluated whether members of the Sp1 transcription family are able to bind to the potential consensus binding sites within the −100 to −60 promoter region. In addition, we analyzed binding of these transcription factors to "B" fragment containing the same mutation used in the transfection assays. EMSA showed that ^{32}P-labeled double-stranded oligonucleotides corresponding to the "B" region of the *PSA* gene promoter and nuclear extracts from LNCaP cells form a prominent nucleo–protein complex. The addition of an excess of double-stranded oligonucleotide suppressed complex formation (Fig. 2A). Simultaneous addition of antibodies against Sp1, Sp2, Sp3, and Sp4 did not produce an additional shift, and supershift of DNA–protein complex was not either observed in the presence of anti-AR antibody. As a control for Sp1 supershift, we used the ^{32}P-labeled double-stranded Sp1 and AR probe (Fig. 2B). Overall, these findings suggest that other factor(s) different from the Sp1 transcription family are responsible for the minimal PSA expression. In agreement with these observations, a previous study has reported a novel 45 kDa protein as responsible of the basal PSA promoter activity [13].

Effect of MAP Kinase on PSA mRNA and Protein Expression

To address whether MAP kinase influences PSA expression, LNCaP cells were cultured in RPMI1640

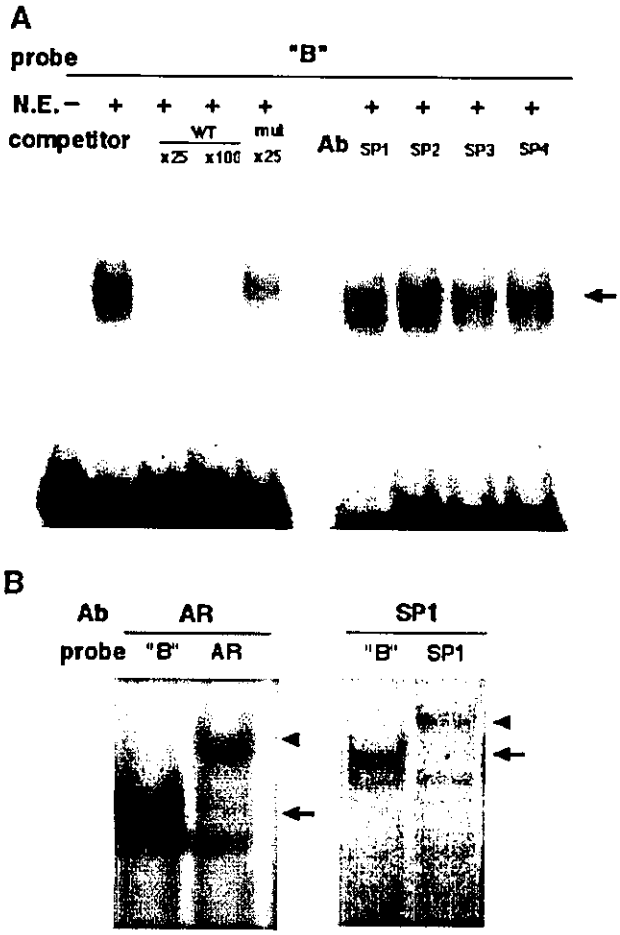

Fig. 2. Formation of DNA–protein complexes on the *PSA* gene proximal promoter. Nuclear extracts (5 μg/lane) from LNCaP cells were incubated with a ^{32}P-labeled −54/−37 ["B"] fragment of the human PSA promoter containing the wild-type sequence ("B"wt) in the absence or presence of 25- or 100-fold molar excess, or the mutant oligonucleotide ("B"mut). Nucleo–protein complexes were analyzed by electrophoresis on a 4% polyacrylamide gel. To identify proteins bound to the "B" consensus site, the reaction mixture was incubated with an anti-Sp1, -Sp2, -Sp3, or -Sp4 antibodies (**A**); as positive control, probes containing AR and Sp1 consensus sequence were used (**B**). Arrows indicate the DNA–protein complexes specific to LNCaP extracts and arrowheads show supershifted bands in the presence of Sp1 and AR antibodies.

containing 10% FBS up to 80% of confluency, and then switched to phenol red free RPMI-1640 with 2% charcoal-stripped FBS; three groups of cells were prepared: (1) Control (transfected with pUSEamp-empty vector), (2) MEK (transfected with MEK1 expression vector), and (3) MEK + U (transfected with MEK1 expression vector and treated with 10 mM U0126). Total cell protein was extracted and conditioned medium collected for radio-immunoassay of the PSA protein 24 hr after treatment. As shown in Figure 3,

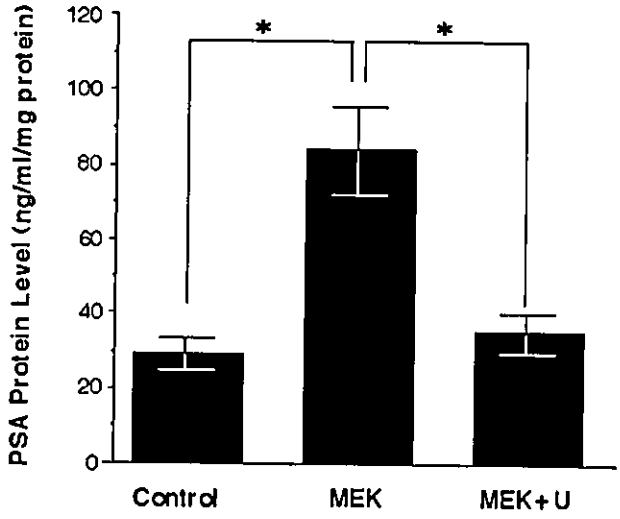

Fig. 3. Immunoassay analysis of PSA protein after MAP kinase stimulation. LNCaP cells were transfected with 0.5 μg of pUSE-empty vector (Control), MEK1-pUSE vector (MEK), and MEK1-pUSE vector plus 10 μM U0126 (MEK + U) in phenol red-free RPMI 1640 medium containing 5% charcoal-treated FBS and incubated for 24 hr. Conditioned media was collected for immunoassay of the PSA protein. PSA protein concentration was normalized by the total protein concentration. Data are means ± SE ($n = 3$). *$P < 0.01$.

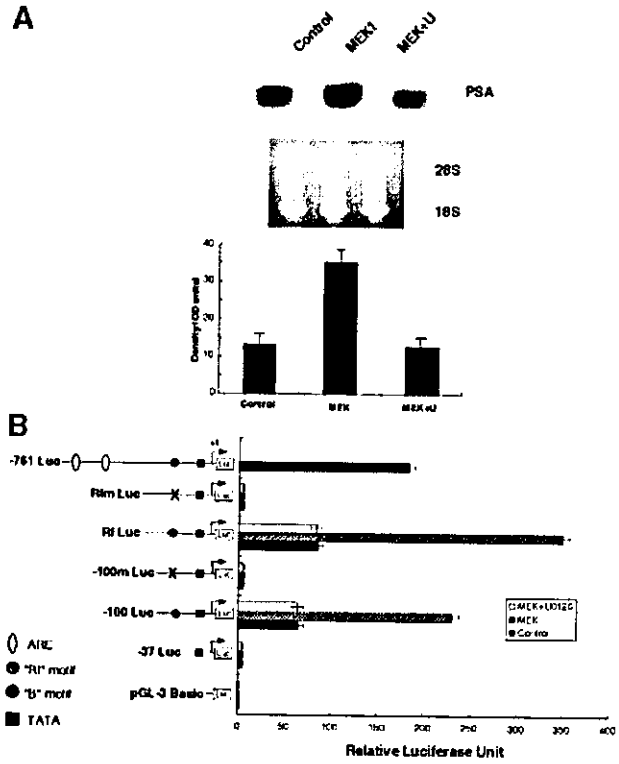

Fig. 4. Effect of MAP kinase on PSA mRNA expression and promoter activity in LNCaP cells. Cells were transfected with 0.5 μg of MEK1 expression vector (MEK1), empty vector (Control), or with MEK1 expression vector plus 10 μM U0126 (MEK + U) for 24 hr. **A**: Fifteen micrograms of total RNAs were analyzed by Northern blot. Density of mRNA was determined from X-ray film using the NIH image software. Data are the means of two separate experiments and normalized to 28S. **B**: The PSA promoter constructs (RILuc, RImLuc, −100Luc, −100m Luc, −37 Luc, and pGL3 Basic) were transfected into LNCaP cells and then cells were treated as described above. The cross represents mutations in the constructs. The data are mean ± SEM values ($n = 3$).

the amount of PSA in the medium was increased in MEK, whereas this effect was abrogated in MEK + U. These results indicate that PSA protein expression may be elevated by MAP kinase stimulation, and that the stimulus is a specific event. Northern blot analysis showed increased mRNA level in MEK1-transfected cells, whereas the transcripts were decreased by further addition of U0126. These results suggest that the expression of PSA by MAP kinase is, at least in part, regulated at the transcription level.

The −60 PSA Promoter Region Is Essential for MAP Kinase Responsiveness

To evaluate the need of MAP kinase for the signaling pathway leading to increased PSA expression in LNCaP cells, we performed luciferase assay using the −100Luc and the RILuc constructs. Cells were transfected with the MEK1 expression vector alone or previously treated with U0126 (described in Fig. 4A). We found that MEK1-mediated PSA expression increased 3.5-fold and 4-fold over control in −100Luc and the RILuc, respectively. This induction was totally abolished when their corresponding mutants were used (Fig. 4B). Therefore, the MAP kinase-sensitive region appears to be involved in the region of the −100Luc construct.

MAP Kinase Stimulates Transcription Factor Binding to PSA Promoter in LNCaP Cells

As shown in Figure 5, MAP kinase significantly stimulates PSA promoter activity in the absence of androgen. To elucidate the function of the region, we examined the mechanism by which MEK1 modulates *PSA* gene expression. We performed EMSA using nuclear protein extracts from LNCaP transfected with MEK1-treated with or without U0126 and control. Probe "B" that corresponds to the −60 region formed a complex, and the intensity of this complex increased with MEK1 and decreased with the U0126 as compared to control (Fig. 5). These results indicate that the binding of nuclear protein(s), which associates preferentially to the "B" site on the PSA promoter, is regulated through MAP kinase pathway in androgen-independent conditions.

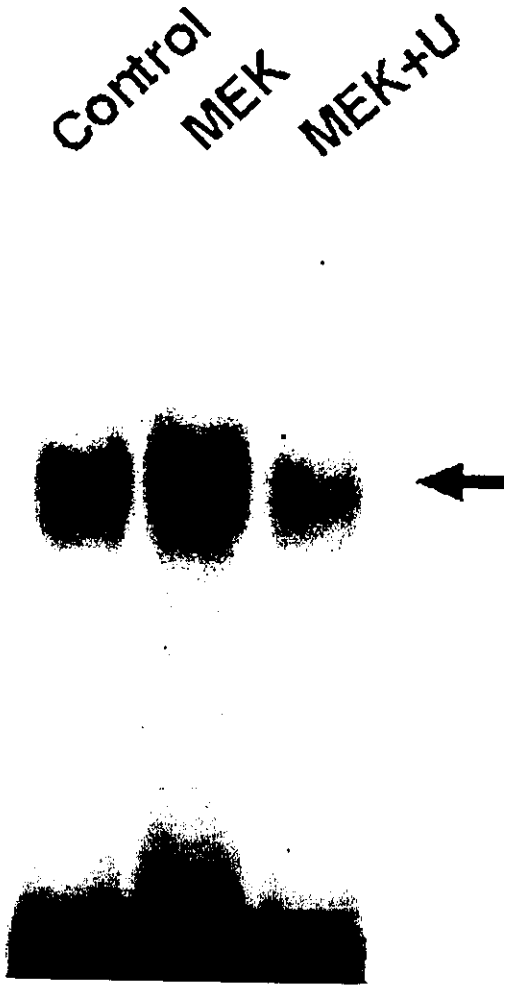

Fig. 5. MAP kinase activation increases the binding of nuclear proteins to the −60 bp region. LNCaP cells were transfected with pUSE-empty vector (**A**), MEK1 expression vector (**B**), or MEK1 expression vector in the presence of 10 μM of U0126 (**C**) in an androgen-deprived environment. Cells were harvested after 24 hr incubation and nuclear proteins were extracted. Five micrograms of nuclear proteins were incubated in the presence of ^{32}P-labeled "B" WT oligonucleotide and analyzed by EMSA on a 4% polyacrilamide gel. The arrow indicates specific bands. Binding complexes were detected by exposing the dried gels to X-ray film at −80°C overnight.

DISCUSSION

PSA is a clinically important androgen-dependent gene that is used for monitoring treatment response, prognosis, and progression in patients with prostate cancer. The transcriptional control of PSA is initially androgen-regulated and decreased after androgen deprivation [15]. However, rebound of serum PSA is consistently observed in patients with hormone-refractory prostate cancer [11]. At present it has not been well studied the mechanism of androgen-independent regulation of PSA.

It has been previously shown that AR is activated by protein kinase A and/or protein kinase C pathway in the absence of androgen [16–19], and that androgen-independent induction of *PSA* gene expression in LNCaP cells is regulated by AR-dependent pathway [17]. However, our results of deletion and mutation analysis of PSA promoter in an androgen-deprived condition revealed that androgen response elements are not required for basal promoter activity. EMSA showed specific binding of nuclear protein to the "B" site. The protein is not AR since supershift band was not observed using anti-AR antibody. A 150-bp pN/H region has been demonstrated to be a strong AR-independent positive-regulatory element and a 17-bp RI site has been identified as the key cis-element of the PSA promoter in LNCaP. Furthermore, a 45 kDa (p45) cell-specific transcription factor associates with RI in LNCaP and may be responsible for the regulation of PSA promoter activity independently from androgen and AR [13]. The cis-element; "B" in our study is identical to the RI site. EMSA and supershift assay showed that specific DNA–protein complex formed using double-stranded oligonucleotide probe contains the "B" sequence. The binding protein observed in our study may be the p45 cell-specific transcription factor, although we did not perform a UV cross-linking. This novel transcription factor might play an important role in the regulation of androgen-independent *PSA* gene transcription.

High levels of MAP kinases expression are detected in more advanced, metastatic, and androgen-independent prostate cancers [20]. Progression of the disease is commonly associated with increase in serum PSA. These findings suggest that MAP kinase pathway is involved in the androgen-independent regulation of *PSA* gene expression. Our data showed that PSA transcription and secretion are up-regulated in LNCaP transfected with MEK expression vector, while the effect is abolished in cells treated with the MAP kinase inhibitor U0126 under androgen-deprived conditions. Binding of the transcription factor to the "B" region is increased by MAP kinase activation. Growth factors such as epidermal growth factor, transforming growth factor-α, insulin-like growth factor, interleukin-6, keratinocyte growth factor, and other fibroblast growth factor family members are expressed in androgen-independent prostate cancers [21]. Elevated levels of MAP kinase activity in androgen-independent prostate cancer cells have been previously demonstrated [22]. These results suggest that secreted growth factors may act in autocrine fashion leading to activation of the MAP kinase pathway. High level of RI–p45 complex in EMSA is seen in the androgen-independent prostate cancer cell line C4-2 [13]. Although we used only LNCaP cells due to the limited availability of PSA

producing androgen-independent prostate cancer cell lines, these results indicate that the transcription of PSA in an androgen-deprived environment could be regulated via MAP kinase pathway. The increased association of the transcription factor to the "B" site by MAP kinase activation might be a possible mechanism by which up-regulation of the *PSA* gene transcription occurs in androgen-independent prostate cancer cells. Prostate cancer cells could bypass the requirement of androgen by the production of autocrine factors, which could activate MAP kinase and lead to progression to a hormone refractory stage and become androgen-independent. Further studies should be carried out to determine if this is a direct effect on transcription factors or an indirect effect involving other MAP kinase-sensitive nuclear factors especially using PSA producing androgen-independent prostate cancer cells.

CONCLUSION

In the present study, we identified a cis-element essential for androgen-independent regulation of PSA promoter and reported the involvement of MAP kinase pathway in androgen-independent *PSA* gene expression. The activation of MAP kinase and increased binding of transcription factor to its binding site may result in androgen-independent up-regulation of the PSA in prostate cancer cell lines.

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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

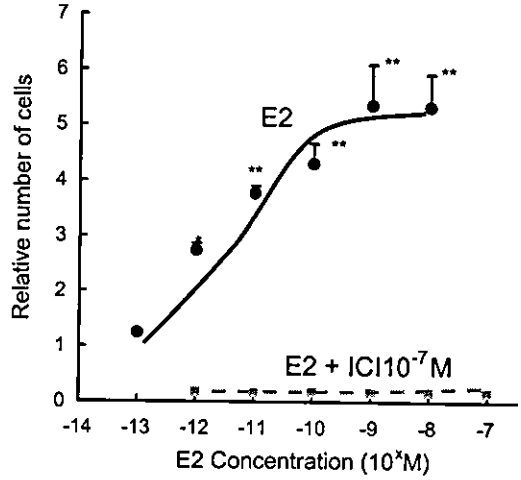

Fig. 1. Effects of 17β-estradiol (E2) and ICI182780 (ICI) on GH3 cell proliferation. Cells were seeded in 24-well plates at 1×10^4 cells per well. After five days treatment with E2 at 10^{-13} to 10^{-9} M alone or with ICI at 10^{-7} M, cell proliferation was measured by a modified MTT assay. Each point 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.

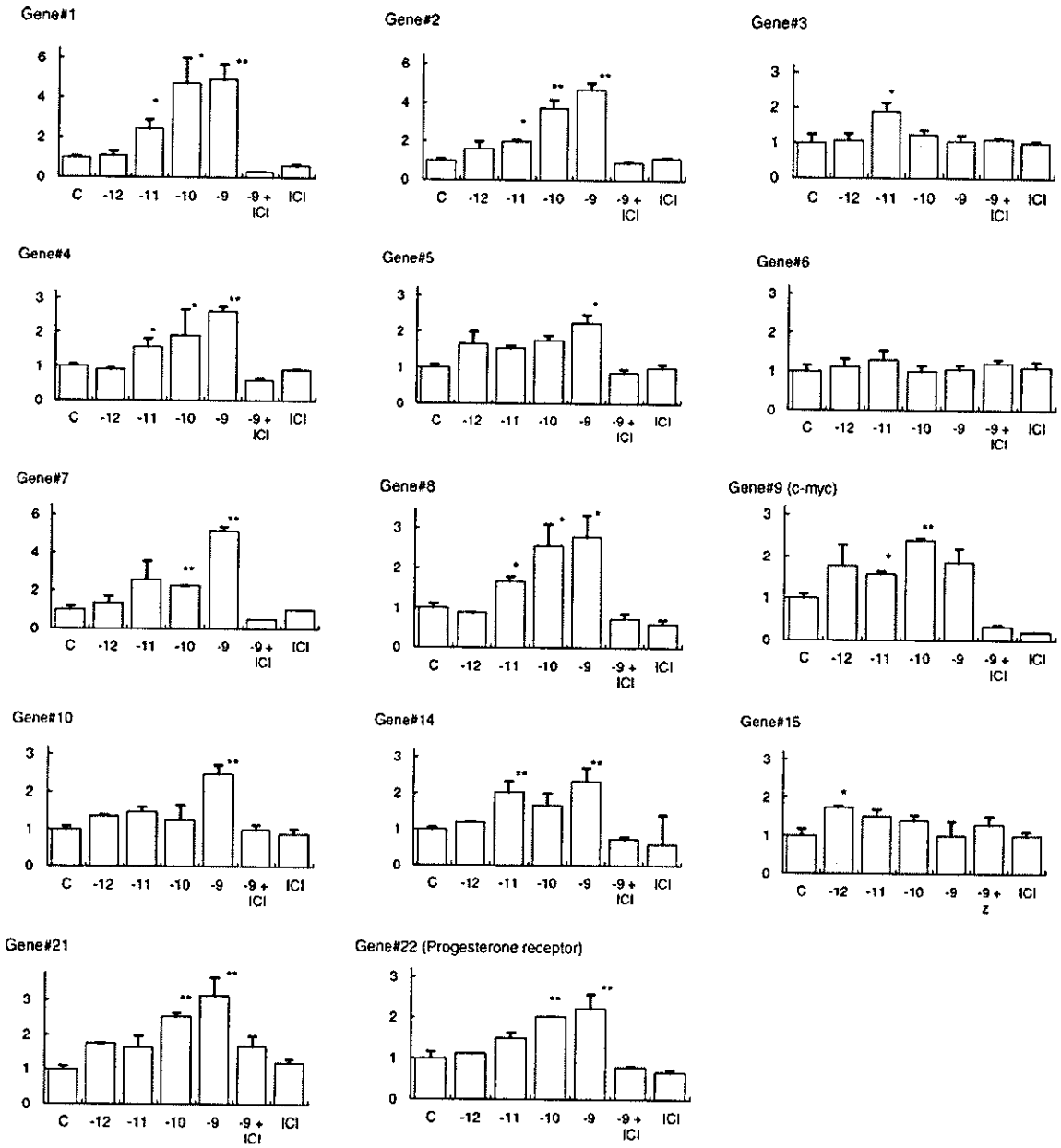

Fig. 2. Dose-dependent changes in gene expression levels measured by quantitative real-time RT-PCR. Cells were treated with different concentrations of E2 at 10^{-12} to 10^{-9} M and/or a single dose of ICI 182780 (ICI) at 10^{-7} M for 24 h. All mRNA contents were normalized with reference to G3PDH 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

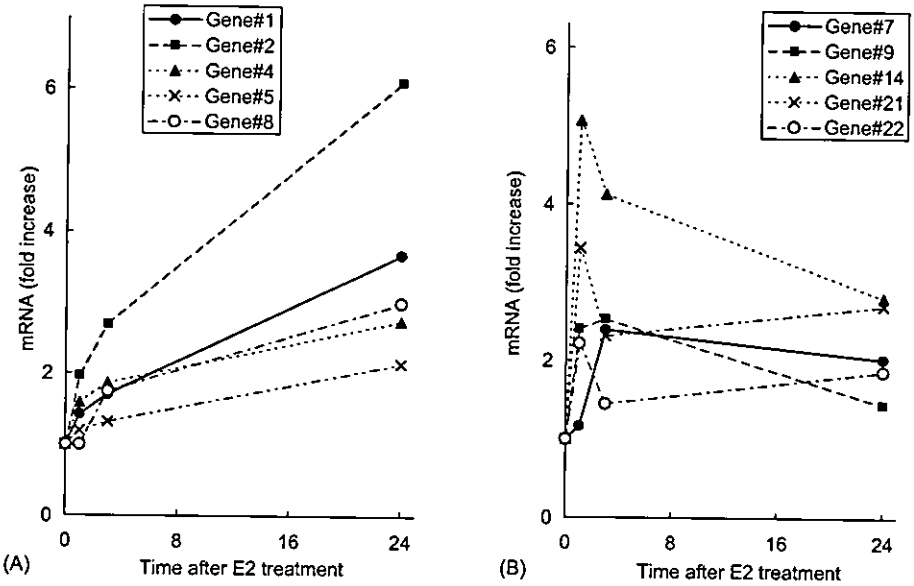

Fig. 3. Time-dependent change in gene expression levels measured by quantitative real-time RT-PCR. All mRNA contents were normalized with reference to G3PDH mRNA. Cells were treated with E2 at 10^{-9} M for 0, 1, 3 and 24 h. Each point represents a mean ± S.E.M. ($n = 4$). *,** Indicates significant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

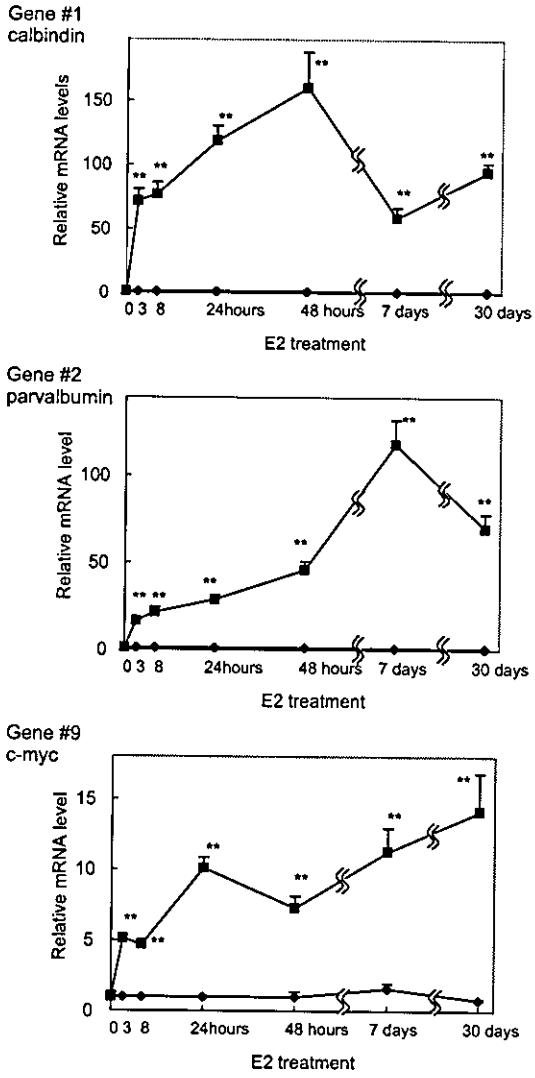

Fig. 4. Time-dependent analysis of three estrogen-responsive genes in the pituitary in vivo. Ovariectomized F344 rats were treated subcutaneously with pellets containing E2 for 3, 8, 24, and 48 h and 7 and 30 days. Gene expression was measured by quantitative real-time RT-PCR in pituitary tissue and fold changes were calculated based on the mRNA level in the ovariectomized controls at time zero. All mRNA contents were normalized with reference to G3PDH mRNA. Each point and bar represent mean ± S.E.M. ($n = 5$), *,** Indicates significant differences from the control values at 0.05 and 0.01, respectively.

regulated by estrogen with confirmation in most cases by quantitative real-time PCR. Suppression by ICI of E2-induced gene expression was also confirmed. The degrees of change were similar with real-time PCR analysis and GeneChip data and although we selected up-regulated genes after 24 h of estrogen exposure, some genes proved to be rapidly regulated (Fig. 3(B)) including these for the progesterone receptor and c-*myc*. Estrogen-responsive induction of progesterone receptor is well documented for the primary target, the uterus, as well as in the anterior pituitary gland [26,27]. Estrogen activation of c-*myc* also has been reported in the anterior pituitary gland and breast cancer cells [28,29]. A total of seven genes could be listed as down-regulated but they were not analyzed further, since all of them displayed relatively small degrees of change to 0.34–0.48 of the control values. Other known estrogen-responsive genes in the pituitary gland, such as prolactin and TGFα were not on the array used in the present study.

Interestingly, the in vivo expression of two genes, calbindin-D9k and parvalbumin, was found to be highly induced by E2 both in the short and longer term, which may suggest that hypothalamus or other indirect endocrine pathways would be involved in regulating genes in addition to the direct transcriptional activation. Calbindin-D9k is a vitamin D-dependent intestinal calcium-binding protein that is detectable in the duodenum, uterus and placenta [30–32]. Another vitamin D-dependent calcium-binding protein, calbindin-D28k, expressed in kidney and brain has no homology with calbindin-D9k either at the nucleotide or at the transcript levels [33]. The calbindin-D9k gene has been reported to contain a 15-base-pair imperfect palindrome with high homology to the estrogen- and glucocorticoid-responsive elements (ERE and GRE) [34]. Although there is no evidence that this protein is regulated by estrogen in the intestine through this motif, it is possible that the imperfect ERE is functional for the hormone-dependent transcription in the pituitary gland. Parvalbumin is another calcium-binding protein that belongs to the EF-hand calcium-binding protein like calbindin-D9k [35]. It is abundant in fast contracting/relaxing muscle fibers, where it plays a role as a calcium buffer and is also found in neurons as well as in endocrine glands including pituitary, thyroid, adrenals, testes and ovaries [36]. It has been postulated that parvalbumin can prevent cell death due to calcium overload in neurons. Although its expression is developmentally regulated in muscle, brain and other tissues, no evidence indicating hormonal regulation has been reported [37,38]. The 5′ flanking region of the gene seems to function as the promoter but it does not contain any motifs for estrogen-dependent transcription [39,40].

Since RNA was extracted from whole anterior pituitary tissue in the present study, it is not clear which types of cell actually contributed to the increase in mRNA levels. Chronic treatment of rats with E2 is known to result in the development of lactotrophic tumors [5]. The F344 strain is the most sensitive to E2 and somatolactotrophs of the pituitary become hyperplastic after exposure for a week and steadily proliferate thereafter. In the present study, major response of GH3 cells was cell proliferation so that some of the identified genes might be expected to be mitosis-related and involved in estrogen-induced pituitary hyperplasia/tumorigenesis. Although up-regulation of the calbindin-D9k and parvalbumin gene are evident on long-term treatment of E2, there was no obvious correlation with the time period for pituitary hyperplasia in contrast to the c-*myc* expression which steadily increase.

In conclusion, the present microarray analysis allowed identification of a number of estrogen-responsive genes in GH3 cells whose regulation appears biologically relevant in the pituitary gland in vivo. The actual significance of two calcium-binding proteins discovered to be prominently induced by E2 remains to be explored in the future.

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Possible enhancing effects of atrazine and nonylphenol on 7,12-dimethylbenz[*a*]anthracene-induced mammary tumor development in human c-Ha-*ras* proto-oncogene transgenic rats

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Our transgenic (Tg) strain carrying copies of the human c-Ha-*ras* proto-oncogene is highly susceptible to 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary carcinogenesis, possibly due to activation of the transgene, and can be used in medium-term bioassay models to test for modifying effects of estrogenic environmental compounds on tumor development. The present study was conducted to assess the influence of dietary feeding of the endocrine disruptors atrazine and nonylphenol on DMBA-induced carcinogenesis in c-Ha-*ras* Tg rats. Animals of both sexes were given a single oral dose of DMBA (25 mg/kg body weight) at 50 days of age and thereafter received soybean-free diet containing 5, 50 or 500 ppm atrazine, or 10, 25, 100 or 250 ppm nonylphenol. In female Tg rats, atrazine at a dose of 5 ppm increased the incidences of mammary adenomas and adenocarcinomas ($P<0.01$ and $P<0.05$), while 50 ppm increased the adenocarcinoma incidence ($P<0.05$). In males, skin tumor development, in contrast, was significantly decreased at the highest dose. Nonylphenol at 10 ppm increased adenocarcinoma and total mammary tumor multiplicity in female Tg rats ($P<0.05$), but there was no dose dependence, a significant quadratic dose-response trend rather being observed ($P<0.05$). *In vitro*, atrazine did not cause proliferation of MCF-7 cells at any of a range of doses tested. These results suggest that endocrine disruptors may enhance mammary carcinogenesis, but only in a certain limited dose range under the present experimental conditions. The doses applied, moreover, were all extremely high compared to the possible environmental human exposure levels. (Cancer Sci 2004; 95: 404–410)

It is well documented that estrogen is an obligatory factor for development of the mammary gland.[1,2] The hormone also plays an important role in both the etiology and treatment of mammary cancer and accumulating evidence indicates that slightly elevated levels of circulating estrogens may predispose to tumorigenesis.[3,4] Exogenous estrogens may similarly increase mammary cancer risk.[5–7] This may be of considerable importance, since recently, a number of environmental chemicals impacting on the endocrine system (endocrine disruptors) have been shown to exhibit estrogenic activity, these including triazine derivatives and alkylphenolic compounds.

Among the symmetrical triazine-type herbicides, atrazine (6-chloro-N^2-ethyl-N^4-isopropyl-1,3,5-triazine-2,4-diamine) is one of the most widely and heavily used herbicides all over the world. It is employed in agriculture as a selective pre- and post-emergence agent for annual control of grass and broad-leaved weeds. A number of studies have suggested that atrazine is an endocrine disruptor[8–15] and in female Sprague-Dawley, but not other strains of rats, high doses (50–1000 ppm) in the diet have been found to be associated with an increased incidence and/or an earlier onset of mammary gland tumors.[16–20]

Alkylphenol polyethoxylates are the second largest group of nonionic surfactants in commercial production. They find practical application, not only in detergents, but also in paints, herbicides, pesticides, and many other formulated products. Alkylphenol polyethoxylates with 8–12 ethoxylate groups are commonly used, with nonylphenol polyethoxylates accounting for about 80% of the total. It has been estimated that 60% of alkylphenol polyethoxylates end up in the aquatic environment,[21] most entering via sewage treatment works, where they are readily degraded to form relatively stable metabolites.[22,23] Some of these metabolites are hydrophobic (e.g. the alkylphenols, nonylphenol, and octhylphenol) and tend to accumulate in sewage sludge and river sediment. In 1991, Soto *et al.*[24] reported estrogenicity for nonylphenol released from plastic tubes. The compound has been implicated in the disruption of endocrine functions in wildlife,[25–28] but despite its estrogenic activity, there have been only a limited number of reports concerning effects on carcinogenesis *in vivo*.[29–31] Whether it might influence neoplasia of the breast is of particular interest.

We have established a rat line carrying copies of the human c-Ha-*ras* proto-oncogene with its own promoter region, which is highly susceptible to *N*-methyl-*N*-nitrosourea (MNU)- and 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary carcinogenesis.[32,33] Tumors develop in almost all females within as short a period as 8 to 12 weeks after a single MNU or DMBA treatment. Most of the tumor cells contained mutated transgene, but not endogenous Hras gene, indicating that activation of the transgene plays an important role in such rapid development of tumors. The animals have also been found to be susceptible to *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine urinary bladder,[34] *N*-nitrosomethylbenzylamine esophageal,[35] and DMBA skin carcinogenesis.[36] We recently showed that the transgenic (Tg) rats have a potential for use in medium-term bioassay models to test for the modifying effects of estrogenic environmental compounds on mammary tumor development.[37] In the present study, they were employed to investigate potential modifying effects of atrazine and nonylphenol on DMBA mammary and skin carcinogenesis.

Materials and Methods

Animals. Human c-Ha-*ras* proto-oncogene Tg and non-Tg rats

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bred by CLEA Japan Inc., Tokyo[32,34] at 7 weeks of age were maintained in plastic cages in an air conditioned room at 22±2°C and 55±10% humidity. The animals were all allowed free access to pellet basal diet (Oriental Yeast Co., Ltd., Tokyo), from which soy constituents, isoflavones which were known to act as estrogens, had been eliminated (Table 1), and to tap water. The experiments were conducted according to the "Guidelines for Animal Experiments in National Cancer Center, Japan."

Experimental protocols. A total of 276 female and male Tg and non-Tg rats received a single dose of DMBA (25 mg/kg body weight) by gavage at 50 days of age (Fig. 1). One day thereafter, they were placed on powdered basal diet containing nonylphenol (Sigma-Aldrich Japan, Tokyo), atrazine (Wako Pure Chemical Industries, Ltd., Osaka) or no supplement. Palpation and observation were regularly performed to monitor the development of mammary and skin tumors after DMBA treatment. The surviving animals were sacrificed by exsanguination under deep ether anesthesia at the end of week 8 for Tg females and week 20 for non-Tg females and both groups of males. Numbers of grossly visible tumors were recorded before they were measured and sampled for histological examination. Values are expressed as average tumor volume of total tumors for each rat. Body, liver, kidney, ovary, uterus, and testis weights were also recorded.

Measurement of serum atrazine and nonylphenol. Blood samples were centrifuged and measurement of serum atrazine and nonylphenol was performed by Japan Food Research Laboratories (Tokyo) using liquid chromatography/mass spectrometry (LC/MS). The limit of detection was 50 ppb for both compounds.

Histological examination. At autopsy, visible mammary tumors were excised and immediately fixed in ice-cold acetone, trimmed, and embedded in paraffin. Histopathological examinations were then performed according to the criteria for classification of mammary tumors of Squartini and Pingitore[38] and Boorman *et al.*[39]

Cell culture and proliferation assay. The estrogen receptor-positive human mammary cancer cell line MCF-7 was kindly provided by Dr. K. Sakabe, Kitazato University. For routine maintenance, cells were grown in Dulbecco's modified Eagle's medium (DMEM) with high glucose, supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 25 m*M* HEPES. Cells were grown at 37°C in an atmosphere of 5% CO_2/95% air under saturating humidity and routinely passaged at ~70% confluence.

Cell proliferation assay. Cells were seeded in 96-well plates (2500 cells/well), and then incubated for 1 day (37°C, in an atmosphere of 5% CO_2/95% air under saturating humidity). The seeding medium (DMEM) was removed and changed to phenol red-free DMEM medium supplemented with low protein solution, "TCH" (CELOX, St. Paun, MN), instead of charcoal-dextran-treated fetal bovine serum. Test chemicals were then added to each well. The plates were incubated for 3 days and cell proliferation was measured by WST-1 assay[26] using a Cell Counting Kit (Dojindo, Kumamoto). The absorbance was measured (460 nm, reference 595 nm) using a Bio-Rad Model 550 microplate reader. The estrogenic activity of chemicals was assessed by calculating the absorbance (*A*) value of cells grown in the presence of the test chemical/*A* value for the control.

Statistics. The data were analyzed using Student's *t* test and the χ^2 test with the JMP software package (version 3.1) (SAS Institute, Inc., Cary, NC).

Results

General observations in experiment I. Modifying effects of atrazine on DMBA-induced mammary and skin carcinogenesis in

Table 1. Components of the basal diet

corn	30%
wheat	30%
bran	12%
fish powder	10%
skim milk	5%
lucerne meal	4%
gluten meal	3%
corn oil	1%
beer yeast	2%
syrup	0.7%
minerals, vitamins	2.3%
Total	100%

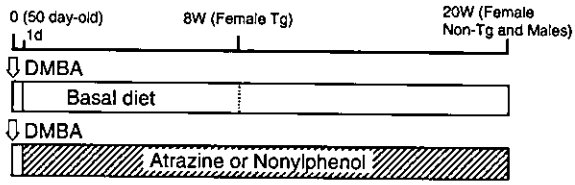

Fig. 1. Experimental protocol. Fifty-day-old female and male Tg rats received a single dose of DMBA, 25 mg/kg, by gavage. The rats were fed diets containing atrazine (5, 50, 500 ppm in basal diet) in experiment I, or nonylphenol (10, 25, 100, 250 ppm) in experiment II from 1 day after DMBA administration to week 8 for Tg females or for week 20 for non-Tg females and for both groups of males.

Table 2. Final body weights and food consumption in Tg

Sex	Atrazine (ppm)	Initial No. of rats	Effective No. of rats	Final body[1] weight (g)	Daily intakes	
					Food (g/day/rat)	Atrazine (mg/day/rat)
Female	0	10	6	299.3±21.3	20.8±1.18	—
	5	10	6	286.2±19.8	31.3±1.05*	0.157
	50	10	6	276.3±19.8	34.2±1.47*	1.71
	500	10	7	258.3±18.5**	18.8±0.80*	9.40
Male	0	10	8	598.4±60.7	28.8±1.46	—
	5	10	9	608.1±46.5	31.6±2.05*	0.158
	50	10	8	642.6±55.2	35.3±4.41*	1.77
	500	10	10	586.0±85.2	28.4±3.47	14.2

1) Mean±SD.
*, ** Significantly different from the control group at $P<0.05$ and $P<0.01$ (Student's *t* test), respectively.

female and male Tg rats were examined in experiment I. Only 2 rats died before the termination, for reasons unrelated to the treatment. Data for final body weights and intake of food in Tg rats are summarized in Table 1. In females, final body weights in rats fed 500 ppm atrazine were significantly decreased as compared to 0 ppm (control) ($P<0.01$). There were no consistent significant differences noted in the weights of liver, kidneys, ovaries/testes, and uterus. The daily food intake of groups given 5 and 50 ppm atrazine was significantly greater than that of controls in both females ($P<0.01$) and males ($P<0.005$). In contrast, food intake in the 500 ppm group of females was decreased ($P<0.05$).

Effects of atrazine on mammary tumor induction. In female Tg rats, palpable mammary tumors were firstly observed at 4 weeks after administration of DMBA, after which the incidence increased rapidly, especially in rats fed the 5 or 50 ppm doses. No tumors were observed in non-Tg rats within 8 weeks. In Tg males, palpable mammary tumors were firstly observed at week 11 and the incidence increased with time, apparently independently of the atrazine treatment. In the male non-Tg rats, only

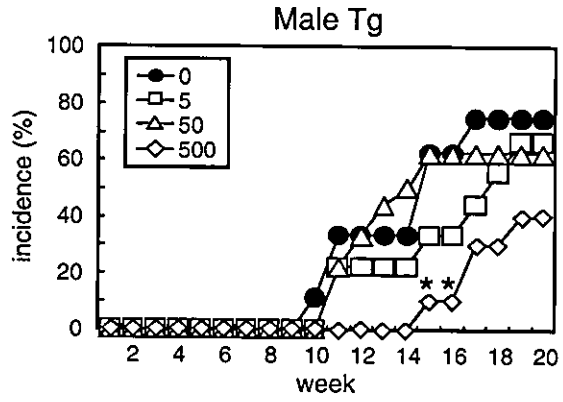

Fig. 2. Periodic observation of skin tumors in male Tg rats fed atrazine (5, 50, 500 ppm). * Significantly different from 0 ppm by the χ^2 test at $P<0.05$.

Table 3. Effects of atrazine on mammary and skin tumor induction in Tg rats (experiment I)

Sex	Atrazine (ppm)	Effective No. of rats	Mammary adenoma		Mammary adenocarcinoma		Skin tumors	
			Incidence (%)	No./rat[1)]	Incidence (%)	No./rat[1)]	Incidence (%)	No./rat[1)]
Female	0	6	1 (16.7)	0.10±0.42	3 (50.0)	3.00±3.69	0	0
	5	6	4 (66.7)**	0.67±0.52	6 (100)*	7.17±6.08	0	0
	50	6	0	0	6 (100)*	10.33±7.94	0	0
	500	7	2 (28.6)	0.43±0.79	6 (85.7)	5.57±5.31	0	0
Male	0	8	3 (33.3)	0.67±1.32	5 (55.6)	1.89±2.32	6 (66.7)	2.13±1.73
	5	9	2 (22.2)	0.33±0.71	6 (66.7)	2.56±3.24	6 (66.7)	1.00±1.00
	50	8	2 (22.2)	0.22±0.44	6 (66.7)	3.00±3.81	6 (66.7)	1.75±1.75
	500	10	1 (10.0)	0.10±0.32	10 (100)	2.70±2.06	4 (40.0)	0.50±0.71*

1) Mean±SD.
*, ** Significantly different from the control group at $P<0.05$ and $P<0.01$ (χ^2 test), respectively.

Table 4. Final body weight and food consumption (experiment II)

Sex	Line	Nonylphenol (ppm)	Effective No. of rats	Final body[1)] weight (g)	Daily intakes	
					Food[1)] (g/day/rat)	Nonylphenol (mg/day/rat)
Female	Tg	0	10	262.4±16.4	19.4±3.5	—
		10	9	259.6±15.3	27.5±6.5**	0.275
		25	9	263.1±15.3	20.8±6.8	0.520
		100	9	270.1±16.4	22.9±7.1	2.29
		250	10	248.9±18.7	21.1±4.2	5.28
	Non-Tg	0	8	306.3±17.1	18.4±5.4	—
		10	8	320.9±22.4	21.1±6.1	0.211
		25	7	319.8±17.4	20.9±4.0	0.523
		100	7	319.2±37.6	18.9±5.8	1.89
		250	8	325.9±43.8	18.5±3.9	4.63
Male	Tg	0	9	628.0±58.2	29.3±3.7	—
		10	8	626.7±66.5	29.7±6.1	0.297
		25	7	613.6±96.1	30.6±6.5	0.765
		100	7	656.0±41.0	36.5±9.4**	3.65
		250	9	654.1±73.6	33.7±6.4**	8.43
	Non-Tg	0	10	619.8±56.2	31.7±8.3	—
		10	7	576.4±42.1	28.3±5.5**	0.283
		25	7	614.7±36.7	28.2±3.9**	0.705
		100	8	626.8±73.0	30.8±5.9	3.08
		250	7	580.1±53.7	31.3±10.9	7.83

1) Mean±SD.
** Significantly different from the control group by Student's t test ($P<0.005$).