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Estrogen and thyroid hormone interaction on regulation of gene expression

(enkephalin/hypothalamus/estrogen receptor/thyroid hormone receptors)

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ABSTRACT **Estrogen receptor (ER) and thyroid hormone receptors (TRs) are ligand-dependent nuclear transcription factors that can bind to an identical half-site, AGGTCA, of their cognate hormone response elements. By *in vitro* transfection analysis in CV-1 cells, we show that estrogen induction of chloramphenicol acetyltransferase (CAT) activity in a construct containing a CAT reporter gene under the control of a minimal thymidine kinase (*tk*) promoter and a copy of the consensus ER response element was attenuated by cotransfection of TRα1 plus triiodothyronine treatment. This inhibitory effect of TR was ligand-dependent and isoform-specific. Neither TRβ1 nor TRβ2 cotransfection inhibited estrogen-induced CAT activity, although both TRα and TRβ can bind to a consensus ER response element. Furthermore, cotransfection of a mutated TRα1 that lacks binding to the AGGTCA sequence also inhibited the estrogen effect. Thus, the repression of estrogen action by liganded TRα1 may involve protein–protein interactions although competition of ER and TR at the DNA level cannot be excluded. A similar inhibitory effect of liganded TRα1 on estrogen induction of CAT activity was observed in a construct containing the preproenkephalin (PPE) promoter. A study in hypophysectomized female rats demonstrated that the estrogen-induced increase in PPE mRNA levels in the ventromedial hypothalamus was diminished by coadministration of triiodothyronine. These results suggest that ER and TR may interact to modulate estrogen-sensitive gene expression, such as for PPE, in the hypothalamus.**

Many genomic actions of estrogen or thyroid hormones [triiodothyronine (T_3)] are mediated through the interactions of ligand–receptor complexes with DNA hormone response elements of target genes (1–3). The consensus DNA sequences of estrogen response elements (ERE) and glucocorticoid response elements (GRE) comprise two major categories of DNA hormone response elements. It has been demonstrated, for example, that the glucocorticoid receptor and progesterone receptor can interact with the same DNA sequence, the GRE (1, 3). Importantly, the half-site of a consensus ERE, AGGTCA, is identical to that of a thyroid hormone response element (TRE), vitamin D response, and retinoic acid response elements, but functionally distinct from GRE (2–4). Based on structural similarities, estrogen receptor (ER) and thyroid hormone receptor (TR) belong to the same nuclear receptor superfamily and provide an important model for receptor interactions. There are two major TR subtypes, TRα and TRβ, that are encoded by different genes (5, 6). With alternative splicing, three functional isoforms, TRα1, TRβ1, and TRβ2, have been identified in mammalian systems. These TRs can bind to TRE as monomers, homodimers, and heterodimers (6). In the present study, we have examined potential estrogen–thyroid hormone interactions on the regulation of estrogen-sensitive genes, such as for preproenkephalin (PPE), expressed in certain hypothalamic neurons.

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MATERIALS AND METHODS

Plasmid Constructs. An expression vector for ER encoding the wild-type human ER in pSG5 plasmid was kindly provided by P. Chambon (Institut National de la Santé et de la Recherche Médicale, Strasbourg, France). Other vectors used in the experiments have been described before: pSG-TRα1 containing a rat wild-type TRα1 in pSG5X (7); TRα1-p containing a P-box mutated rat TRα1 in pSG5X (8); rTRα1, rTRβ1, and rTRβ2 containing wild-type rat TRα1, TRβ1, and TRβ2, respectively, in pcDNAI/Amp vector (Invitrogen) (9). The reporter plasmid (ERE-*tk*-CAT) was constructed by insertion of a double-stranded consensus ERE (T*AGGTCA*CTGT-*GACCT*T) in pUTKAT4 [a kind gift from B. Pentecost and S. X. Wu-Peng (10)] with the consensus ERE directly located upstream of the herpes simplex virus-TK (*t*hymidine *k*inase) promoter driving the bacterial chloramphenicol acetyltransferase (CAT) gene. A CAT reporter plasmid (-437PPE-CAT) containing a PPE genomic insert from −437 to +53 of the rat PPE gene was constructed by ligation of PCR-amplified DNA to pPLFCAT [a kind gift from S. L. Sabol, National Institutes of Health, Bethesda (11)].

Cell Culture and Cotransfections. CV-1 cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle's phenol-free medium (DMEM) supplemented with 5% stripped fetal bovine serum (FBS), 20 mM L-glutamine, 50 units/ml of penicillin, and 50 μg/ml of streptomycin. The serum was stripped of steroid hormones and thyroid hormones by use of dextran T-70-coated activated charcoal. The cells were plated on 60-mm dishes with a density of 0.4×10^6 cells per dish, and transfected by calcium phosphate precipitation method (ProFection, Promega) with expression (1 μg) and reporter (4 μg ERE-*tk*-CAT or 5 μg −437PPE-CAT) plasmids, as well as 2 μg Rous sarcoma virus-β-galactosidase plasmid and pBluescript-SK plasmid to a total of 15 μg DNA per dish. After 12–16 h of transfection, the cells were washed and continued to grow in the absence or presence of 17β-estradiol (E_2, 10^{-7} M), T_3 (10^{-7} or 10^{-6} M), or E_2 plus T_3 for 48 h before harvesting. Cell extracts were prepared by three rapid freeze/thaw cycles in 0.25 M Tris·HCl, pH 8.0, and

Abbreviations: CAT, chloramphenicol acetyltransferase; E_2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen response element; HYPOX, hypophysectomy; OVX, ovariectomy; pERE, putative ERE; T_3, triiodothyronine; tk, thymidine kinase; TR, thyroid hormone receptor; VMH, ventromedial hypothalamus; GRE, glucocorticoid response element; TRE, thyroid hormone response element; PPE, preproenkephalin.

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analyzed for CAT activity using an assay kit (Promega). The conversion of chloramphenicol to acetylated forms was quantified by liquid scintillation counting of TLC-separated material. The transfection efficiencies were normalized by measuring β-galactosidase activity in the extracts.

Animals and Hormone Treatments. Female Sprague Dawley rats (175–200 g, Charles River Breeding Laboratories) were ovariectomized (OVX) or hypophysectomized (HYPOX) by the supplier and maintained on a 12-h light/12-h dark cycle. Hormone treatments were carried out at least 2 weeks after surgery. Estradiol benzoate (20 μg per rat) and T_3 (500 μg/kg per day) were dissolved in sesame oil and 50% saline/50% ethanol, respectively, and given s.c. and i.p., respectively. All steroids were obtained from Sigma.

Preparation of Nuclear Protein Extracts and Total Cellular RNA and Determination of PPE mRNA. After CO_2 narcosis and decapitation of the rats, the whole hypothalamus or ventromedial hypothalamus (VMH) was dissected (12, 13), and nuclear protein extracts from dissected tissues were prepared by using conditions described (12, 14). The concentrations of nuclear proteins were determined by the use of Bradford method (15). Total cellular RNA from dissected VMH was extracted by use of TRIzol (Life Technologies, Grand Island, NY) following the manufacturer's instructions and the concentration was determined by UV absorbance. The levels of PPE mRNA were determined by using slot-blot hybridization with ^{32}P-labeled PPE cDNA probe, were densitometrically analyzed in a Zenith soft laser densitometer (model SLR-2D/1D), and were normalized with oligo$(dT)_{15}$ signals as previously described (13).

Electrophoretic Mobility-Shift Assay. Oligonucleotides containing a consensus ERE from vitellogenin A2 gene and a putative ERE (pERE) from the PPE promoter [−381 to −417 from transcription start site (16)] were labeled with [^{32}P]dATP by use of a fill-in reaction with Klenow enzyme (Boehringer Mannheim) (14). Nuclear protein extracts or baculovirus-expressed TRα1 and TRβ1 were incubated with DNA probe at room temperature at conditions as previously described (12, 14). The DNA–protein complexes were separated on a 4% nondenaturing polyacrylamide gel (37.5:1 acrylamide/bis) with 5% glycerol by electrophoresis at room temperature. After electrophoresis, the gel was dried and exposed to Kodak X-Omat film with an intensifying screen at −70°C. For competition assays, the cold oligonucleotides were preincubated for 15 min before the addition of a probe. The supershift assay

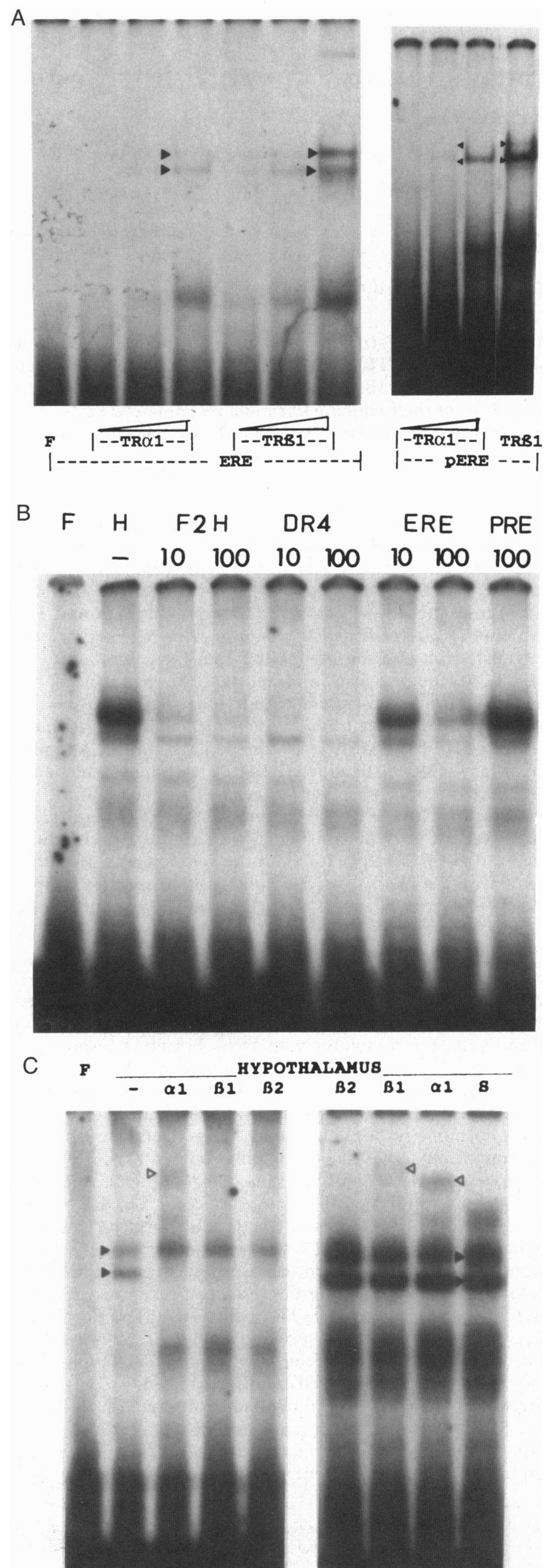

FIG. 1. The interactions of TR with a consensus ERE as well as a pERE from the rat PPE gene. The protein–DNA interactions were analyzed by electrophoretic mobility-shift assay using a consensus ERE from vitellogenin gene or a pERE from rat PPE gene as described (12, 14). F, free probe. (*A*) Purified baculovirus-expressed TRα1 and TRβ1 bound to a consensus ERE as well as a pERE from the rat PPE promoter in a concentration-dependent manner. The solid arrowheads indicate the specific TR-ERE complexes. (*B*) Competition analysis of ERE binding activity in the hypothalamic nuclear protein extracts from the OVX female rats. The numbers above each lane indicate the molar excess of cold oligonucleotide added. F2H and DR4 are two high-affinity TRE oligonucleotides (17); ERE, an oligonucleotide containing a consensus ERE; PRE, an oligonucleotide containing a consensus progesterone response element (12). Lane H, a DNA binding assay of hypothalamic nuclear extracts without addition of any cold oligonucleotides. (*C*) Supershift analysis of ERE binding activity in the hypothalamic nuclear extracts from the OVX female rats using a consensus ERE probe. (*Left*) The specific antibody against to TRα1 (α1), TRβ1 (β1), and TRβ2 (β2), respectively, was preincubated with the nuclear extracts at 4°C overnight. (*Right*) The specific antibody was added after the DNA binding assay and incubated on ice for 2 h. S, the addition of preimmune serum in the DNA binding assay. The solid arrowheads indicate the specific ERE binding complexes and the open arrowheads denote the supershifted complexes.

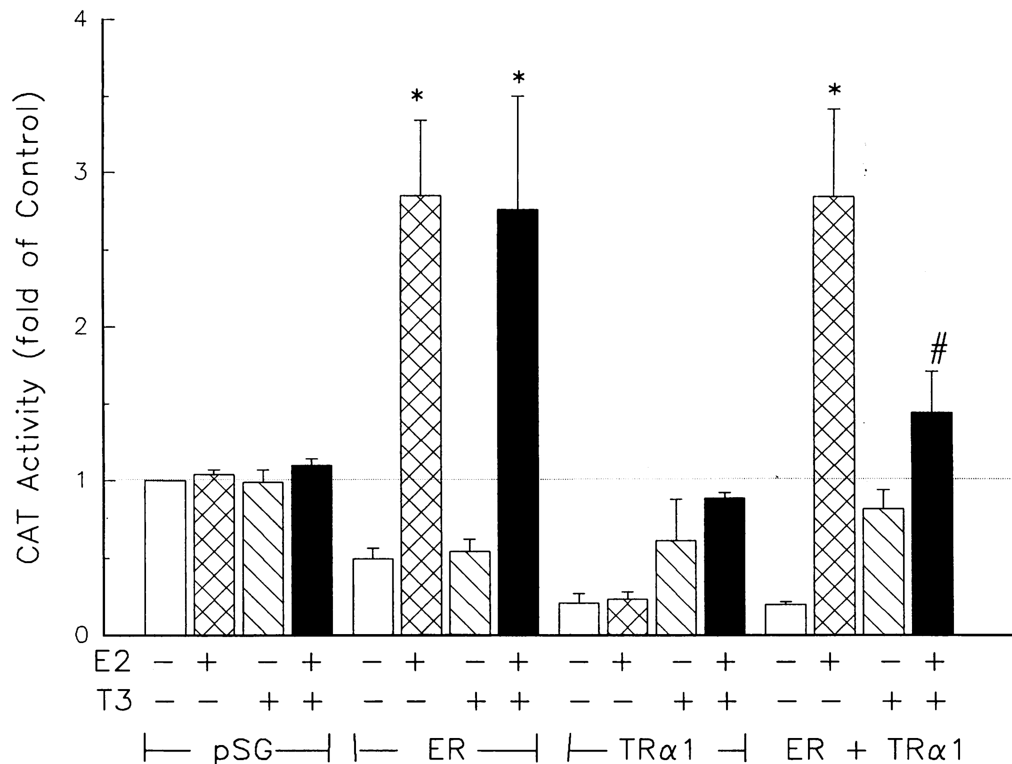

FIG. 2. The ability of TRα1 to inhibit the estrogen-induced gene expression in a ligand-dependent manner. Cotransfections of expression vectors (1 μg) of ER, TRα1, control vector pSG5X (pSG), or ER plus TRα1 with ERE-*tk*-CAT reporter plasmid in CV-1 cells were performed as described. The transfected cells were treated with E_2 (10^{-7} M), T_3 (10^{-6} M), E_2 plus T_3, or ethanol-vehicle control for 48 h before harvesting. CAT activity was determined and normalized relative to the β-galactosidase activity for each sample and expressed as fold of the basal activity. The basal activity (1-fold) was defined as the CAT activity of cotransfection of pSG and ERE-*tk*-CAT reporter plasmids with ethanol-vehicle treatment. The values were presented as the mean ± SEM of three to seven samples. *, $P < 0.05$ compared with corresponding vehicle control and corresponding treatment in pSG-transfected groups; #, $P < 0.05$ compared with E_2 treatment in ER plus TRα1 group.

was carried out by preincubating the nuclear protein extracts with an antibody at 4°C overnight with gentle shaking, or by postincubating an antibody with the DNA reaction mixture on ice for 2 h.

Statistics. The data are presented as mean ± SEM. One-way analysis of variance (ANOVA) following post hoc Student–Newman–Keuls test was used to determine the difference among multiple groups. Student's *t* test was used for analyzing difference between two groups. A $P < 0.05$ was accepted as the level of statistical significance.

RESULTS

TR Can Bind to a Consensus ERE as well as the PPE Promoter. Using electrophoretic mobility-shift assay, the protein–DNA interactions between TR or ER and a consensus ERE as well as PPE promoter were assessed. As shown in Fig. 1*A*, baculovirus-expressed TRα1 and TRβ1 specifically bound to a consensus ERE as well as a pERE from the rat PPE promoter in a concentration-dependent manner. As a positive control, specific binding complexes were displayed between baculovirus-expressed ER and the consensus ERE as well as pERE (data not shown). Analysis of nuclear protein extracts from hypothalamus of OVX female rats displayed specific TR-ERE interactions as demonstrated by competition assay (Fig. 1*B*) with high affinity TREs, DR4 and F2H (17, 18), and by supershift assay (Fig. 1*C*) with specific antibodies against TRα1, TRβ1, and TRβ2. Thus, like ER, TR can also bind to the consensus ERE as well as the pERE from the rat PPE promoter, and the specific ERE binding complexes in hypothalamic nuclear extracts from female rats contain not only ER (14) but also TR.

Liganded TRα1 Produces Inhibition of Estrogen-Induced Gene Expression. If both ER and TR can interact with an ERE, then what is the functional significance of these interactions? This was evaluated by *in vitro* cotransfection assays in CV-1 cells. As shown in Fig. 2, E_2 (10^{-7} M), in the presence of ER and ERE-*tk*-CAT, produced a significant induction of CAT activity, which was not altered by cotransfection of TRα1 alone. However, the addition of T_3 (either 10^{-6} or 10^{-7} M) significantly decreased the estrogen induction of CAT activity when both ER and TRα1 were present. The addition of T_3 alone without cotransfection of TRα1 did not affect the estrogen induction of CAT activity. The omission of the consensus ERE from the reporter construct failed to show any alterations in CAT activity compared with vehicle control when treated with either E_2 or T_3 in the cotransfection assay (data not shown). Thus, the estrogen effect is dependent on ER as well as ERE; the inhibition of estrogen induction of CAT activity by T_3 requires ligand (T_3)-receptor (TRα1) interaction.

We next tested whether the binding of TRα1 to AGGTCA sequences is critical for the TRα1 inhibition of estrogen effect. Cotransfection of ER and ERE-*tk*-CAT with a P-box mutated TRα1, which is incapable of binding to the AGGTCA sequence in the consensus TREs (8), also displayed a ligand-dependent inhibition of estrogen-induced increase in CAT activity (Fig. 3), indicating that direct DNA binding by TR is not required for T_3 inhibition of estrogen mediated gene expression.

Inhibition of Estrogen Induction of Transcriptional Activity by TR Is Isoform-Specific. There are multiple isoforms of TR deriving from two genes with alternative splicing and promoter choice. The functional import of each isoform of TR is not well-understood, and thus we evaluated the interactive effects

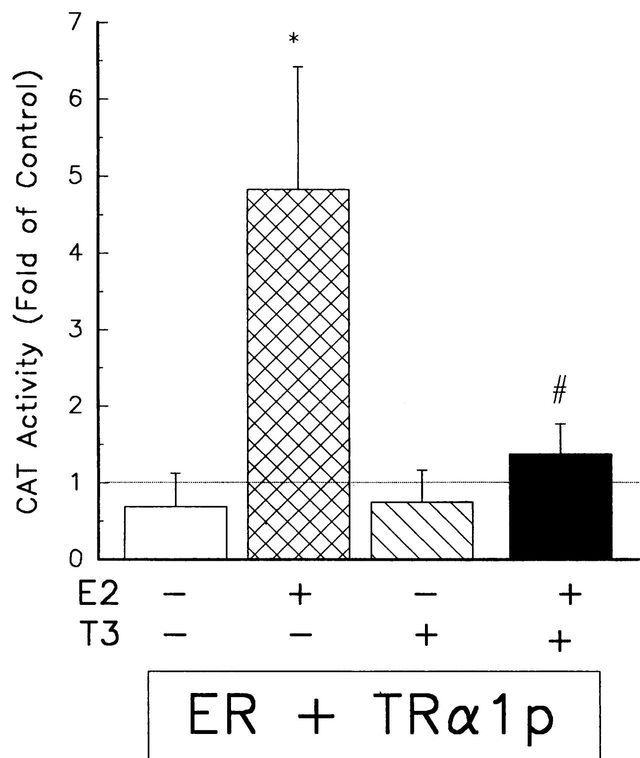

FIG. 3. Inhibition of estrogen induction of CAT activity by liganded TRα1 mutant. Cotransfections of ERE-*tk*-CAT reporter gene with ER and mutated TRα1 expression vector (TRα1p) were carried out as described. The values are mean ± SEM of four samples. *, $P < 0.05$ compared with vehicle control; #, $P < 0.05$ compared with E_2 treatment. See Fig. 2 legend for details.

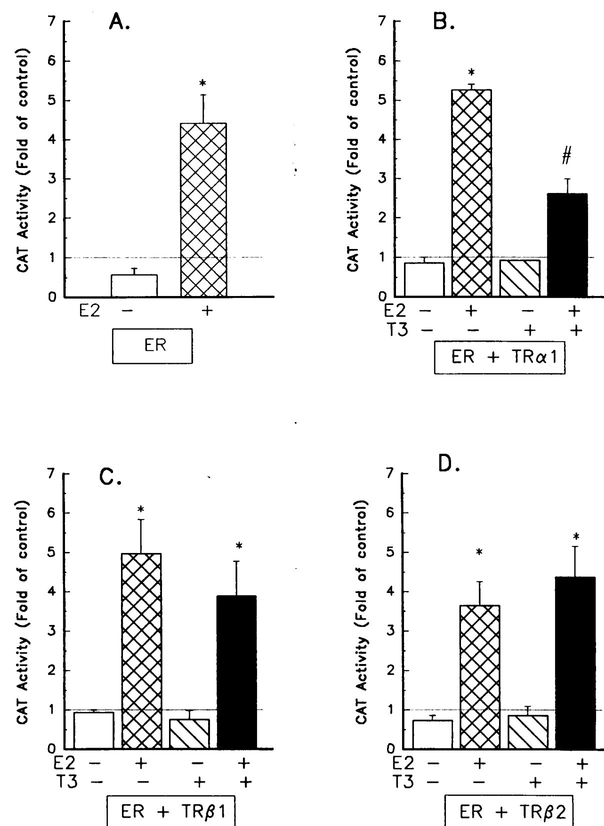

FIG. 4. Isoform-specific inhibition of estrogen induction of CAT activity by liganded TR. Expression vectors for ER (pSG-ER), and rTRα1, rTRβ1, rTRβ2 in pcDNAI/Amp were used for the cotransfection studies as described. CAT activity was determined and normalized relative to the β-gal activity for each sample and expressed as fold of the basal activity. The basal activity (1-fold) was defined as the CAT activity of cotransfection of pcDNAI/Amp blank vector and ERE-*tk*-CAT reporter plasmids with ethanol-vehicle treatment. The values are mean ± SEM of two to seven samples. *, $P < 0.05$ compared with corresponding vehicle control. #, $P < 0.05$ compared with estrogen treatment in ER + TRα1 group.

of TR isoforms on estrogen-induced gene expression by cotransfection assays. Unlike TRα1 (see Figs. 2 and 4*B*), cotransfection of either TRβ1 (Fig. 4*C*), or TRβ2 (Fig. 4*D*) with ER and ERE-*tk*-CAT did not manifest ligand-dependent inhibition of estrogen induction of CAT activity (as shown in Fig. 4).

Estrogen Induction of PPE Promoter Activity Is Attenuated by Liganded TRα1. The significance of TR-ER interaction on regulation of gene expression was further illustrated by using a cotransfection assay of PPE promoter-driven CAT reporter gene expression in CV-1 cells. Functional EREs are present within 437 bases of the promoter region of the rat PPE gene (§and unpublished data). Cotransfection of TRα1 with ER and −437PPE-CAT exhibited T_3-dependent inhibition of estrogen induction of CAT activity (Fig. 5 *Left*), indicating that ligand-TRα1 can modulate estrogen induction of a specific gene with neuroendocrine import.

T_3 Treatment Inhibits Estrogen Induction of PPE mRNA in Hypothalamus of HYPOX Female Rats. The significance of TR–ER interactions on regulation of gene expression was further assessed in an *in vivo* animal study. Treatment with estrogen produced a significant increase in PPE mRNA level in VMH of HYPOX female rats, and this elevation was significantly reduced by pretreatment with T_3 (500 μg/kg per day for 5 days) as shown in Fig. 5 *Right*. T_3 alone also produced a smaller but a significant increase in PPE mRNA level. These results additionally show that the effects of estrogen and T_3 are mediated directly at central nervous system without dependence on the pituitary.

§Zhu, Y.-S., Yen, P. M., Chin, W. W. & Pfaff, D. W., 10th International Congress of Endocrinology, June 1996, San Francisco, p. 138, abstr. P1–17.

DISCUSSION

The nuclear thyroid/steroid receptor superfamily comprises a large number of proteins (1, 2, 19), which share similarities in amino acid composition, protein structure, and cognate DNA recognition elements. Among these, the DNA binding domain of these receptors, a "zinc finger" structure, and their cognate DNA response elements can be divided to two major categories according to the core sequences. These are represented by GREs and EREs (1). A challenge is to determine how these receptors in nerve cell nuclei regulate gene expression and related behavioral responses. Using estrogen–ER and T_3–TR systems, we have studied the interactions of these nuclear receptors in the regulation of gene expression *in vitro* using cotransfection analysis. Also, we have studied hypothalamic PPE gene expression in intact animals as well as the regulation of behavioral responses such as lordosis in female rats [see the accompanying paper (20)]. We show that thyroid hormone, T_3, can inhibit estrogen-induced gene expression in cotransfection assays (see Fig. 2), similar to Glass *et al.* (21) and Segars *et al.* (22). This inhibition is TR-dependent as well as ERE-dependent since in the absence of TRα1, T_3 does not affect estrogen-induced CAT activity (see Fig. 2). Without an ERE in the reporter construct, both estrogen and T_3 do not alter CAT activity regardless the presence of corresponding receptors (data not shown). This interaction could occur at either the DNA level or the protein level. At the DNA level, a T_3–TR

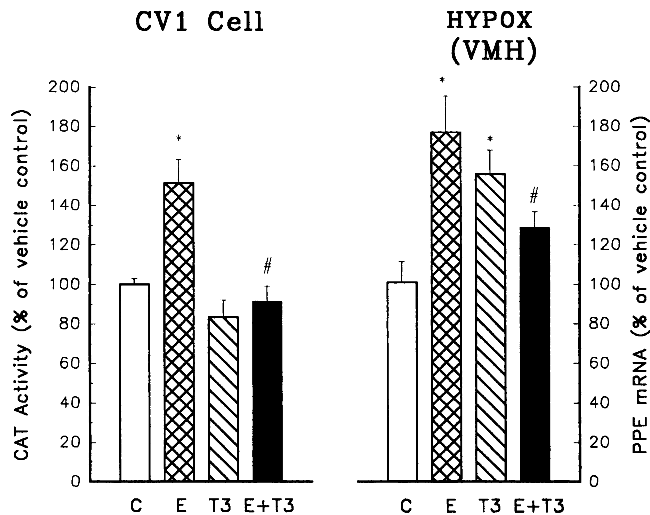

FIG. 5. Inhibition of estrogen induction of PPE gene expression by thyroid hormone in cotransfected CV-1 cells (*Left*) and in hypothalamus of HYPOX female rats (*Right*) (*Left*) Liganded-TRα1 blocked estrogen-induced increase in PPE promoter activity. Cotransfections of −437PPE-CAT reporter gene with ER and TRα1 expression vector were carried out as described. E_2 (10^{-7} M), T_3 (10^{-6} M), or E_2 plus T_3 was added in the cells for 48 h before harvesting. CAT activity is expressed as percent of vehicle control (C), and the values are mean ± SEM of six samples. *, $P < 0.05$ compared with vehicle control; #, $P < 0.05$ compared with estrogen treatment (E). (*Right*) Treatment with T_3 inhibited estrogen induction of PPE mRNA in VMH of HYPOX female rats. HYPOX female rats ($n = 5$ for each group) were treated with vehicle or T_3 (500 μg/kg per day i.p.) for 5 days, and at day 3, a single dose of estradiol benzoate (20 μg per rat sc) or vehicle was administrated. The animals were killed 48 h after estradiol benzoate treatment. The VMH was dissected, and total cellular RNA was extracted. PPE mRNA levels were analyzed by slot-blot hybridization, and presented as percent of vehicle control. *, $P < 0.05$ compared with vehicle control (C); #, $P < 0.05$ compared with estradiol benzoate treated group (E).

complex could compete with the estrogen–ER complex to bind to an ERE. At the protein level, there is no evidence that ER and TR can form heterodimers (18, 23) to attenuate ER-mediated action. Graupner *et al.* (23) have shown that inhibition of ER activity on TRE by TRs does not result from heterodimer formation by using transient transfection assay with mutated TRs, which lack the DNA binding domain but contain the ligand binding/dimerization domain, and by using gel mobility-shift assay of ER and TRs interacting with a TRE. It is more probable that estrogen–ER and T_3–TR systems interact with common transcription factors or coactivators. If these factors are the limiting factors for estrogen-induced gene expression, the course of these factors by the T_3–TR system would diminish the estrogen-mediated gene expression. We currently favor this "squelching model" based on the available evidence: (*i*) a TRα1 mutant lacking the ability to bind to AGGTCA sequences (8) produced similar inhibition of estrogen-induced gene expression as the wild type (see Figs. 1 and 3), suggesting that TR binding to these sequences is not required for this inhibition; (*ii*) although both TRα1 and TRβ1 can bind to ERE in *in vitro* assays, only TRα1 produced ligand-dependent inhibition of estrogen effect (see Figs. 2 and 4), suggesting that TR binding to ERE alone is not sufficient for this inhibition. Such a squelching model has been reported to be operative in other systems (17, 24). Associated proteins related to ER and TR have been reported (25–29), and a common coactivator for steroid hormone and thyroid hormone/retinoic acid receptors has recently been identified (30). Whether the same coactivator is involved in the hypothalamic ER–TR interaction is currently under investigation.

Strikingly, this T_3–TR mediated inhibition of the estrogen effect is TR isoform-specific; it was only observed when TRα1 was cotransfected, but not TRβ1 and TRβ2 (see Fig. 4). This TR isoform-specific action has been reported previously in other systems such as for TR interference of glucocorticoid-mediated gene expression (31). There are at least three functional isoforms of TR, TRα1, TRβ1, and TRβ2, in mammalian systems (4, 6) generated by alternative promoter choice and RNA splicing of transcripts of two TR genes, TRα and TRβ. These TR isoforms have high homology in amino acid composition, especially in the DNA binding domain and hormone binding domain. The most diversified region between TRα isoforms and TRβ isoforms is located in the N-terminal area, related to their trans-activation activity (4, 6). Thus, further mutation analysis could identify the specific region of TRα responsible for the ligand-dependent TR inhibition of estrogen–ER-mediated gene expression.

The potential significance of ER–TR interactions on regulation of estrogen-sensitive promoters is exemplified by the study of PPE gene expression both in cotransfection assay and in intact animals, as well as by estrogen-induced lordosis behavior in female rats [see accompanying paper (20)]. The PPE gene encodes enkephalin peptides that have been postulated to function as neuromodulators and have been implicated in estrogen-mediated female reproductive behavior (32–34). We and others have demonstrated that estrogen can produce tissue-specific, sex-specific, and time- and dose-dependent induction of PPE gene expression in VMH of OVX female rats (35–37) that may be correlated with estrogen-induced lordosis behavior in the female rat (32, 34, 36). Estrogen-induced PPE gene expression may be directly mediated by an estrogen–ER complex interacting with functional EREs in the PPE promoter region (Zhu *et al.*, Program Abstr., 10th International Congress of Endocrinology, p138, 1996 and unpublished data). ER has been shown to be expressed in the hypothalamus including the VMH (38), and the ER and PPE genes have been reported to be expressed in the same hypothalamic neurons (39, 40). Furthermore, specific ERE-like binding activity is detected between the hypothalamic nuclear proteins from female rats and a consensus ERE as well as the putative ERE from the rat PPE promoter region (14). These complexes contain ER (14) as well as TRs (see Fig. 1). All of the TR isoforms are expressed in the rat hypothalamus (41, 42); however, the predominant isoforms are TRα1 and the nonfunctional c-erbα2 (41). In as much as c-erbα2 does not bind to T_3, and TRβ subtypes do not produce ligand-dependent inhibition of estrogen effect in cotransfection study, we propose that T_3 inhibition of estrogen-induced PPE mRNA in VMH of OVX female rats is mediated through TRα1, if this interaction occurs in the nuclei of hypothalamic neurons.

Like estrogen-induced lordosis behavior (32), estrogen induction of PPE mRNA in the VMH and T_3 inhibition of the estrogen effect are not dependent on the pituitary (see Fig. 5). Furthermore, this T_3 inhibition of estrogen-induced gene expression is not due to changes in plasma estradiol concentration, to changes in estrogen bioactivity, or to decreases in hypothalamic ER levels [see accompanying paper (20)]. Taking these results together, we conclude that T_3 inhibition of estrogen-induced hypothalamic gene expression may be mediated directly through liganded ER–TR interaction in the nuclei of hypothalamic neurons.

Finally, T_3 inhibition of estrogen induction of hypothalamic gene expression, such as for PPE, may have behavioral consequences. Both exogenous and endogenous T_3 display an inhibitory effect on estrogenic induction of lordosis behavior, a well-defined female reproductive behavior in rodents [see accompanying paper (20)]. These phenomena could relate to the alteration of environmental conditions such as stress, cold temperature, and food availability signaled by thyroid hormones to reproductive function (43).

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