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A distal estrogen responsive element upstream the cap site of human transthyretin gene is an enhancer-like element upon ERα and/or ERβ transactivation

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ABSTRACT

Previous studies reported that 17 beta-estradiol (E2) is responsible for the up-regulation of transthyretin (TTR) expression *via* an estrogen receptor (ER)-dependent pathway in rat choroid plexus (CP) and liver. A computer-assisted homology search identified a putative estrogen-responsive element (ERE) in the 5′ flanking region of the human TTR (hTTR) gene (ERETTR), with the sequence aAGTCAAAGTGACCa, between −3406 and −3392 bp. Luciferase reporter assays and electrophoretic mobility shift (EMSA) and supershift analysis were carried out to investigate if E2 regulates TTR transcription *via* this putative ERE. Luciferase reporter assays in COS-7 cells were carried out with a plasmid construction where the TTR fragment containing the putative ERETTR was cloned in pGL2-promoter vector (pGL2-P) (pGL2-P/TTR), co-transfected with estrogen receptor α (ERα) and/or estrogen receptor β (ERβ) expression vectors. These assays demonstrated that, upon incubation with E2, one or both ERs (α and/or β) transactivate the reporter gene. The pGL2-P/TTR showed a significant transactivation of up to 6.8-fold, by E2, when co-transfected with ERβ, and up to 4-fold with ERα. Specific binding of ER (α and/or β) to ERETTR was demonstrated by EMSA and supershift assays confirmed the binding to ERα and/or ERβ. Our findings further suggest a mechanism underlying the regulation of TTR expression through the identification of a novel ERE in the TTR gene, which functions as an E2-dependent enhancer-like element.

1. Introduction

Estrogens, as 17β-estradiol (E2), are steroid hormones that mediate many biological activities by the regulation of gene transcription through two classes of estrogen receptors (ER), α and β (Green et al., 1986; Warner et al., 1999). These receptors are tissue and cell specific and are genetically and functionally distinct (Mueller and Korach, 2001). However, both receptors share a similar domain with estrogen-binding affinity (Kuiper et al., 1997) and recognize the same DNA motifs, the estrogen-responsive elements (ERE) (Klein-Hitpass et al., 1986) in regulatory regions of estrogen responsive genes (Pace et al., 1997). Generally, E2 diffuses into cells and activates its nuclear receptors through homo and/or heterodimerization of ER that bind to ERE (Bourdeau et al., 2004), increasing or decreasing the transcription of genes (Kuiper et al., 1996; Nilsson et al., 2001). Alternatively, E2–ER complexes transactivate genes, indirectly, through protein–protein interactions (Safe, 2001; Safe and Kim, 2008), or trigger quick responses *via* non-genomic mechanisms through the ER located in or adjacent to the plasma membrane or other non-ER plasma membrane-associated estrogen binding proteins (Bjornstrom and Sjoberg, 2005; Manavathi and Kumar, 2006).

Transthyretin (TTR) has long being recognized as a transporter for thyroxin (T4) and retinol binding protein (RBP) complexed with retinol (Monaco, 2000; Raz and Goodman, 1969). More recently, its capacity to bind other molecules, as amyloid-beta peptides and metallothioneins (Goncalves et al., 2008b; Martinho et al., 2010; Schwarzman and Goldgaber, 1996), its proteolytic activity (Costa et al., 2008; Liz et al., 2004) and its involvement in nerve repair (Liz et al., 2009) have widened the current view of TTR functions.

TTR is mostly produced and secreted by the liver to the peripheral circulation (Felding and Fex, 1982) and by the epithelial cells of the choroid plexus (CP) to the cerebrospinal fluid (CSF) (Soprano et al., 1985). This homotetrameric protein of 127 amino acids is encoded by a single copy gene about 7.0 Kb in length located in the long arm of chromosome 18 (Wallace et al., 1986; Whitehead et al., 1984) in the region q11.2-q12.1 (Sparkes et al., 1987). In the 5′-flanking region upstream the transcription initiation site, a TATA box sequence (at −30 to −24 base pairs), a CAAT box sequence (from −101 to −96 base pairs) and several consensus sequences are present. Previous studies showed that the mouse TTR gene is regulated by two major regulatory

Abbreviations: E2, 17 beta-estradiol; B-gal, beta-galactosidase; CSF, cerebrospinal fluid; CP, choroid plexus; EREcon, consensus oligonucleotide for ERE; EMSA, electrophoretic mobility shift assay; pGL2-P/TTR, ERETTR cloned in pGL2 vector; ER, estrogen receptor; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; ERE, estrogen-responsive element; pGL2-P, pGL2-promoter vector; RBP, retinol binding protein; TTR, transthyretin.

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regions in the 5′-flanking region: a promoter sequence (−50 to −150 bp) and an enhancer sequence (at −1860 to −1960 bp). In both regions, several putative regulatory sites were identified (Costa et al., 1986, 1990). These regulatory regions in the TTR gene are also found in humans (hTTR) and are sufficient to drive TTR gene expression in liver (Costa et al., 1986; Yan et al., 1990). Nevertheless, the regulation of TTR expression in CP is not well clarified, and the few studies carried out suggested that the TTR gene is differentially regulated in liver and in CP (Motojima and Goto, 1989; Nagata et al., 1995; Yan et al., 1990). In fact, some studies showed that the presence of a sequence shorter than 1 Kb upstream the cap site is sufficient to drive TTR expression in the liver, while TTR CP expression requires a further upstream sequence located above 3 Kb (Nagata et al., 1995; Yan et al., 1990). TTR is a target of E2 as it regulates TTR expression in mice liver and CP (Goncalves et al., 2008a) *via* ER (Quintela et al., 2009). Furthermore, other hormones, as progesterone, 5alpha-dihydrotestosterone and glucocorticoids, also regulate TTR expression, particularly in CP (Martinho et al., 2012; Quintela et al., 2008, 2011).

In an attempt to understand the molecular mechanisms underlying the regulation of TTR expression by E2, we performed a computer-assisted homology search that identified a putative distal ERE (aAGTCAAAGTGACCa) in the 5′-flanking region of hTTR gene (ERETTR). All these observations prompted us to investigate if ERETTR specifically binds ER (α and/or β) and is essential for TTR transactivation by E2.

2. Material and methods

2.1. Material

The pGL2 luciferase reporter vectors (Promega, Madison, WI, USA) were used in the luciferase reporter assays: pGL2-promoter (pGL2-P) contains the SV40 promoter upstream of the luciferase gene and, pGL2-basic that lacks promoter and enhancer sequences. The ERETTR element was inserted in pGL2-P (pGL2-P/TTR) upstream the SV40 promotor (Fig. 1A and B). The ER expression vectors, pSG5-hERα and pSG5-hERβ, were kindly provided by Dr. Pierre Chambon (*Institut de Genétique et de Biologie Moleculaire et Cellulaire* – Strasbourg, France) and Dr. Jan-Ake Gustafsson (*Karolinska Institutet* – Sweden), respectively.

The COS-7 and MCF-7 cell lines were obtained from the American Type Culture Collection (Manassas-ATCC, VA, USA).

2.2. Methods

2.2.1. In silico analysis of the 5′-flanking region of hTTR gene

In order to investigate the presence of putative palindromic ERE consensus sequences in the TTR gene, an *in silico* analysis was performed on a ~6 Kb fragment in the 5′-flanking region of hTTR gene (NCBI Accession number: NG_009490.1), using the MatInspector software version 7.3 (Genomatix).

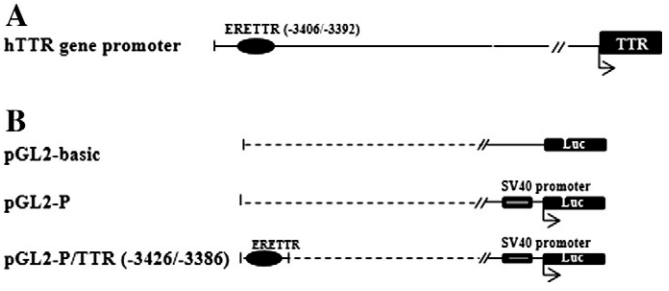

Fig. 1. Schematic representation of the human TTR gene and plasmid vectors used in luciferase reporter assays. A) 5′-flanking region of human TTR gene. B) Plasmid vectors included in the study; pGL2-P/TTR vector was generated from pGL2-P vector (Promega) and contains the putative ERETTR (from −3406 to −3392 bp).

2.2.2. Constructs, transfection and luciferase reporter ssays

2.2.2.1. Reporter plasmid constructs. A fragment in the 5′flanking region of the hTTR gene, containing a putative ERE, was amplified from genomic DNA and subsequently, introduced in the luciferase reporter vector pGL2-P (Promega). Briefly, the hTTR fragment comprising the region between −3426 bp and −3366 bp was amplified using primers A and B containing adapter sequences (Table 1), purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega), digested with *Kpn* I and *Bgl* II and introduced between *Kpn* I and *Bgl* II sites of pGL2-P. The pGL2-P/TTR was sequenced to confirm that cloning had been successful.

2.2.2.2. Cells and transfection. COS-7 cell line was used for reporter assays because it does not express ERα nor ERβ endogenously (Kahlert et al., 2000). Cells were cultured in 75 or 150 cm^2 flasks in DMEM with 10% fetal bovine serum and 100 U/mL antibiotic at 37 °C in a humidified incubator under an atmosphere of 5% CO_2/95% air. One to two days before transfection, COS-7 cells were seeded in six-well cell culture plates (150,000 cells per well) and cultured in DMEM containing 10% fetal bovine serum, without antibiotics. The luciferase reporter plasmid pGL2-P/TTR, pGL2-P (Fig. 1) and hERα and/or hERβ expression vectors (pSG5-hERα or pSG5-hERβ, respectively) were used to transfect approximately 95% confluent COS-7 cells (Table 2), using Lipofectamine 2000 (Invitrogen – Life Technologies Ltd, Carlsbad, CA, USA), according to the manufacturer's protocol. All co-transfections performed included a total amount of 2.5 μg of DNA and, control cells, without hERα and hERβ constructs, were co-transfected with pGL2-basic (Promega) to reach a similar total DNA mass. Six hours post-transfection, cells were washed and cultured in DMEM with 100 U/mL antibiotic, without fetal bovine serum for 12 h. Then, medium was changed to DMEM with 100 U/mL antibiotic and 100nM 17β-estradiol or vehicle (DMEM with 100 U/mL antibiotic and EtOH 1%) alone. After 24 h, cells were washed twice with phosphate buffer saline (PBS; 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH of 7.4), and 170 μL of Reporter Lysis Buffer 1× (Promega) were added to each well. After incubation for 1 h at RT, cells were scrapped and the lysates were centrifuged at 9000 *g* for 2 min. The supernatants were transferred to fresh microtubes and kept on ice.

2.2.2.3. Luciferase reporter assays. Luciferase activity was measured using the Britelite Plus Kit (PerkinElmer, Santa Clara, CA, USA): 100 μL of COS-7 cells supernatants and an equal volume of Britelite Plus reagent (PerkinElmer) were added to each well of a 96-microwell plate (Luminunc Plates, Nunc, ThermoScientific, Penfield, NY, USA). After an incubation of 10 min, the luciferase activity was measured using the Top Count NXT Scintillation and Luminescence Microplate Counter (Packard Instrument Company, Meriden, CT, USA). Luciferase activities were normalized to β-galactosidase (β-gal) activities, to correct for differences in transfection efficiency. β-gal measurement assays were performed using the β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer (Promega), according the manufacturer's indications. Briefly, in a 96-microwell plate, 50 μL of Assay Buffer 2X (Promega), which contains the substrate ONPG (o-nitrophenyl-β-D-galactopyranoside), were added to an equal volume of each sample. Samples were incubated for 30 min and 150 μL of sodium carbonate 1 M were added to each well to stop the reaction. The absorbance was

Table 1
Primer sequences containing adapter sequences to restriction endonucleases designed to amplify the hTTR fragment containing the putative ERE. The adapter sequences to restriction sites are underlined in both primer sequences.

Designation	Sequence (5′-3′)	Restriction endonuclease
A	5′-ttatggtacctgatagttcagaaagcaagc-3′	*Kpn* I
B	5′-tacaagatcttagaacagttttcaggccat-3′	*Bgl* II