Estrogen regulation of transferrin gene expression in MCF-7 human breast cancer cells

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Abstract

Transferrin (Tf) is an iron transport protein expressed in MCF-7 human breast cancer cells. In nuclear run-on assays, 17 β -estradiol (E2) increased the rate of Tf gene expression approximately 3-fold within 1 h after treatment and reporter gene activity was also induced in MCF-7 cells transfected with a construct containing a –3600 to +39 Tf gene promoter insert. Deletion and mutation analysis identified an E2-responsive promoter region between –811 and –762, which was GC-rich (80%) and contained two nonconsensus estrogen response elements (EREs). E2-responsiveness of this region was associated with a GGACA(N)₃TGGCC motif (–803 to –791) which bound human estrogen receptor α (hER α) in gel mobility shift assays. In *Drosophila* Schneider SL-2 cells, the –811 to –752 was E2-responsive after cotransfection with hER α expression plasmid plus E2, whereas Sp1 protein did not induce transactivation. These studies confirm that E2 induces Tf gene expression through a nonconsensus distal ERE.

Journal of Molecular Endocrinology (2002) **29**, 305–317

Introduction

Iron is essential for cell growth and metabolic processes including oxygen transport, DNA synthesis and electron transport (Conrad & Umbreit 2000). However, iron must be bound by proteins to prevent tissue damage from free radical formation (McCord 1998). Transferrin (Tf) is an iron-transport protein that is an essential component for iron metabolism. Tf is a member of a family of conserved proteins that include serum transferrin, lactotransferrin and melanotransferrin. Tf is an essential growth factor necessary for cell proliferation, differentiation and cell function and is predominantly synthesized in the fetal and adult liver. However, Tf secretion has been identified in Sertoli cells of the testes (Kissinger *et al.* 1982, Skinner & Griswold 1982), oligodendrocytes and astrocytes in adult brain (Connor *et al.* 1990), normal mammary tissues (Bradshaw & White 1985, Lee *et al.* 1987), chicken oviduct (Vandewalle *et al.* 1989), T lymphoma cells (Morrone *et al.* 1988),

lung cancer cells (Vostrejs *et al.* 1988) and MCF-7 human breast cancer cells (Schaeffer *et al.* 1989).

In the liver, regulation of Tf gene expression has been extensively studied and four functional regions of the Tf 5' promoter region have been identified: (i) a tissue-specific promoter between –125 to –45; (ii) a distal promoter region between –620 and –125; (iii) a negative regulatory region between –1000 and –620; and (iv) an enhancer region between –3600 and –3300 (Schaeffer *et al.* 1989). Tf gene expression is regulated by iron and hypoxia in hepatoma cells. Induction by hypoxia occurs through hypoxia-inducible factor-1 (HIF-1) interactions with sequences in the enhancer between –3600 to –3300 (Rolfs *et al.* 1997).

In Sertoli cells, Tf gene expression is regulated by follicle-stimulating hormone (FSH), retinoic acid, testosterone and insulin (Skinner & Griswold 1982, Huggenvik *et al.* 1987), whereas in the chicken oviduct, Tf gene expression is regulated by 17 β -estradiol (E2) (Lee *et al.* 1987). Previous studies

have extensively investigated hormonal regulation of lactotransferrin, which is expressed in multiple tissues and is estrogen-responsive in the rodent reproductive tract (Newbold *et al.* 1992, Teng 1995). The molecular biology of E2-induced transactivation of mouse and human lactotransferrin is complex and includes interactions of the estrogen receptor (ER) and the ER-related receptor $\alpha 1$ with compounded estrogen response elements (EREs) and a steroidogenic factor-binding element (Liu & Teng 1991, 1996, Liu *et al.* 1993, Yang *et al.* 1996, Zhang & Teng 2000). Vandewalle *et al.* (1989) showed that secretion of Tf by MCF-7 breast cancer cells was induced by E2, and 4'-hydroxytamoxifen reduced the enhanced secretion. This study is focused on the mechanism of hormonal regulation of Tf in MCF-7 human breast cancer cells. The rate of Tf gene expression is increased by E2 in MCF-7 cells in nuclear run-on assays, and analysis of the Tf gene promoter identified a functional nonconsensus ERE at -803 to -791 that was primarily responsible for E2-induced transactivation.

Materials and methods

Chemicals, oligonucleotides and antibodies

DME-F12, RPMI-1640, Dulbecco's phosphate-buffered saline (DPBS), acetyl coenzyme A, and E2 were purchased from Sigma Chemical Company (St Louis, MO, USA). MEM and Schneider's *Drosophila* medium were obtained from Life Technologies (Grand Island, NY, USA). Fetal bovine serum was purchased from InVitrogen (Carlsbad, CA, USA), Summit Biologicals (Boulder, CO, USA) or JRH (Lenexa, KS, USA). Restriction enzymes, T4 ligase and T4 polynucleotide kinase were obtained from Promega Corporation (Madison, WI, USA). Antibodies to human ER α (hER α) (C-314) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and recombinant hER α was obtained from Panvera (Madison, WI, USA). [32 P]ATP and [32 P]UTP were purchased from NEN (Boston, MA, USA). Oligonucleotides were synthesized by the Gene Technologies Laboratory (Texas A&M University, College Station, TX, USA), the Institute for Developmental and Molecular Biology (Texas A&M University, College Station, TX), or Genosys

(The Woodlands, TX, USA). Synthetic oligonucleotides used in these studies are indicated in Table 1. All other chemicals and biochemicals were the highest quality available from commercial sources.

Cell maintenance

MCF-7 and MDA-MB-231 human breast cancer cell lines and Schneider SL-2 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). MCF-7 cells were maintained in MEM supplemented with 2.2 g/l sodium bicarbonate, 10 μ g/ml insulin, 0.11 g/l pyruvic acid, 1 g/l glucose, 2.38 g/l Hepes, 10 ml/l antibiotic/antimycotic solution (Sigma) and 10% fetal bovine serum. MDA-MB-231 cells were cultured in DME-F12 with phenol red supplemented with 2.2 g/l sodium bicarbonate, 10 ml/l antibiotic/antimycotic solution (Sigma) and 5% fetal bovine serum. Both breast cancer cell lines were cultured at 37 °C in a 5% CO₂:95% atmosphere environment.

Schneider's SL-2 cells were cultured at room temperature in Schneider's *Drosophila* medium supplemented with 10% heat-inactivated fetal bovine serum. For transient transfection experiments, MCF-7 and MDA-MB-231 cells were seeded in 12-well tissue culture plates at a density of 2×10^5 cells per well for luciferase assays or in 60 mm tissue culture plates at a density of 6×10^5 for chloramphenicol acetyl transferase (CAT) assays in DME-F12 without phenol red supplemented with 2.2 g/l sodium bicarbonate, 10 ml/l antibiotic/antimycotic solution (Sigma) and 2.5% charcoal-stripped fetal bovine serum. Schneider's SL-2 cells were seeded in 12-well tissue culture plates in Schneider's *Drosophila* medium supplemented with 10% heat-inactivated fetal bovine serum.

Plasmids

The hER α expression plasmid was kindly provided by Dr Ming-Jer Tsai (Baylor College of Medicine, Houston, TX, USA), and HE11, the DNA-binding domain deletion mutant of hER α , was provided by Dr Pierre Chambon (Illkirch, France). hER β expression plasmid was provided by Dr J-A Gustafsson (Karolinska Institute, Huddinge,

Table 1 Synthetic oligonucleotides used for transferrin studies^a

–1600 forward	5'-CCAAGCTTCTGTAGTGGACTCTAAAG-3'
–1078 reverse	5'-TTTGGATCCACGAAGTAAATCCAGACTTCAGAG-3'
–1078 forward	5'-TTTAAGCTTCTCTGAAGTCTGGATTACTTCGTG-3'
–620 reverse	5'-TTTGGATCCTTCCTGTGGGACACCTTAGACTAG-3'
–831 reverse	5'-TTTAAGCTTCTGGTTAGGAGACTCATCGACTAGAG-3'
–762 forward	5'-TTTAAGCTTCACTGGGCGCTTGC-3'
–831 mut1 forward	5'-TTAAGCTTCTGGTTAGGAGACTCATCGACTAGAGGGT*GAT*ATGGTGGCCC-3'
–831 mut2 forward	5'-TTAAGCTTCTGGTTAGGAGACTCATCGACTAGAGGGGGACATGGTT*GCT*C-3'
–831 mutERE forward	5'-TTAAGCTTCTGGTTAGGAGACTCATCGACTAGAGGGT*GAT*ATGGTT*GCT*C-3'
–831/–762	5'-AGCTTCTGGTTAGGAGACTCATCGACTAGAGGGGGACATGGTGGCCCCAGGCTGT AAGAACAGGCCACACCGTCG-3'
–811/–752	5'-AGCTTCTAGAGGGGGACATGGTGGCCCCAGGCTGTAAGAACAGGCCACACCGTCC ACTGGGCCGCG-3'
–811/–752 pERE	5'-AGCTTCTAGAGGGGGACATGGTGA*CCCCAGGCTGTAAGAACAGGCCACACCGTC CACTGGGCCGCG-3'
–811/–752 mERE	5'-AGCTTCTAGAGGGGGACATGGTGGT*T*CCAGGCTGTAAGAACAGGCCACACCGTC CACTGGGCCGCG-3'
–811/–762	5'-AGCTTCTAGAGGGGGACATGGTGGCCCCAGGCTGTAAGAACAGGCCACACCGTCG-3'
–811/–772	5'-AGCTTCTAGAGGGGGACATGGTGGCCCCAGGCTGTAAGAACAGGCCG-3'
–811/–782	5'-AGCTTCTAGAGGGGGACATGGTGGCCCCAGGCTGTG-3'
–801/–752	5'-AGCTTACATGGTGGCCCCAGGCTGTAAGAACAGGCCACACCGTCCACTGGGCCGCG-3'
–801/–772	5'-AGCTTACATGGTGGCCCCAGGCTGTAAGAACAGGCCG-3'
–801/–782	5'-AGCTTACATGGTGGCCCCAGGCTGTG-3'
–791/–752	5'-AGCTTCCCAGGCTGTAAGAACAGGCCACACCGTCCACTGGGCCGCG-3'

^aMutations (m or mut) are indicated by asterisks.

Sweden). The pPAC and pPAC/Sp1 expression vectors were kindly provided by Dr Robert Tjian (University of California-Berkeley, Berkeley, CA, USA). The pPAC/hER α expression plasmid was made in this laboratory by releasing the hER α -coding sequence from the hER α expression plasmid and cloned into the XhoI site of pPAC. The pTATA-luc construct was made by inserting an oligonucleotide containing the E1B TATA box into BamHI-XhoI sites of pXP2 vector as described (Vyhldal *et al.* 2000). The pSp1₃ plasmid contains three tandem consensus GC-rich Sp1-binding sites linked to a luciferase reporter gene (Dong *et al.* 1999).

The pTf–3600/+39-CAT and pTf–620/+39-CAT reporter constructs were kindly provided by Dr Mario Zakin (Institut Pasteur, Paris, France). The pTf–1600/–620-CAT and pTf–1600/–620-luc constructs were made by digestion of pTf–3600/+39-CAT with HindIII and ligation of the 1 kb fragment into pBLTATA-CAT or pTATA-luc. pTf–1600/–1078-CAT/luc, pTf–1078/–620-CAT/luc, pTf–831/–620-CAT/luc and pTf–762/–620-CAT/luc were made by PCR of the appropriate fragment using

primers as listed in Table 1. The PCR fragments were gel-purified, cut with BamHI and HindIII, and ligated into pBLTATA-CAT. The pTf–831/–752-CAT and smaller constructs were made by annealing, phosphorylation and ligation of synthetic oligonucleotides (Table 1) into pBLTATA-CAT.

Transient transfections and reporter assays

Twenty-four hours after seeding, cells were transfected with the indicated reporter constructs, wild-type or variant hER α expression plasmid and pCDNA3/His/lacZ using calcium phosphate. Six hours following transfection, breast cancer cells were shocked with 25% glycerol in sterile DPBS for 1 min, washed twice with DPBS and serum-free DME-F12 without phenol red supplemented with 2.2 g/l sodium bicarbonate, 0.2 g/l BSA, 0.01 g/l apotransferrin and 10 ml/l antibiotic/antimycotic solution was added containing the indicated compounds. Six hours after transfection, Schneider cells were treated with the appropriate compounds in the same medium used for transfection. Cells were allowed to grow for an additional 6 h

(Schneider's cells) or 24–48 h (mammalian cells) before harvesting.

Cells were harvested in $1 \times$ Reporter Lysis Buffer (Promega, Madison, WI). CAT activity was determined on 75–100 μ g protein using 0.2 mCi *d*-threo-[dichloroacetyl-1- 14 C] chloramphenicol and 4 mM acetyl-coenzyme A. Reactions were incubated overnight at 37 °C. Acetylated products were extracted with ethyl acetate and separated on thin-layer chromatography (TLC) in a 95:5 chloroform:methanol solvent mixture. After TLC, acetylated products were visualized and quantitated on a Packard Instant Imager (Downers Grove, IL, USA). Luciferase activity was determined on 25 μ l cell extract using the Luciferase Assay Reagent from Promega. β -Galactosidase activity was detected using the luminescent Galacton-Plus Assay system from Tropix (Bedford, MA, USA) after a 15 min incubation of 25 μ l cell extract and the Galacton-Plus. Intensity of light emission from luciferase and β -galactosidase assays was determined using a Packard LumiCount luminometer. Percent conversion from CAT assays was divided by relative light units (RLUs) of β -galactosidase assays to correct for relative transfection efficiencies. The experiments were carried out at least in triplicate and Scheffé's tests using SuperAnova were performed to determine statistical significance.

Electrophoretic mobility shift assay

Synthetic oligonucleotides were annealed and end-labeled using T4-polynucleotide kinase and [γ - 32 P]ATP. Gel shift assays using recombinant hER α were carried out in $1 \times$ binding buffer (20 mM Hepes, 100 mM KCl, 1 mM EDTA, 5% glycerol and 2.5 mM dithiothreitol (DTT)) in 30 μ l final volume. The indicated amount of protein was incubated in $1 \times$ binding buffer on ice for 15 min. Five microliters of probe mix (5 fmol 32 P-labeled oligonucleotide DNA, 1 μ l 10% Ficoll and 500 ng poly-dIC in $1 \times$ binding buffer) were added and the reaction continued at 25 °C for 15 min. Reaction mixtures were loaded onto a 5% polyacrylamide gel and run at 110 V in $1 \times$ TBE (0.9 M Tris, 0.9 M borate, 2 mM EDTA (pH 8.0)). Gels were dried and protein–DNA complexes were visualized by autoradiography or phosphor imaging on a Molecular Dynamics (Sunnyvale, CA, USA) Storm. For supershift assays, hER α antibody or normal IgG was incubated with proteins and oligo-

nucleotides for 30 min on ice before electrophoresis. The extracts were then subjected to electrophoresis and detected as described above.

Nuclear run-on assay

Cells (5×10^7) were treated with different concentrations of solvent or E2 for 1 or 2 h as indicated. Cells were then washed once with ice-cold DPBS and then scraped into 5 ml ice-cold DPBS, transferred to a 50 ml conical tube, and collected by centrifugation at 500 *g* for 5 min at 4 °C. The cell pellet was loosened by gentle vortexing for 5 s and 5 ml ice-cold lysis buffer B (10 mM Tris–HCl (pH 7.4), 3 mM CaCl₂, 2 mM MgCl₂) were added and the pellet was fully resuspended. An additional 30 ml lysis buffer B was added and tubes were rocked for 1 min to swell the cells. Cells were collected by centrifugation at 500 *g* for 5 min at 4 °C. Cells were resuspended by gentle vortexing in 1 ml lysis buffer. One milliliter of NP-40 lysis buffer B (lysis buffer B, 1% (v/v) NP-40) and cells were homogenized in a Dounce homogenizer with a B-type pestle. Intact nuclei were detected by trypan blue staining. Nuclei were collected by centrifugation at 500 *g* for 5 min at 4 °C and nuclei were resuspended in 200 μ l glycerol storage buffer (50 mM Tris–HCl (pH 8.3), 40% (v/v) glycerol, 5 mM MgCl₂, 0.1 mM EDTA).

Two hundred microliters of $2 \times$ reaction buffer (10 mM Tris–HCl (pH 8.0), 5 mM MgCl₂, 0.3 M KCl, 5 mM DTT, and 1 mM each ATP, CTP, GTP) and 10 μ l [α - 32 P]UTP (800 Ci/mmol) were added to 200 μ l freshly isolated nuclei and incubated at 30 °C for 30 min. After incubation, 40 U of RNase-free DNase were added and incubated at room temperature for 5 min. Following the addition of 100 μ g yeast tRNA, labeled RNA was isolated by the addition of 1 ml RNazol B (Tel-Test, Friendswood, TX, USA) and 100 μ l chloroform. RNA was precipitated at –20 °C in isopropanol for 1 h. RNA was collected by centrifugation at 16 000 *g* for 15 min at 4 °C and washed with 70% ethanol. The RNA pellet was dissolved in 500 μ l 20 mM Tris–HCl (pH 7.9) and 20 mM EDTA.

Tf and β -actin cDNA plasmids (5 μ g, linearized) were immobilized on Zeta-Probe GT membranes (Bio-Rad, Hercules, CA, USA). Membranes were prehybridized at 55 °C for 30 min in ULTRAhyb

Figure 1 Hormone regulation of Tf gene/gene promoter constructs in MCF-7 cells. (A) Nuclear run-on assays. Nuclear run-on assays were performed as described in Materials and methods with nuclei isolated from MCF-7 cells treated with 10 nM E2 for 1 or 2 h. Bands were quantitated with a Scanalytics ZeroD Scanner (Sunnyvale, CA) and are graphed as relative Tf/ β -tubulin with ratios in the DMSO treatment group set at 100%. Results are expressed as the mean of duplicate determinations. (B) E2-responsiveness of Tf promoter constructs in MCF-7 cells. MCF-7 cells were transiently transfected with the indicated Tf constructs, wild-type hER α expression plasmid and pCDNA/His3/lacZ. Transfected cells were dosed for 48 h, after which cells were harvested and CAT activity was determined as described in Materials and methods. Results are expressed as means $\pm$ s.d. for three replicate determinations. *Significant induction by E2 ($P<0.05$).

A

B

pTf-811/-752 in MDA-MB-231

hybridization buffer (Ambion, Austin, TX, USA). After prehybridization, 4×10^6 c.p.m. labeled RNA were added and hybridization continued at 55 °C for 48 h. Membranes were washed for 15 min at 55 °C in $2 \times$ SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0), followed by an additional wash with $2 \times$ SSC containing $10 \mu\text{g/ml}$ RNase A for 15 min at room temperature. Membranes were exposed to film for 6 weeks.

Statistical analysis

Statistical significance was determined by ANOVA and Scheffé's test and the confidence levels are indicated. Results are expressed as means $\pm$ S.D. for at least three determinations.

Results

Induction of Tf gene and gene promoter constructs in MCF-7 and MDA-MB-231 cells

MCF-7 cells were treated with 10 nM E2 for 1 or 2 h and the rate of Tf gene expression was determined in a nuclear run-on assay using nuclei from hormone-treated cells. The rate of gene expression was increased approximately 2.5- and 3-fold in the 1 and 2 h treatment groups (Fig. 1A), and this was consistent with a previous report showing a 3-fold increase of Tf secretion in the media after treating MCF-7 cells with E2 (Batistuzzo de Medeiros *et al.* 1997). Transient transfection studies with promoter-reporter constructs containing Tf gene promoter inserts were carried out in both MCF-7 and MDA-MB-231 cells cotransfected with hER α expression plasmid. Previous studies have also reported the requirement for cotransfected hER α , even in ER-positive MCF-7 cells, due to overexpression of the promoter-reporter constructs resulting in limiting

levels of endogenous ER α . These requirements for cotransfected hER α have been reported in studies with other E2-responsive promoters from the pS2, c-myc, progesterone receptor, cathepsin D and retinoic acid receptor $\alpha 1$ genes and promoters containing one or two consensus EREs (Savouret *et al.* 1991, Dubik & Shiu 1992, Cavailles *et al.* 1993, Krishnan *et al.* 1994, Zacharewski *et al.* 1994, Rishi *et al.* 1995, Sathya *et al.* 1997).

E2 induced reporter gene activity 17-fold over control levels in MCF-7 cells transfected with a reporter gene construct containing 3.6 kb of the Tf gene promoter (Tf-3600/+39) and an ER α expression plasmid (Fig. 1B). In contrast, E2 did not affect reporter gene activity in MCF-7 cells transiently transfected with pTf-620/+39, suggesting that the downstream region of the promoter was not E2-responsive. Transfection of MCF-7 cells with pTf-1600/-620 confirmed that E2-responsiveness was associated with this region of the promoter and further 3' (pTf-1600/-1078) and 5' (pTf-1078/-620) deletions showed that elements within the -1078 to -620 region of the promoter were E2-inducible.

Deletion and mutation analysis of the Tf gene promoter

A series of constructs containing Tf gene promoter inserts with 5'- and 3'-deletions of the -1078 to -620 region was prepared and their E2-dependent activities were determined in MCF-7 cells (Fig. 2A). In transient transfection studies, the following constructs were E2-responsive: pTf-831/-620, pTf-831/-763, pTf-811/-752, pTf-811/-762, pTf-811/-772, pTf-811/-782 and pTf-801/-752. E2 induced a 3- to 9-fold increase in luciferase activity.

Figure 2 Analysis of hormone-responsiveness of the Tf gene promoter in MCF-7 and MDA-MB-231 cells. (A) Deletion analysis of the Tf gene promoter. MCF-7 cells were transiently transfected with the indicated Tf constructs, wild-type hER α expression plasmid and pCDNA/His3/lacZ. Transfected cells were treated for 48 h; cells were harvested and CAT activity was determined as described in Materials and methods. Results are expressed as the means $\pm$ S.D. for three replicate determinations. *Significant induction by E2 ($P < 0.05$). The -811 to -762 transferrin promoter sequence is shown in the figure. ERE half sites are underlined; bases mutated in pTf -811/-752 ml (TT) and pTf -811/-752 m² (A) are indicated. (B) Transfection of MDA-MB-231 cells with pTf-811/-752. MDA-MB-231 cells were cotransfected with $1 \mu\text{g}$ pTf-811/-752, $0.2 \mu\text{g}$ pCDNA3/His3/lacZ, and $0.5 \mu\text{g}$ wild-type hER α , hER β , hE11 or pCDNA3 (empty vector). Cells were treated with DMSO or 10 nM E2 for 48 h, after which cells were harvested and luciferase and β -galactosidase activity was detected as described in Materials and methods. Results are expressed as mean corrected luciferase activity $\pm$ S.D. *Significant induction by E2 ($P < 0.05$).

The active -811 to -752 region of the promoter contained an upstream nonconsensus ERE at -803 to -791 (GGACA TGG TGGCC) with one base in each half-site differing from a consensus sequence and a downstream noncon-

sensus sequence (GGCCA CAC CGTCC) with three bases differing from a consensus ERE. We therefore mutated the upstream half-site (TGGCC→TGGTT) to give the construct pTf-811/-752_{M1} and, in transient transfection

studies with the mutant construct, E2-responsiveness was lost. In contrast, when the TGGCC motif was mutated to give a consensus ERE half-site (TGACC), the resulting construct (pTf-811/-752_{M2}) was highly E2-responsive after transient transfection in MCF-7 cells. These results suggest that the nonconsensus ERE at -803 to -791 is important for hormone activation of this region of the Tf gene promoter; however, this region also contains G/GC-rich sequences which can be hormone activated through interactions with ER α /Sp1 (Porter *et al.* 1996, Duan *et al.* 1998, Sun *et al.* 1998, Wang *et al.* 1998, 1999, Dong *et al.* 1999, Qin *et al.* 1999, Xie *et al.* 1999, 2000). Therefore, we further investigated the E2-responsiveness in ER-negative MDA-MB-231 breast cancer cells transfected with pTf-811/-752 and expression plasmids for hER α , hER β or HE11, an hER α mutant with a DNA-binding domain deletion that activates hER α /Sp1 (Duan *et al.* 1998). E2 induced luciferase activity in cells transfected with hER α but not hER β or HE11, and the latter response suggests that hER α /Sp1-mediated transactivation of pTf-811/-751 through G/GC-rich sequences is not observed.

Comparative activation of pTf-811/-752 by ER α or Sp1 in SL-2 cells

To confirm that Sp1 is not required for the E2-responsiveness of pTf-811/-752, the Sp1- (and hER α -) deficient *Drosophila* SL2 cells were transiently transfected with pTf-811/-752 and expression plasmids for hER α or Sp1. These transcription factors are not expressed in SL-2 cells; however, their interactions on GC-rich promoters can be investigated in transient transfection studies (Dong *et al.* 1999). In cells transiently cotransfected with pTf-811/-752 and increasing amounts of the hER α expression plasmid, E2 induced up to a

45-fold increase in luciferase activity in a dose-dependent manner (Fig. 3A). Cotransfection of pTf-811/-752 and increasing amounts of the Sp1 expression plasmid resulted in no change in luciferase activity over cotransfection with pPAC empty vector. In contrast, cotransfection of increasing amounts of the Sp1 expression plasmid with pSp1₃ (contains three tandem GC-rich Sp1-binding sites) (Dong *et al.* 1999) resulted in increased luciferase activity over cotransfection with pPAC alone (Fig. 3B). These results indicate that Sp1 is not necessary for E2-responsiveness of the -811 to -752 region of the Tf gene promoter.

Interaction of hER α with [³²P]Tf-811/-752

Preliminary studies with nuclear extracts from MCF-7 cells incubated with [³²P]Tf-811/-752 gave diffuse retarded bands in gel mobility shift assays (data not shown). The concentration of hER α in nuclear extracts is relatively low, and binding to nonconsensus EREs can be observed using recombinant or *in vitro*-translated hER α (Lin *et al.* 2002). The results in Fig. 4 show that a consensus [³²P]ERE binds recombinant hER α to give an intense retarded band (lane 2) that is supershifted by ER α antibodies (lane 4) but not by nonspecific IgG (lane 3). Excess unlabeled ERE oligonucleotide competitively decreased intensity of the retarded band (lane 5). Incubation of [³²P]Tf with hER α gave a retarded band (lane 7) with mobility similar to that observed for the hER α -ERE band and a slightly less mobile band. hER α antibodies (lane 9) but not IgG (lane 8) supershifted the more mobile band, which was also decreased after competition with excess unlabeled ERE (lane 10) or Tf (lane 11). These results show that hER α binds the -811 to -752 region of the Tf gene promoter confirming that a nonconsensus ERE within this region is necessary for hormone activation of Tf gene expression.

Figure 3 Activation of Tf gene promoter constructs in SL-2 cells. (A) Transfection of pTf-811/-752 into SL2 cells. SL2 cells were transfected as described in Materials and methods with 500 ng pTf-811/-752 and pPAC empty vector or different amounts of hER α expression plasmids as indicated. Cells were treated with 10 nM E2 18 h after transfection and were harvested 6 h after treatment and luciferase activity was determined. Results are expressed as mean RLU $\pm$ S.D. for four replicate determinations. *Significant induction ($P<0.05$) by E2. (B) Sp1 does not transactivate pTf-811/-752. SL2 cells were transfected as described in Materials and methods with 50 ng pTf-811/-752 or pSp1₃ and pPAC empty vector or Sp1 expression plasmid at the amounts indicated. Cells were harvested 24 h after transfection and luciferase activity was determined. Results are expressed as mean RLU $\pm$ S.D. for four replicate determinations. *Significant ($P<0.05$) induction over pPAC alone.

Figure 4 Binding of hERα to [³²P]ERE and [³²P]Tf–811/–752. Recombinant hERα, radiolabeled oligonucleotides and antibodies or unlabeled oligonucleotides were incubated and analyzed by gel mobility shift assays as described in the Materials and methods. The specifically bound ER-DNA retarded band and ERα antibody supershifted complexes are indicated with arrows.

Discussion

Studies with Tf promoter constructs indicate that complex protein–protein interactions regulate Tf gene expression in a cell type-specific manner. In hepatoma cells, four distinct functional regions have been identified in the Tf gene promoter (Schaeffer *et al.* 1989), and subsequent studies have investigated protein interactions with these elements in hepatoma, Sertoli, HeLa and neuronal cell lines. In hepatoma cell lines, the proximal regions I and II (PRI and PRII respectively) between –100 and the start site are required for full promoter activity in transient transfection assays, and hepatocyte nuclear factor-4 bound PRI, whereas CCAAT-binding protein bound PRII (Schaeffer *et al.* 1989). In contrast, Sertoli cell-specific factors (SP-A and SP-D) bound PRI, and SP- α and SP- β interacted with PRII to regulate Sertoli cell-specific transcription (Schaeffer *et al.* 1993). An enhancer region between –3600 and –3300 was inactive in Sertoli and neuronal cells (Sawaya *et al.* 1996), but in liver cells, HIF-1 bound two HIF-1-binding sites in the distal region of the promoter to induce Tf gene expression in response to low levels of oxygen (Boissier *et al.* 1991, Rolfs *et al.* 1997). Two distal and one central regulatory regions (DRI, DRII and CR) (–620 to –100) have also been identified in the Tf gene promoter and a member of the CTF/NF-1 family interacts with the CR in liver cells (Schaeffer *et al.* 1993). However, the identities of proteins that bind DRI or DRII are unknown (Brunel *et al.* 1988, Schaeffer *et al.* 1993). In addition to the regulatory regions for Sertoli and liver cells, two upstream regions have been identified between –1000 and –819 in B103 neuronal cells and these elements bind proteins belonging to the steroid/retinoid receptor or NF-1 families of transcription factors respectively (Sawaya *et al.* 1996).

Vandewalle *et al.* (1989) reported that Tf secretion in MCF-7 cells was increased approximately 3-fold after treatment with E2. Nuclear run-on assays demonstrated that transcription of the Tf gene is increased 2- to 3-fold in MCF-7 cells treated with 10 nM E2 (Fig. 1A). E2 induced reporter gene activity from MCF-7 cells transiently transfected with a construct containing a 3.6 kb Tf gene promoter insert (Fig. 1B). These data indicate that regulation of Tf gene expression by E2 occurs at the level of transcription. Interestingly, pTf –620/+39

was nonresponsive to E2, whereas previous studies showed that this region was important for regulation of Tf gene expression in Sertoli cells in response to FSH and cAMP and for high basal level transcription in hepatoma cell lines (Chaudhary *et al.* 1996, Chaudhary & Skinner 1998).

Transient transfection of MCF-7 cells with Tf gene promoter deletion constructs (Fig. 2) have identified an E2-responsive enhancer element between –811 and –752. This region of the promoter also contains G/GC-rich sequences which have been identified in other E2-responsive gene promoters and interact with ER α /Sp1 to mediate hormone-induced transactivation (Duan *et al.* 1998, Sun *et al.* 1998, Wang *et al.* 1998, 1999, Dong *et al.* 1999, Qin *et al.* 1999, Xie *et al.* 1999, 2000). However, results of transfection studies in SL-2 cells indicate that ER α but not Sp1 plays a role in hormonal activation of pTF –811/–752 (Fig. 3A). Within the –811 to –752 region is a nonconsensus ERE, and mutation or deletion of the downstream ERE half-site resulted in the loss of E2-responsiveness (Fig. 2B). Gel mobility shift assays showed that [³²P]Tf –811/–752 bound hER α to form a specifically bound retarded band. This nonconsensus ERE contains two bases that are different from a consensus sequence; however, the GGACA (5') and TGGCC (3') ERE half-sites have previously been identified in E2-responsive nonconsensus EREs from the rat luteinizing hormone B gene (Shupnik & Rosenzweig 1991) and rabbit uteroglobin gene promoters (Slater *et al.* 1990). hER α activation of the nonconsensus ERE in the Tf gene promoter was confirmed in Schneider SL-2 insect cells, indicating that hormone regulation of Tf gene expression in MCF-7 cells is ERE-dependent. This study complements an increasing number of reports showing that nonconsensus EREs play an important role in regulating E2-induced transactivation of multiple genes (Stancel *et al.* 1995, Sathya *et al.* 1997, Lascombe *et al.* 1998).

Acknowledgements

The financial assistance of the National Institutes of Health (ES09106 and CA76636) and the Texas Agricultural Experiment Station is gratefully acknowledged. S.S. is a Sid Kyle Professor of Toxicology.

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Received 16 April 2002

Accepted 22 July 2002