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

C Vyhlidal, X Li and S Safe

Department of Veterinary Physiology and Pharmacology and Department of Biochemistry and Biophysics, Texas A&M University, 4466 TAMU, College Station, Texas 77843-4466, USA

(Requests for offprints should be addressed to S Safe; Email: ssafe@cvm.tamu.edu)

Abstract

Transferrin (Tf) is an iron transport protein expressed in MCF-7 human breast cancer cells. In nuclear run-on assays, 17β-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 (hERα) in gel mobility shift assays. In Drosophila Schneider SL-2 cells, the −811 to −752 was E2-responsive after cotransfection with hERα 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 (Rolls 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 17β-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 α1 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 (hERα) (C-314) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and recombinant hERα 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 µg/ml insulin, 0·11 g/l pyruvic acid, 1 g/l glucose, 2·38 g/l Heps, 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% CO\(_2\)-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 hERα 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 hERα, was provided by Dr Pierre Chambon (Illkirch, France). hERβ expression plasmid was provided by Dr J-A Gustafsson (Karolinska Institute, Huddinge,
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\(_{\text{afii9825}}\) expression plasmid was made in this laboratory by releasing the hER\(_{\text{afii9825}}\)-coding sequence from the hER\(_{\text{afii9825}}\) 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 (Vyhlidal 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\(_{\text{p13600/+39-CAT}}\) and pTf\(_{\text{p16200}}\) reporter constructs were kindly provided by Dr Mario Zakin (Institut Pasteur, Paris, France). The pTf\(_{\text{p11600/+39-CAT}}\) and pTf\(_{\text{p16200}}\) reporter constructs were made by digestion of pTf\(_{\text{p13600/+39-CAT}}\) with HindIII and ligation into pBLTATA-CAT. pTf\(_{\text{p11600/+39-CAT}}\) 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\(_{\alpha}\) 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

---

**Table 1** Synthetic oligonucleotides used for transferrin studies

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>−1600 forward</td>
<td>5′-CCAAGCTTCTGTAGTGGACTCTAAAG-3′</td>
</tr>
<tr>
<td>−1078 reverse</td>
<td>5′-TTTGGATCCACGAAAGTAAATCCCAGCTTCAGGAGG-3′</td>
</tr>
<tr>
<td>−1078 forward</td>
<td>5′-TTTAGCTTCTGTTAGGAGACTCATGACACTAGGAGGTCATGTTCAGTGC-3′</td>
</tr>
<tr>
<td>−620 reverse</td>
<td>5′-TTTAGCTTCTGTTAGGAGACTCATGACACTAGGAGGTCATGTTCAGTGC-3′</td>
</tr>
<tr>
<td>−831 reverse</td>
<td>5′-TTTAGCTTCTGTTAGGAGACTCATGACACTAGGAGGTCATGTTCAGTGC-3′</td>
</tr>
<tr>
<td>−762 forward</td>
<td>5′-TTTAGCTTCTGTTAGGAGACTCATGACACTAGGAGGTCATGTTCAGTGC-3′</td>
</tr>
<tr>
<td>−831 mut1 forward</td>
<td>5′-TTTAGCTTCTGTTAGGAGACTCATGACACTAGGAGGTCATGTTCAGTGC-3′</td>
</tr>
<tr>
<td>−831 mut2 forward</td>
<td>5′-TTTAGCTTCTGTTAGGAGACTCATGACACTAGGAGGTCATGTTCAGTGC-3′</td>
</tr>
<tr>
<td>−831−762 forward</td>
<td>5′-AGCTTCTGTTAGGAGACTCATGACACTAGGAGGTCATGTTCAGTGC-3′</td>
</tr>
</tbody>
</table>
| −811−752        | 5′-AGCTTCTAGAGGGAGCAGTGGGCCCAGCTGAAGACACGCAACACAGTC
| −811−752 pERE   | 5′-AGCTTCTAGAGGGAGCAGTGGGCCCAGCTGAAGACACGCAACACAGTC
| −811−752 mERE   | 5′-AGCTTCTAGAGGGAGCAGTGGGCCCAGCTGAAGACACGCAACACAGTC
| −811−762        | 5′-AGCTTCTAGAGGGAGCAGTGGGCCCAGCTGAAGACACGCAACACAGTC
| −801−762        | 5′-AGCTTCTAGAGGGAGCAGTGGGCCCAGCTGAAGACACGCAACACAGTC
| −801−772        | 5′-AGCTTCTAGAGGGAGCAGTGGGCCCAGCTGAAGACACGCAACACAGTC
| −791−752        | 5′-AGCTTCTAGAGGGAGCAGTGGGCCCAGCTGAAGACACGCAACACAGTC

*a* Mutations (m or mut) are indicated by asterisks.
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 × 10⁷) 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 µl 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 × 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 µl [α-³²P]UTP (800 Ci/mmol) were added to 200 µl 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 µg yeast tRNA, labeled RNA was isolated by the addition of 1 ml RNAzol B (Tel-Test, Friendswood, TX, USA) and 100 µl 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 µl 20 mM Tris–HCl (pH 7·9) and 20 mM EDTA.

TF and β-actin cDNA plasmids (5 µg, 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 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 hERα 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±s.d. for three replicate determinations. *Significant induction by E2 (P<0.05).
Estrogen regulation of transferrin in MCF-7 cells  ·  C VYHLIDAL and others

hybridization buffer (Ambion, Austin, TX, USA). After prehybridization, 4 × 10⁶ 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 × SSC (0·3 M NaCl, 30 mM sodium citrate, pH 7·0), followed by an additional wash with 2 × SSC containing 10 µg/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 Scheffe’s test and the confidence levels are indicated. Results are expressed as means ± 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 hERα expression plasmid. Previous studies have also reported the requirement for cotransfected hERα, even in ER-positive MCF-7 cells, due to overexpression of the promoter-reporter constructs resulting in limiting levels of endogenous ERα. These requirements for cotransfected hERα have been reported in studies with other E2-responsive promoters from the pS2, c-myc, progesterone receptor, cathepsin D and retinoic acid receptor α1 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 ERα 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 hERα 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±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 µg pTf−811/−752, 0·2 µg pCDNA3/His/lacZ, and 0·5 µg wild-type hERα, hERβ, 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±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 nonconsensus 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/−752M2) 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 hormonally activated through interactions with ERα/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 or pSp13 and pPAC empty vector or Sp1 expression plasmid at the amounts indicated. Cells were transactivated pTf−811/−752. SL2 cells were transfected as described in Materials and methods with 50 ng pTf−811/−752 and 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 hERα 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 hERα in nuclear extracts is relatively low, and binding to nonconsensus EREs can be observed using recombinant or in vitro-translated hERα (Lin et al. 2002). The results in Fig. 4 show that a consensus [32P]ERE binds recombinant hERα to give an intense retarded band (lane 2) that is supershifted by ERα 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 hERα gave a retarded band (lane 7) with mobility similar to that observed for the hERα-ERE band and a slightly less mobile band. hERα 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 hERα 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.

**Comparative activation of pTf−811/−752 by ERα or Sp1 in SL-2 cells**

To confirm that Sp1 is not required for the E2-responsiveness of pTf−811/−752, the Sp1- (and hERα-) deficient Drosophila SL2 cells were transiently transfected with pTf−811/−752 and expression plasmids for hERα 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 hERα 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 gave a dose-dependent decrease in 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.
Figure 4 Binding of hERα to \(^{32}\text{P}]\text{ERE}\) and \(^{32}\text{P}]\text{Tf–811–752}\). 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 ERα/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 ERα 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 hERα 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). hERα 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.
References


Lee EY, Barcellos-Hoff MH, Chen LH, Parry G & Bissell MJ 1987 Transferrin is a major mouse milk protein and is synthesized by mammary epithelial cells. *In Vitro Cellular and Developmental Biology* 23 221–226.


Received 16 April 2002
Accepted 22 July 2002