Transcriptional activation of transforming growth factor α by estradiol: requirement for both a GC-rich site and an estrogen response element half-site

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ABSTRACT

17 β -Estradiol (E2) induces transforming growth factor α (TGF α) gene expression in MCF-7 cells and previous studies have identified a 53 bp (–252 to –200) sequence containing two imperfect estrogen responsive elements (EREs) that contribute to E2 responsiveness. Deletion analysis of the TGF α gene promoter in this study identified a second upstream region of the promoter (–623 to –549) that is also E2 responsive. This sequence contains three GC-rich sites and an imperfect ERE half-site, and the specific cis-elements and trans-acting factors were determined by promoter analysis in transient transfection experiments, gel mobility shift assays and *in vitro* DNA footprinting. The results are consistent with an estrogen receptor α

(ER α)/Sp1 complex interacting with an Sp1(N)₃₀ ERE half-site (½) motif in which both ER α and Sp1 bind promoter DNA. The ER/Sp1–DNA complex is formed using nuclear extracts from MCF-7 cells but not with recombinant human ER α or Sp1 proteins, suggesting that other nuclear factor(s) are required for complex stabilization. The E2-responsive Sp1(N)_xERE½ motif identified in the TGF α gene promoter has also been characterized in the cathepsin D and heat shock protein 27 gene promoters; however, in the latter two promoters the numbers of intervening nucleotides are 23 and 10 respectively.

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INTRODUCTION

Transforming growth factor α (TGF α) and other polypeptide growth factors play an important role in oncogenesis in multiple tissues (Sporn & Roberts 1985, Keski-Oja *et al.* 1988, Aaronson 1991, Cross & Dexter 1991, Dickson & Lippman 1995, Ethier 1995). TGF α acts as a mitogen in most cell types and interacts with the transmembrane epidermal growth factor receptor to initiate intracellular signaling pathways (Derynck 1988). TGF α is expressed in multiple breast cancer cell lines and treatment of estrogen receptor (ER)-positive breast and endometrial cancer cell lines with 17 β -estradiol (E2) induces TGF α gene expression and/or protein secretion. Induction of TGF α by E2 may be important for mediating the mitogenic effects of estrogen through paracrine and autocrine pathways

(Bates *et al.* 1988, Clarke *et al.* 1989, Manni *et al.* 1990, Gong *et al.* 1992).

In transient transfection studies, E2 induces reporter gene activity using constructs containing –2813 to –5, –1565 to –5, and –1145 to –5 inserts from the TGF α gene promoter (Saeki *et al.* 1991). Constructs containing the more proximal region from this promoter (–370 to –5 and –77 to –5) exhibited low basal activity and the sequences from –370 to –77 were required for transcriptional activation of TGF α by E2 in MCF-7 cells (Saeki *et al.* 1991). El-Ashry and coworkers (1996) recently identified a 53 bp sequence (–252 to –200) in the TGF α gene promoter containing two imperfect palindromic estrogen responsive elements (EREs) that bound ER α .

Studies in this laboratory have been investigating the mechanisms of ER α -mediated expression of

TABLE 1. The synthetic oligonucleotides used in this study. GC-rich sequences and ERE half-sites are underlined and mutations are noted with an asterisk

	Sequence (sense)
Oligonucleotide	
– 625/– 549	5'CCCCGCCCCAGAGATGCCCCAGGAGCGGCCCTCGGTGTAGGTAACGGGTGCCC CGGGCGGCTCCCGTCCGCCGCCTG 3'
– 625/– 549m1	5'CT*CT*GT*A*A* <u>CAGAGATGCCCCAGGAGCGGCCCTCGGTGTAGGTAACGGGTGCCCC</u> GGGCGGCTCCCGTCCGCCGCCTG 3'
– 625/– 549m2	5'CCCCGCCCCAGAGATGCCCCAGGAGCGGCCCTCGGTGTAGGTAACGGGTGCCCC T*T*A*CA*GCTCCCGTCCGCCGCCTG 3'
– 625/– 549mERE	5'CCCCGCCCCAGAGATGCCCCAGGAGCGGCCCTCGGTGTAA*A*TAACGGGTGCCCC GGGCGGCTCCCGTCCGCCGCCTG 3'
– 625/– 561	5'CCCCGCCCCAGAGATGCCCCAGGAGCGGCCCTCGGTGTAGGTAACGGGTGCCCC GGGCGGCTCCG 3'
– 625/– 561m2	5'CCCCGCCCCAGAGATGCCCCAGGAGCGGCCCTCGGTGTAGGTAACGGGTGCCCC T*T*A*CA*GTCCG 3'
– 625/– 580	5'CCCCGCCCCAGAGATGCCCCAGGAGCGGCCCTCGGTGTAGGTAACG 3'
Consensus ERE	5'GTCAAAGT <u>CAGGTCACAGTGACCT</u> GATCAAAGTT 3'
Consensus Sp1	5'CGATCGGGGCGGAGCTTATTGGCGAGC 3'
TATA	5'CATCCGTCGACGCTGTAGGGTATATAATGGATCCGGATC 3'
Xenobiotic response element (XRE)	5'GATCTCCGGTCCTTCTCACGCAACGCCTGGGG 3'
Sp1 ₃	5'AGCTTATTTCGATCGGGGCGGGGCGAGCATTCGATCGGGGCGGGGCGAGCA TTCGATCGGGGCGGGGCGAGCG 3'

several genes and their interactions with other regulatory pathways and receptors including the aryl hydrocarbon receptor (AhR) (Krishnan *et al.* 1995, Safe 1995, Gillesby *et al.* 1997, Zacharewski & Safe 1998). Based on results of preliminary studies showing inhibitory AhR-ER crosstalk with TGFα, we have further analyzed the 5'-flanking region of the TGFα gene promoter to delineate trans-acting factors and genomic elements required for E2 responsiveness. Deletion analysis of the upstream region (–1145 to –377) of the TGFα gene promoter identified an E2-responsive sequence (–625 to –549) containing three GC-rich sequences and a GGTA A imperfect ERE half-site (ERE½). Further analysis showed that E2-induced transactivation was associated with an ERα/Sp1 complex interaction with an Sp1(N)₃₀ERE½ motif in which both the Sp1 and ERE½ motif were required. Thus, the TGFα gene promoter contains at least two functional enhancer sequences at –625 to –549 and –252 to –200 that are required for E2 responsiveness in MCF-7 cells.

MATERIALS AND METHODS

Chemicals and biochemicals

Recombinant human Sp1 protein was purchased from Promega Corp. (Madison, WI, USA). Pure recombinant ER human protein was purchased from Pan Vera (Madison, WI, USA). The TGFα

oligonucleotides were synthesized by the Gene Technologies Laboratory or the Institute for Developmental and Molecular Biology, Texas A&M University (College Station, TX, USA). Human ERα antibody (C-314) and human Sp1 antibody (1C6) were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). All other chemicals and biochemicals were the highest quality available from commercial sources. Wild-type human (h) ERα and mutant hE11 expression plasmid were kindly provided by Dr Pierre Chambon (Illkirch, France). The synthetic oligonucleotides used in this study are shown in Table 1. The GC-rich sequences and ERE½ are underlined and mutations are noted with an asterisk.

Plasmids

The pTGFα–1145/–5 was kindly provided by Dr David S Salomon (National Cancer Institute, Bethesda, MD, USA). The pXP1 luciferase reporter vector was obtained from the American Type Culture Collection (Rockville, MD, USA). The TATA-luc construct was made in this laboratory by inserting an E1B TATA oligonucleotide into the pXP1 vector. The pTGFα–1145/–377, pTGFα–1145/–919, and pTGFα–919/–377 plasmids were made by ligation of the appropriate restriction fragments from pTGFα–1145/–5 into TATA-luc. Constructs containing synthetic oligonucleotides were made by annealing, phosphorylating, and cloning into TATA-luc.

Cell maintenance

MCF-7 and MDA-MB-231 cells were obtained from the American Type Culture Collection. MCF-7 cells were grown in DME-F12 (Sigma Chemical Co., St Louis, MO, USA) supplemented with 2.2 g/l sodium bicarbonate, 10% fetal calf serum, and 10 ml/l antibiotic/antimycotic solution (Sigma). MDA-MB-231 cells were maintained in DME-F12 supplemented with 2.2 g/l sodium bicarbonate, 5% fetal calf serum, and 10 ml/l antibiotic/antimycotic solution. All cells were grown in 150 cm² tissue culture dishes at 37 °C in 5% CO₂:95% air. For transfection experiments, MCF-7 cells were seeded onto 12-well tissue culture plates in DME-F12 without phenol red supplemented with 2.2 g/l sodium bicarbonate, 5% charcoal-stripped fetal calf serum, and 10 ml/l antibiotic/antimycotic solution, and MDA-MB-231 cells were seeded onto 12-well tissue culture plates in DME-F12 without phenol red supplemented with 2.2 g/l sodium bicarbonate, 2.5% charcoal-stripped fetal calf serum, and 10 ml/l antibiotic/antimycotic solution.

Transient transfection assay

Twenty-four hours after seeding, MCF-7 or MDA-MB-231 cells were transfected by the calcium phosphate method with 500 ng of the TGF α -luciferase reporter constructs, 100 ng ER α expression vector and 100 ng pCDNA3/His/lacZ (Invitrogen, Carlsbad, CA, USA) that was used as a standard reference control plasmid. After 20 h, media were removed and fresh DME-F12 without phenol red supplemented with 2.5% charcoal-stripped fetal calf serum containing DMSO or 10 nM E2 in DMSO were added to the cells for 42 h. Cells were washed twice with PBS and harvested in 1 × Reporter lysis buffer (Promega). Luciferase assays were performed on 20 µl cell extract using the Luciferase assay system (Promega). Light emission was detected on a Lumicount luminometer (Packard, Meriden, CT, USA). β -Galactosidase assays were performed on 20 µl cell extract using the luminescent Galacton-Plus assay kit (Tropix, Bedford, MA, USA). The experiments were carried out at least in triplicate and significance ($P < 0.05$) was determined by Anova and Student's *t*-test.

Gel mobility shift assays

Oligonucleotides were annealed and end-labeled using T4-polynucleotide kinase and [γ -³²P]ATP. Nuclear extracts from MCF-7 cells were prepared as described previously (Krishnan *et al.* 1995). Gel

shift reactions with nuclear proteins were carried out in 25 mM HEPES, 1.5 mM EDTA, 10% glycerol, 1.0 mM dithiothreitol (DTT), and 150 mM KCl in a final volume of 25 µl. Eight micrograms nuclear extracts from cells treated with DMSO or 10 nM E2 for 2 h were incubated with 1 µg polydeoxyinosinic-deoxycytidylic acid for 15 min at 25 °C to bind nonspecific DNA binding proteins. After addition of ³²P-labeled DNA, the mixture was incubated for 15 min at 25 °C. For competition assays, excess specific or nonspecific competitor oligo DNA was added (100- to 1000-fold) 5 min before the addition of ³²P-labeled DNA. Reaction mixtures were loaded onto a 5% polyacrylamide gel and run at 110 V in 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. For the supershift assays, ER antibody (C-134), Sp1 antibody (IC6) or normal mouse IgG was added to gel shift reactions as indicated and incubated for 20 min at 4 °C. Extracts were then subjected to electrophoresis and detection as described above.

Schneider cell experiments

Drosophila Schneider's SL2 cells were maintained in Schneider's medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal calf serum. SL2 cells were grown in 150-cm² tissue culture flasks in the dark at 22 °C. SL2 cells were seeded onto 12-well tissue culture plates and transfected 24 h after seeding with 500 ng of the reporter construct and the indicated amounts of pPAC empty vector, pPAC/hER α (Dong *et al.* 1999) or pPAC/Sp1 (provided by R Tjian, University of California, Berkeley, CA, USA) expression plasmids. After transfection, cells were grown overnight and then dosed with 10 nM E2 without changing the medium. Cells were allowed to grow an additional 6 h and then harvested in 1 × Reporter lysis buffer (Promega). Luciferase activity of the cell extracts was detected as described for the mammalian cells.

SssI footprinting

Ten nanograms pTGF α - 1145/ - 5/luc linearized with HindIII were incubated with varying concentrations of MCF-7 nuclear extract treated with 10 nM E2 in 1 × MS buffer (5% glycerol, 17.65 mM MgCl₂, 0.18 mM S-adenosylmethionine, 5 mM DTT). Proteins were allowed to bind to the DNA for 5 min on ice followed by 20 min at 20 °C. SssI (0.5 U, New England Biolabs, Beverly, MA, USA) was added to the reaction and incubated at 30 °C for

FIGURE 1. Analysis of the TGF α gene promoter. MCF-7 cells were transiently transfected with several constructs including empty vectors (TATA-luc and pXP1) (A) and those containing TGF α gene promoter inserts from -1145 to -5 (A, B), and wild-type and mutant constructs containing sequences from -625 to -549 (C). Luciferase activities were determined as described in Materials and Methods. Cells were cotransfected with wild-type hER α , and treated with DMSO or 10 nM E2. (D) Effects of wild-type vs mutant ER α . MDA-MB-231 breast cancer cells were transfected with pTGF α - 625/-549 as described above; however, the comparative effects of wild-type (W.T.) vs variant (hE11) ER α on E2 inducibility were determined. Results are expressed as means $\pm$ s.d. for at least 3 separate experiments for each treatment group. Significant induction ($P < 0.05$) by E2 is indicated with an asterisk. RLU refers to units of relative luciferase activity.

15 min followed by heat inactivation for 15 min at 75 °C. Following methylation, deamination and PCR amplification were carried out as previously described by Kladde and coworkers (1996). PCR primers used were TGF α A1b (AAAAACAAAAACACCTAACTTCAAAC) and TGF α A2 (GGA TTATTTTTTTTTTGAGTTAAGTTTTTG).

RESULTS

Deletion analysis of the TGF α gene promoter and transactivation studies in breast cancer cells

Initial studies showed that transfection of the TATA-luc (empty vector) or pXP1 vector alone or in combination with ER α expression plasmid and 10 nM E2 did not result in induction of luciferase activity (Fig. 1A). Moreover, hormone-induced transactivation of TGF α - 1145/-5-luc was only observed after cotransfection with ER α . In MCF-7 cells transiently transfected with pTGF α - 1145/-5-luc and pTGF α - 1145/-377-luc, E2 induced reporter gene activity confirming that the -1145

to -377 region of the TGF α gene promoter was E2 responsive (Fig. 1B). Further analysis of this gene promoter demonstrated that constructs containing the upstream region (-1145 to -919) were not E2 responsive, whereas E2 induced luciferase activity in MCF-7 cells transfected with pTGF α - 919/-377 and pTGF α - 625/-549. The 77 bp sequence (-625 to -549) contains three GC-rich Sp1 binding sites and an imperfect ERE $\frac{1}{2}$ (GGTAA) and this region of the TGF α gene promoter was further analyzed using several deletion and mutant constructs (Fig. 1C). Sequential mutation of the GC-rich sites and ERE $\frac{1}{2}$ motif (TGF α - mERE-luc) demonstrated that only the upstream (5'-) GC-rich binding site and the imperfect ERE $\frac{1}{2}$ motif were required for E2 responsiveness. Transcriptional activation of pTGF α - 625/-549 by E2 was observed using wild-type ER α in MCF-7 and MDA-MB-231 cells, whereas no induction was observed using variant hE11 containing deletions in the DNA binding domain (Fig. 1D). These results suggest that DNA binding of ER α is required for E2 activation.

Gel mobility shift assays

Protein binding to the $-625/-580$ region of the *TGF α* gene promoter that contains the active Sp1(N)₃₀ERE_{1/2} motif was investigated by gel mobility shift assays using [³²P]*TGF α* $-625/-580$ and various proteins and MCF-7 nuclear extracts.

The results illustrated in Fig. 2A compare binding of recombinant Sp1 and ER α proteins to [³²P]*TGF α* $-625/-580$ and their cognate sequences. Recombinant human ER α binds [³²P]ERE to form an intense retarded band (lanes 2 and 3) and human recombinant Sp1 protein also binds a consensus [³²P]Sp1 oligonucleotide to give an intense retarded band (lanes 5 and 6). Using the same concentrations of ER α (lanes 8 and 9) or Sp1 (lanes 10 through 12) proteins, weak binding to [³²P]*TGF α* $-625/-580$ was observed and binding of ER α and Sp1 proteins (combined) (lanes 13 and 14) gave the same weak retarded bands. In contrast, [³²P]*TGF α* $-625/-580$ binds nuclear extracts from MCF-7 cells (Fig. 2B) to form an intense broad retarded band that is significantly decreased in intensity after coincubation with 200- to 1000-fold excess unlabeled Sp1 (lanes 3 through 5), 100- to 400-fold excess ERE (lanes 6 through 8) or 100-fold excess *TGF α* $-625/-580$ (lane 9) oligonucleotides. In contrast, competition with a non-specific xenobiotic response element (XRE) oligonucleotide (lane 10) did not decrease the intensity of the retarded band. Interaction of ER and Sp1 proteins with [³²P]*TGF α* $-625/-580$ was further investigated using nuclear extracts from MCF-7 cells treated with 10 nM E2 (Fig. 3, lane 1), followed by incubation with antibodies to Sp1 (lanes 2 and 3) and ER (lane 4) proteins and nonspecific IgG (lane 5). Incubation with 5 μ g Sp1 antibody gave a supershifted band (SS-band) (lane 3) and a second supershifted band with slightly higher mobility was observed after coincubation with ER antibody (lane 4). In contrast, coincubation with non-specific IgG did not result in formation of a supershifted complex.

FIGURE 2. Gel mobility shift assays. (A) Comparative binding of recombinant ER α and Sp1 proteins to ³²P-labeled ERE, Sp1, and *TGF α* $-625/-580$ oligonucleotides. Radiolabeled oligonucleotides were incubated with different amounts of recombinant human (rh) ER α (lanes 2, 3, 8, 9) or Sp1 (5, 6, 10–12) proteins or their combination (lanes 13 and 14) and analyzed by gel mobility shift assay as described in Materials and Methods. ER α and Sp1 proteins bound weakly to *TGF α* $-625/-580$ and only individual bands for each complex were observed. (B) Binding of nuclear extracts to [³²P]*TGF α* $-625/-580$. Nuclear extracts from MCF-7 cells were incubated with [³²P]*TGF α* $-625/-580$ and unlabeled oligonucleotides and analyzed by gel mobility shift assays as described in Materials and Methods. Unlabeled Sp1 (lanes 3–5), ERE (lanes 6–8) and *TGF α* $-626/-580$ (lane 9) competitively decreased retarded band intensity whereas the nonspecific XRE oligonucleotide (lane 10) did not affect the broad protein–DNA complex (Bound DNA).

FIGURE 3. ERα and Sp1 antibody supershifts. [³²P]TGFα – 625/– 580 was incubated with nuclear extracts from MCF-7 cells, and the effects of Sp1 or ERα antibodies or nonspecific IgG on the broad retarded band (Bound DNA) were determined as described in Materials and Methods. Supershifted bands (SS-bands) with different mobilities were observed after incubation with Sp1 (lane 3) or ERα (lane 4) antibodies (Ab) but not with IgG (lane 5). Immunodepletion experiments with ER and Sp1 antibodies also decreased retarded band intensity (data not shown).

In vitro SssI footprinting

Figure 4 summarizes *in vitro* SssI DNA methylation footprinting of the – 625 to – 580 region of the TGFα gene promoter (lane 1) and incubation with nuclear extracts from MCF-7 cells significantly enhanced binding (decreased methylation) at the ERE½ (lanes 2 and 3). Only minimal footprinting was observed at the GC-rich site;

FIGURE 4. *In vitro* footprinting of the proximal region of the TGFα gene promoter by SssI DNA methyltransferase-mediated methylation (Kladde *et al.* 1996). A restriction fragment of the TGFα gene promoter was incubated alone or in combination with nuclear extracts from MCF-7 cells, and DNA-methylation patterns in the – 625 to – 580 region were determined as described in Materials and Methods. Incubation with nuclear extracts markedly inhibited DNA methylation in the region of the ERE½ but only slight decreases in DNA methylation were observed at the GC-rich sequence (lanes 1–3, left to right).

however, this decreased methylation was observed in replicate experiments.

Interactions of Sp1 and ERα with pTGFα – 625/– 549, pTGFα – 625/– 549-mERE in Schneider SL2 cells

Schneider SL2 cells do not express Sp1 or ERα proteins, and functional interactions of both transcription factors were investigated using constructs containing the – 625/– 549 region of the TGFα gene promoter. Cotransfection of TATA-luc (empty vector) with Sp1 expression plasmid did not enhance luciferase activity, whereas a dose-dependent response was observed using pTGFα – 625/– 549 (Fig. 5A). In SL2 cells transfected with pTGFα – 625/– 549, ERα expression plasmid

FIGURE 5. Transfection of *Drosophila* Schneider SL2 cells. (A) SL2 cells were cotransfected with pTATA-luc or pTGF α -625/-549, and the indicated amounts of Sp1 expression plasmid. The effects of ER α on (B) pTGF α -625/-549, (C) pTGF α -625/-549-mERE, or (D) p(Sp1)₃-luc are shown. SL2 cells were transfected with 500 ng reporter and the indicated amounts of ER α and/or Sp1 expression plasmids, and luciferase activities were determined as described in Materials and Methods. Results are expressed as means $\pm$ s.d. for three replicate determinations. Significant differences ($P < 0.05$) in activity with respect to the empty vector pPAC are indicated with an asterisk. RLU refers to units of relative luciferase activity.

(10 or 100 ng) plus pPAC (empty vector) increased luciferase activity and, in cotransfection studies (ER α plus Sp1 expression plasmids), the response was essentially additive (Fig. 5B). The role of the ERE $\frac{1}{2}$ site was investigated using pTGF α -625/-549-mERE (Fig. 5C); the results showed that ER α expression plasmid alone did not increase luciferase activity, whereas expression of 50 ng Sp1 expression plasmid increased luciferase activity comparable to that observed using the wild-type construct (Fig. 5B). Cotransfection of Sp1 and ER α expression plasmids (using pTGF α -625/-549-mERE) gave results that were not significantly different from those observed with Sp1 alone. Previous studies using a construct containing two GC-rich sites from the bcl-2 gene promoter showed that cotransfection with Sp1 and ER α expression plasmids gave enhanced (more than additive) transactivation (Dong *et al.* 1999), and the results in Fig. 5D using a construct containing three GC-rich sites also gave enhanced activity using ER α and Sp1 expression plasmids. Thus, results obtained using pTGF α -625/-549 are consistent with both ER α and Sp1 action in this region of the TGF α gene promoter in which ER α -ERE $\frac{1}{2}$ binding is required.

DISCUSSION

Growth of breast and endometrial tumors is dependent on multiple mitogenic factors including estrogenic hormones and polypeptide growth factors such as TGF α . In many hormone-independent tumors, growth factors play an important autocrine and/or paracrine role in stimulating cancer cell proliferation (Sporn & Roberts 1985, Keski-Oja *et al.* 1988, Aaronson 1991, Cross & Dexter 1991, Dickson & Lippman 1995, Ethier 1995). Several studies have demonstrated that in E2-responsive breast and endometrial tumors, TGF α gene expression and protein secretion are induced by E2 and TGF α stimulates breast cancer cell growth via an endocrine-related autocrine loop (Bates *et al.* 1988, Clarke *et al.* 1989, Manni *et al.* 1990, Gong *et al.* 1992). Reddy and coworkers (1994) confirmed a linkage between E2/TGF α and growth of T47D breast cancer cells using cells stably transfected with metallothionein promoter-dependent antisense TGF α . E2 induced DNA synthesis and growth of stably transfected T47D cells; however, cotreatment with cadmium increased expression of antisense TGF α mRNA and decreased hormone-induced mitogenic activity. The data confirmed results of an

earlier study showing that antisense TGF α mRNA oligonucleotide inhibited comparable E2-induced responses in breast cancer cells (Kenney *et al.* 1993).

Analysis of the TGF α gene promoter has shown that an 1140 bp sequence (–1145 to –5) conferred E2 responsiveness in transient transfection assays utilizing the –1145 to –5 promoter insert linked to a luciferase reporter gene (Fig. 1B) (Sacki *et al.* 1991). E2 responsiveness was observed only after cotransfection of ER α since levels of endogenous ER α were not sufficient in cells that highly overexpress the promoter–reporter constructs. This has been observed in other studies with plasmids containing promoter inserts from other E2-responsive genes, including c-fos, progesterone receptor, cathepsin D, pS2, heat shock protein 27, E2F1, insulin-like growth factor binding protein-4, adenosine deaminase, and retinoic acid receptor α (Cavailles *et al.* 1989, 1993, Weisz & Rosales 1990, Savouret *et al.* 1991, Zacharewski *et al.* 1994, Duan *et al.* 1998, Sun *et al.* 1998, Qin *et al.* 1999, Wang *et al.* 1999, Xie *et al.* 1999). Previous studies showed that E2-responsiveness of this promoter in MCF-7 cells was due, in part, to a 53 bp sequence (–252 to –200) containing two imperfect palindromic EREs (El-Ashry *et al.* 1996). However, further analysis (Fig. 1) showed that upstream promoter sequences (–1145 to –377) also contribute to E2 responsiveness of TGF α . This region of the TGF α gene promoter contains multiple AP-1 and Sp1 protein binding sites and ERE half-sites, and E2 responsiveness was associated with a 77 bp region (–625 to –549) containing three GC-rich Sp1 binding sites and an ERE half-site (Fig. 1C).

E2-dependent transactivation via ER α /Sp1 interactions have been characterized in promoters of several E2-responsive genes, including creatine kinase B, c-myc, cathepsin D, heat shock protein 27, c-fos, retinoic acid receptor α 1, E2F1, adenosine deaminase, insulin-like growth factor binding protein 4 and bcl-2 (Dubik & Shiu 1992, Krishnan *et al.* 1994, Rishi *et al.* 1995, Porter *et al.* 1996, 1997, 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). Both ER α and Sp1 proteins physically interact (Porter *et al.* 1997); however, transactivation is mediated by at least two pathways, namely (i) interaction of ER α /Sp1 proteins with GC-rich genomic sites in which only Sp1 protein binds DNA and (ii) interaction of ER α /Sp1 with Sp1(N)₃₀ERE half-site motifs in which both proteins bind DNA. Mutational analysis of the three GC-rich sites within pTGF α –625/–549 (Fig. 1C) showed that the upstream GC-rich site was required for E2 responsiveness since no induction was observed after mutation of this site (pTGF α –625/–549-

m1). Mutation of the downstream (5'-) GC-rich sites within this region did not affect E2 responsiveness. Previous studies showed that ER/Sp1 action through binding to GC-rich sites can be observed in MCF-7 or ER-negative MDA-MB-231 cells cotransfected with wild-type ER or mutant hE11 that does not contain the DNA binding domain (Duan *et al.* 1998, Sun *et al.* 1998, Dong *et al.* 1999, Qin *et al.* 1999, Wang *et al.* 1999, Xie *et al.* 1999). However, E2 induced luciferase activity in MDA-MB-231 cells transfected with pTGF α –625/–549 plus wild-type hER expression plasmid but not variant hE11 expression plasmid (Fig. 1D), suggesting that Sp1 binding alone was not sufficient for ER α action. Mutation of the ERE half-site (pTGF α –625/–459-mERE) also resulted in loss of transcriptional activation by E2 (Fig. 1C). This result suggests that the Sp1(N)₃₀ERE half-site motif was a functional E2 enhancer sequence and that ER α may be interacting with ERE $\frac{1}{2}$ as a monomer. It is possible that more than one GC-rich site within the –625 to –549 region of the promoter may contribute to ER α /Sp1 action.

Interactions of ER α /Sp1 with Sp1(N)_xERE half sites have previously been analyzed by gel mobility shift assays to give a broad retarded band that binds both proteins but does not exhibit the expected supershifted band (Krishnan *et al.* 1994, Rishi *et al.* 1995, Porter *et al.* 1996, Dong *et al.* 1999). Similar results were observed with [³²P]TGF α –625/–580 oligonucleotide (Figs 2 and 3), and since the retarded band was observed only with nuclear extracts and not with recombinant ER α plus Sp1 proteins, it is likely that other endogenous nuclear factors are required for ER α /Sp1–DNA complex formation. *In vitro* SssI methylation footprinting, a highly sensitive assay for detecting protein–DNA interactions (Kladde *et al.* 1996, Dong *et al.* 1999), was also used to investigate protein interactions with the Sp1(N)₃₀ERE $\frac{1}{2}$ motif in the TGF α gene promoter (Fig. 4). Three major methylated bands were observed in the –625 to –580 region and, in the presence of nuclear extracts from MCF-7 cells (treated with E2), methylation at the ERE $\frac{1}{2}$ was decreased and a small but consistent decrease was also observed at the GC-rich site. Thus, *in vitro* footprinting in the –625 to –580 region of the TGF α gene promoter was consistent with the gel mobility shift results and was similar to results of *in vivo* footprinting of the –304 to –207 region of the uteroglobin gene promoter which also contains ERE and GC-rich motifs required for E2 action (Scholz *et al.* 1998).

Interactions of ER α and Sp1 proteins with a GC-rich region of the bcl-2 gene promoter (two Sp1 binding sites) showed that cotransfection of ER α

FIGURE 6. Model for transcriptional activation of *TGF α* by E2 in breast cancer cells (El-Ashry *et al.* 1996 and this study). Upstream factors interact with downstream general transcription initiation factors (GTFs), TATA binding protein (TBP) associated with RNA polymerase II (pol II).

plus Sp1 expression plasmids resulted in enhanced transactivation in Schneider SL2 cells (Dong *et al.* 1999). Similar results were observed in this study (Fig. 5D) using a plasmid containing a promoter insert with three consensus GC-rich sites. A recent report also showed that transcriptional activation of the vitellogenin A1 *io* gene promoter by E2 in *Drosophila*-derived SL2 cells involved enhanced or synergistic interactions between ER α and Sp1 bound to ERE and GC-rich sites that are separated by >1000 bp (Batistuzzo de Medeiros *et al.* 1997). In contrast, our results with pTGF α - 625/-549 and ERE $\frac{1}{2}$ mutant constructs showed that both GC-rich and ERE $\frac{1}{2}$ sites were required for ER α /Sp1 action and their interactions were essentially additive (Fig. 5). These results were similar to deletion/mutant analysis of the *TGF α* gene promoter in breast cancer cells (Fig. 1) but do not exclude a role for unknown nuclear factors in mammalian cells required for enhanced ER α /Sp1 interactions at Sp1(N)_xERE $\frac{1}{2}$ motifs that are not expressed in SL2 cells.

Thus, hormone-inducibility via ER α /Sp1 action is highly complex and involves GC-rich ERE $\frac{1}{2}$ and ERE motifs in multiple configurations that are promoter dependent (Fig. 6). Moreover, ER α /Sp1 functionality may also depend on the cell context, and ongoing studies in this laboratory are investigating promoter- and cell-specific ER α /Sp1 activation of several gene promoter constructs, including *TGF α* .

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REFERENCES

- Aaronson SA 1991 Growth factors and cancer. *Science* **254** 1146–1153.
- Bates SE, Davidson NE, Valverius EM, Freter CE, Dickson RB, Tam JP, Kudlow JE, Lippman ME & Salomon DS 1988 Expression of transforming growth factor- α and its messenger ribonucleic acid in human breast cancer: its regulation by estrogen and its possible functional significance. *Molecular Endocrinology* **2** 543–555.
- Batistuzzo de Medeiros SR, Krey G, Hihi AK & Wahli W 1997 Functional interactions between the estrogen receptor and the transcription activator Sp1 regulate the estrogen-dependent transcriptional activity of the vitellogenin A1 *io* promoter. *Journal of Biological Chemistry* **272** 18250–18260.
- Cavaillès V, Garcia M & Rochefort H 1989 Regulation of cathepsin D and pS2 gene expression by growth factors in MCF-7 human breast cancer cells. *Molecular Endocrinology* **3** 552–558.
- Cavaillès V, Augereau P & Rochefort H 1993 Cathepsin D gene is controlled by a mixed promoter, and estrogens stimulate only TATA-dependent transcription. *Proceedings of the National Academy of Sciences of the USA* **90** 203–207.
- Clarke R, Brunner N, Katz D, Glanz P, Dickson RB, Lippman ME & Kern FG 1989 The effects of a constitutive expression of transforming growth factor- α on the growth of MCF-7 human breast cancer cells *in vitro* and *in vivo*. *Molecular Endocrinology* **3** 372–380.
- Cross M & Dexter TM 1991 Growth factors in development, transformation, and tumorigenesis. *Cell* **64** 271–280.
- Derynck R 1988 Transforming growth factor α . *Cell* **54** 593–595.
- Dickson RB & Lippman ME 1995 Growth factors in breast cancer. *Endocrine Reviews* **16** 559–589.
- Dong L, Wang W, Wang F, Stoner M, Reed JC, Harigai M, Klade M, Vyhlidal C & Safe S 1999 Mechanisms of transcriptional activation of *bcl-2* gene expression by

- 17 β -estradiol in breast cancer cells. *Journal of Biological Chemistry* **174** 32099–32107.
- Duan R, Porter W & Safe S 1998 Estrogen-induced c-fos protooncogene expression in MCF-7 human breast cancer cells: role of estrogen receptor Sp1 complex formation. *Endocrinology* **139** 1981–1990.
- Dubik D & Shiu RPC 1992 Mechanism of estrogen activation of c-myc oncogene expression. *Oncogene* **7** 1587–1594.
- El-Ashry D, Chrysogelos SA, Lippman ME & Kern FG 1996 Estrogen induction of TGF- α is mediated by an estrogen response element composed of two imperfect palindromes. *Journal of Steroid Biochemistry and Molecular Biology* **59** 261–269.
- Ethier SP 1995 Growth factor synthesis and human breast cancer progression. *Journal of the National Cancer Institute* **87** 964–973.
- Gillesby B, Santostefano M, Porter W, Wu ZF, Safe S & Zacharewski T 1997 Identification of a motif within the 5'-regulatory region on pS2 which is responsible for Ap1 binding and TCDD-mediated suppression. *Biochemistry* **36** 6080–6089.
- Gong Y, Ballejo G, Murphy LC & Murphy LJ 1992 Differential effects of estrogen and antiestrogen on transforming growth factor gene expression in endometrial adenocarcinoma cells. *Cancer Research* **52** 1704–1709.
- Kenney NJ, Saeki T, Gottardis M, Kim N, Garcia-Morales P, Martin MB, Normanno N, Ciardiello F, Day A & Cutler ML 1993 Expression of transforming growth factor α antisense mRNA inhibits the estrogen-induced production of TGF α and estrogen-induced proliferation of estrogen-responsive human breast cancer cells. *Journal of Cell Physiology* **156** 497–514.
- Keski-Oja J, Postlethwaite AE & Moses HL 1988 Transforming growth factors in the regulation of malignant cell growth and invasion. *Cancer Investigation* **6** 705–724.
- Kladde MP, Xu M & Simpson RT 1996 Direct study of DNA-protein interactions in repressed and active chromatin in living cells. *EMBO Journal* **15** 6290–6300.
- Krishnan V, Wang X & Safe S 1994 Estrogen receptor–Sp1 complexes mediate estrogen-induced cathepsin D gene expression in MCF-7 human breast cancer cells. *Journal of Biological Chemistry* **269** 15912–15917.
- Krishnan V, Porter W, Santostefano M, Wang X & Safe S 1995 Molecular mechanism of inhibition of estrogen-induced cathepsin D gene expression by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in MCF-7 cells. *Molecular Cellular Biology* **15** 6710–6719.
- Manni A, Wright C, Badger B, Bartholomew M, Herlyn M, Mendelsohn J, Masui H & Demers L 1990 Role of transforming growth factor- α -related peptides in the autocrine/paracrine control of experimental breast cancer growth *in vitro* by estradiol, prolactin, and progesterone. *Breast Cancer Research and Treatment* **15** 73–83.
- Porter W, Wang F, Wang W, Duan R & Safe S 1996 Role of estrogen receptor/Sp1 complexes in estrogen-induced heat shock protein 27 gene expression. *Molecular Endocrinology* **10** 1371–1378.
- Porter W, Saville B, Hoivik D & Safe S 1997 Functional synergy between the transcription factor Sp1 and the estrogen receptor. *Molecular Endocrinology* **11** 1569–1580.
- Qin C, Singh P & Safe S 1999 Transcriptional activation of insulin-like growth factor binding protein 4 by 17 β -estradiol in MCF-7 cells: role of estrogen receptor–Sp1 complexes. *Endocrinology* **140** 2501–2508.
- Reddy KB, Yee D, Hilsenbeck SG, Coffey RJ & Osborne CK 1994 Inhibition of estrogen-induced breast cancer cell proliferation by reduction in autocrine transforming growth factor α expression. *Cell Growth and Differentiation* **5** 1275–1282.
- Rishi AK, Shao ZM, Baumann RG, Li XS, Sheikh MS, Kimura S, Bashirelahi N & Fontana JA 1995 Estradiol regulation of the human retinoic acid receptor α gene in human breast carcinoma cells is mediated via an imperfect half-palindromic estrogen response element and Sp1 motifs. *Cancer Research* **55** 4999–5006.
- Saeki T, Cristiano A, Lynch MJ, Brattain M, Kim N, Normanno N, Kenney N, Ciardiello F & Salomon DS 1991 Regulation by estrogen through the 5'-flanking region of the transforming growth factor α gene. *Molecular Endocrinology* **5** 1955–1963.
- Safe S 1995 Modulation of gene expression and endocrine response pathways by 2,3,7,8-tetrachlorodibenzo-p-dioxin and related compounds. *Pharmacology and Therapeutics* **67** 247–281.
- Savouret JF, Bailly A, Misrahi M, Rarch C, Redeuilh G, Chauchereau A & Milgrom E 1991 Characterization of the hormone responsive element involved in the regulation of the progesterone receptor gene. *EMBO Journal* **10** 1875–1883.
- Scholz A, Truss M & Beato M 1998 Hormone-induced recruitment of Sp1 mediates estrogen activation of the rabbit uteroglobin gene in endometrial epithelium. *Journal of Biological Chemistry* **273** 4360–4366.
- Sporn MB & Roberts AB 1985 Autocrine growth factors and cancer. *Nature* **313** 745–747.
- Sun G, Porter W & Safe S 1998 Estrogen-induced retinoic acid receptor α 1 gene expression: role of estrogen receptor–Sp1 complex. *Molecular Endocrinology* **12** 882–890.
- Wang F, Hoivik D, Pollenz R & Safe S 1998 Functional and physical interactions between the estrogen receptor–Sp1 and the nuclear aryl hydrocarbon receptor complexes. *Nucleic Acids Research* **26** 3044–3052.
- Wang W, Dong L, Saville B & Safe S 1999 Transcriptional activation of E2F1 gene expression by 17 β -estradiol in MCF-7 cells is regulated by NF-Y-Sp1/estrogen receptor interactions. *Molecular Endocrinology* **13** 1373–1387.
- Weisz A & Rosales R 1990 Identification of an estrogen response element upstream of the human c-fos gene that binds the estrogen receptor and the AP-1 transcription factor. *Nucleic Acids Research* **18** 5097–5106.
- Xie W, Duan R & Safe S 1999 Estrogen induces adenosine deaminase gene expression in MCF-7 human breast cancer cells: role of estrogen receptor–Sp1 interactions. *Endocrinology* **140** 219–227.
- Zacharewski T & Safe S 1998 Antiestrogenic activity of TCDD and related compounds. In *Reproductive and Developmental Toxicology*, pp 431–448. Ed. KS Korach. New York: Marcel Dekker, Inc.
- Zacharewski TR, Bondy KL, McDonnell P & Wu ZF 1994 Antiestrogenic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on 17 β -estradiol-induced pS2 expression. *Cancer Research* **54** 2707–2713.

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