

Estrogen response element and the promoter context of the human and mouse lactoferrin genes influence estrogen receptor α -mediated transactivation activity in mammary gland cells

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Abstract

A critical step in estrogen action is the recognition of estrogen responsive elements (EREs) by liganded estrogen receptor. Our current studies were designed to determine whether an extended estrogen response element half-site (ERRE) contributes to the differential estrogen responses of the human and mouse lactoferrin overlapping chicken ovalbumin upstream promoter/ERE sequences (estrogen response modules, ERMs) in the context of their natural promoters. Transient transfections of MCF-7 cells show that liganded estrogen receptor α (ER α) activates transcription of the human lactoferrin ERM fourfold higher than the mouse lactoferrin ERM in the context of their natural promoters. Since the ERRE of the human lactoferrin gene naturally occurs 18 bp upstream from the ERM and is absent in the mouse lactoferrin gene promoter, we created a chimeric mouse lactoferrin CAT reporter, which now encodes the ERRE in the identical location as in the human lactoferrin gene. The addition of the ERRE in the mouse lactoferrin gene rendered this reporter extremely responsive to estrogen stimulation. Using limited protease digestions and electrophoretic mobility shift assays, we showed that the binding and protease sensitivity of ER α bound to the mouse ERM with or without the ERRE, differed. Importantly, occupancy of additional nuclear receptors at the ERRE may contribute to ER α binding and activation. Furthermore, the presence of ERRE influences the selectivity of coactivators in liganded ER α -mediated transcriptional activity. When the receptor is bound to human and mouse plus genes, which contain the ERRE, steroid receptor coactivator (SRC)-2 was preferred, while SRC-1 and SRC-3 coactivators selectively enhanced the mouse lactoferrin gene activity. Moreover, peroxisome proliferator activated receptor- γ coactivator-1 (PGC-1 α) and PGC-1-related estrogen receptor coactivator (PERC) robustly increase the transcriptional function of ER α in the presence of the ERRE. In conclusion, these data show that the context of the lactoferrin gene influences the ER α -mediated transcriptional activity.

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Introduction

Estrogens regulate a number of physiological processes in both females and males in target tissues including the reproductive system, mammary gland, cardiovascular system, central nervous system, and skeletal system (Lubahn *et al.* 1993, Krege *et al.* 1998, reviewed in Couse & Korach 1999). The biological actions of estrogens are mediated by the ligand-inducible receptors, estrogen

receptors (ER α and ER β) (Walter *et al.* 1985, Green *et al.* 1986, Kuiper *et al.* 1996, Mosselman *et al.* 1996, Tremblay *et al.* 1997) that are members of the steroid hormone receptor superfamily of nuclear receptors. Binding of the natural estrogen 17 β -estradiol to the estrogen receptors induces conformational changes, resulting in the departure of the receptor from an inhibitory complex with heat shock protein, formation of receptor homodimer, and binding of the receptor homodimer to

estrogen response elements (ERE) in target gene promoters (reviewed in McDonnell & Norris 2002).

The consensus ERE was determined by aligning the promoter regions of the *Xenopus laevis* vitellogenin genes A1, A2, B1, B2 and the chicken apo-VLVLII gene (Walker *et al.* 1984) yielding a minimal 13 bp palindromic sequence 5'GGTCAnnnTGAC C3' (n, any nucleotide) (Klein-Hitpass *et al.* 1988). To date, approximately twenty estrogen responsive genes have been identified and their estrogen responses in transiently transfected cells characterized (reviewed in Klinge 2001). Of these genes, only one, the vitellogenin A2 gene, encodes the consensus palindromic ERE. All other known natural estrogen response elements are imperfect palindromes that differ from the consensus by at least 1 base pair (bp) change, and confer different levels of ER transcriptional activation compared with the vitellogenin ERE (reviewed in Klinge 2001).

Among the genes encoding imperfect EREs in their promoter regions are the human and mouse lactoferrin genes (Liu & Teng 1991, Teng *et al.* 1992). Clinical studies showed that estrogen induces endogenous lactoferrin gene expression in the endometrium of normal cyclic women (Teng *et al.* 1992, 2002b) and in the uterus of immature mice (Pentecost & Teng 1987, Teng *et al.* 1989, 2002a). Molecular studies of the mouse lactoferrin gene showed that hormones and mitogens regulated lactoferrin gene expression in the uterus. Studies of the overlapping chicken ovalbumin upstream promoter (COUP)/ERE (estrogen response module, ERM) binding element in the mouse lactoferrin gene promoter demonstrated that the conserved arrangement of overlapping positive and negative regulatory elements allowed repression of the lactoferrin ERE-mediated estrogen response by COUP-transcription factor (TF) competing with ER α for DNA binding (Liu *et al.* 1993). The mitogen response unit, composed of adjacent cAMP response element (CRE) and epidermal growth factor (EGF) response elements, mediated transcriptional activation in response to EGF, forskolin and 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) in human endometrial cells transfected with the mouse lactoferrin reporter gene (Shi & Teng 1996). In addition to its regulated expression in the uterus, lactoferrin is also highly expressed in neutrophils and milk secreted from mammary epithelial cells (reviewed in Teng 2002). Analysis of milk protein concentrations from several species showed that lactoferrin

expression in human milk is higher than that in mouse milk (Masson & Heremans 1971). Moreover, the concentrations of lactoferrin in the colostrum of these two species are higher than lactoferrin levels in their respective milks, corresponding to an almost tenfold higher level of total circulating estrogens just prior to parturition, when colostrum is secreted, compared with established lactation that produces milk (Nagasawa *et al.* 1972, Lönnerdal *et al.* 1976).

Classical estrogen action is a result of the activities of the receptor, the element and the cell context, which collectively influence transcription (Katzenellenbogen *et al.* 1996). In most natural promoter environments, the ERE is usually located near other regulatory elements that may cooperate during estrogen signaling. This is true of the pS2 gene in which mutation of the AP1 site located 52 bp downstream from the ERE decreases the estrogen response of the gene (Barkhem *et al.* 2002). Multiple copies of EREs also influence the estrogen response of a target gene. Electrophoretic mobility shift assay (EMSA) studies of ER α binding to three or four tandem repeats of the ERE suggested that receptor binding is stabilized by dimers at adjacent sites and this cooperative binding promotes transcriptional synergy (Tyulmenkov *et al.* 2000).

Central to the ability of the ERs to discriminate between an ERE and glucocorticoid receptor (GR), progesterone receptor (PR) and androgen receptor (AR) response elements is a group of six amino acids within the first zinc finger of the DNA binding domain termed the P-box (Schwabe *et al.* 1993, reviewed in Pettersson & Gustafsson 2001). Alignment and comparison of the human and mouse lactoferrin gene promoter sequences have revealed comparable positioning of the ERE and the presence of an extended estrogen response half-site (ERRE, 5'-TCAAGGTCATCT-3') just upstream of the ERE in the human lactoferrin gene, but not in the mouse (Yang & Teng 1994). Since the ER P box sequence (CEGCKA) is very similar to the estrogen-related receptor (ERR α) P box sequence (CEACKA), which recognizes the extended core DNA element ERRE, ER may recognize and bind this extended half-site. Indeed, ER α has been reported to bind the ERRE of the lactoferrin and osteopontin promoters (Vanacker *et al.* 1999b, Zhang & Teng 2000). Considering that most imperfect EREs exhibit weaker ER α binding affinities (Curtis & Korach 1991, Darwish *et al.* 1991,

Table 1 Description of plasmids used in transient transfection experiments

Plasmid	Description	Reference
pCAT-Basic	Cloning vector without promoter	Promege, Madison, WI, USA
pSV40-CAT	Cloning vector with SV40 promoter	Promege, Madison, WI, USA
pCH110	β -gal expression vector	Amersham Biosciences, Piscataway, NJ, USA
0-4 mLF-CAT	Mouse lactoferrin promoter (-396/+1)	Liu & Teng (1991)
0-4 mLF plus-CAT	ERRE inserted in mouse lactoferrin promoter	Current study Materials and methods section
0-4 mLF plus mutant-CAT	Mouse lactoferrin plus ERRE mutant	Current study Materials and methods section
0-4 hLF-CAT	Human lactoferrin promoter (-414/+69)	Yang & Teng (1994)
0-4 hLF-CAT m1	Human lactoferrin ERRE mutant	Yang <i>et al.</i> (1996)
0-4 hLF-CAT m6	Human lactoferrin ERE mutant	Yang <i>et al.</i> (1996)
0-4 hLF-CAT m1/m6	Human lactoferrin ERRE and ERE mutant	Yang <i>et al.</i> (1996)
hLF ERM	Human lactoferrin COUP/ERE linked to SV40	Teng <i>et al.</i> (1992)
mLF ERM	Mouse lactoferrin COUP/ERE linked to SV40	Liu & Teng (1992)
pSG5-hER α	Human ER α expression plasmid	Migliaccio <i>et al.</i> (1991)
pCR3.1-hSRC-1	Human SRC-1 expression plasmid	Onate <i>et al.</i> (1995)
pSG5-mSRC-2	Mouse SRC-2 expression plasmid	Chen <i>et al.</i> (1999)
pSG5-hSRC-3	Mouse SRC-3 expression plasmid	Chen <i>et al.</i> (1997)
pcDNA3-hPGC-1 α	Human PGC-1 α expression plasmid	Knutti <i>et al.</i> (2000)
pcDNA3-hPERC	Human PERC expression plasmid	Kressler <i>et al.</i> (2002)

Wood *et al.* 2001, Hall & Korach 2002, reviewed in Klinge 2001), it is possible that additional upstream or downstream sequences are required to confer maximal estrogen responses in the context of their natural gene promoters. A candidate sequence in the lactoferrin gene promoter is the ERRE. In this report, we focus on the role of the estrogen responsive module in ER α -mediated transcription of the lactoferrin gene. We examined whether a 400 bp promoter region of the human and mouse lactoferrin genes containing an imperfect ERE could function to enhance estrogen responses alone and in the presence of an ERRE positioned upstream of the ERE. We established that both the imperfect ERE and ERRE cooperate to achieve potent ER-mediated transcriptional activity. Furthermore, we show that the addition of the ERRE in the natural promoter sequence influences receptor binding and the recruitment pattern of coactivators to the liganded receptor.

Materials and methods

Reagents

Diethylstilbestrol (DES), 17 β -estradiol and α -chymotrypsin were purchased from Sigma

(St Louis, MO, USA). Proteinase K was purchased from Pierce Biotechnology (Rockford, IL, USA). [¹⁴C]Chloramphenicol was purchased from NEN Life Sciences (Perkin Elmer, Boston, MA, USA) and [³²P]dCTP was purchased from Amersham Biosciences (Piscataway, NJ, USA). Antibodies to human estrogen receptor α were purchased from the following sources: H222 (ER-ICA) Abbott Laboratories, (Abbott Park, IL, USA); ER Ab-10 (clone TE111:5D11) from NeoMarkers (Fremont, CA, USA); ER α H184 from Santa Cruz Biothechnology (Santa Cruz, CA, USA).

Plasmids and oligonucleotides

Lactoferrin 5' flanking regions cloned upstream of the polylinker region in the pCAT-Basic plasmid and all other plasmids used in transient transfection assays are described in Table 1. The DNA oligonucleotides used in electrophoresis mobility shift assay (EMSA) contain 5' HindIII (top strand) and XhoI (bottom strand) sites (underlined) and were synthesized by Sigma Genosys (The Woodlands, TX, USA) and the sequences are as follows: *Xenopus laevis* vitellogenin A2 (vitA2) top strand 3'CCCG AAGCTTCCTAGGTCACAGTGAC3' and bottom strand 5'CGCTCGAGGTCACTGTGACCTAG

AAG3'; human lactoferrin (hLF) top strand 5'CCCAAGCTTGGCACCTTCAAGGTCATCTGCTGAAGAAGATAGCAGTCTCACAGGTCAAGGCGATCTT3' and bottom strand 5'CCCTCGAGTGAAGATCGCCTTGACCTGTGAGACTGCTATCTTCTTCAGCAGATGACCTTGAAGGTG3'; mouse lactoferrin (mLF) top strand 5'CCGAAGCTTAGTGTACAGGTCAAGGTAACCCACAAATCT3' and bottom strand 5'CGCTCGAGATTTGTGGGTTACCTTGACCTGTGACACTAAG3'; and mouse lactoferrin plus (mLF plus) top strand 5'CCGAAGCTTATTTGCTTCAAGGTCATCTTGCTCCATGCAGCTTAAGTGTACAGGTCAAGGTAACCCA3' and bottom strand 5'CCCCTCGAGATTTGTGGGTACCTTGACCTGTGACACTTAAGCTGCATGGAGCAAGATGACCTTGAAG3'. The double-stranded vitA2 (33 bp), hLF (46 bp), mLF (46 bp), and mLF plus (82 bp) oligonucleotides were completed by fill-in reaction.

Site-directed mutagenesis

To create the chimeric reporter 0.4 mLF plus-CAT, the 12 bp ERRE half-site sequence located 18 bp upstream of the imperfect ERE in the human lactoferrin promoter was inserted in the same position relative to the imperfect ERE in the 0.4 mLF-CAT reporter using the QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). The selected primers were synthesized by Sigma Genosys with the sequences (forward primer 5'GGAAGGGGATTTGCTTCAAGGTCATCTTGCTCCATGCAGC3' and reverse primer 5'GCTGCATGGAGCAAGATGACCTTGAAGCAAATCCCCTTCC3') spanning the region -381 to -354 of the mouse lactoferrin promoter and the underlined nucleotides represent the 12 bp half-site ERRE inserts; the PCR-based mutagenesis reaction followed the manufacturer's instructions for inserting multiple nucleotides. The resulting mutagenic DNA (50 ng) was then transformed into competent cells, plated on LB-ampicillin plates, colonies were amplified in LB-ampicillin broth medium, and then plasmid DNA was purified and sequenced. The 0.4 mLF plus-CAT mutant was constructed by mutating the underlined guanines in the 12 bp ERRE sequence TCAAGGTCATCT to adenines in the 0.4 mLF plus reporter using the following mutated primer set: forward 5'GGGGATTTGCTTCAAATCA

TCTTGCTCCATGC3' and reverse 5'GCATG GAGCAAGATGATTTTGAAGCAAATCCCC3'. The mutagenesis reaction was carried out following the manufacturer's PCR parameters for creating point mutations.

Cell culture and transient transfection

MCF-7 cells (ATCC #HTB-22, ATCC, Manassas, VA, USA) were cultured in Eagle's minimum essential medium supplemented with 10 ng/ml insulin and 10% FBS. MCF-10a cells (ATCC #CRL-10317, ATCC) were cultured in a 1:1 mixture of Ham's F12:Dulbecco's modified Eagle's medium supplemented with 10 ng/ml insulin, 500 ng/ml hydrocortisone, 20 ng/ml EGF and 5% FBS. All media were supplemented with 100 U/ml penicillin and 0.1 μ g/ml streptomycin and cells were maintained at 37 °C in 5% CO₂. For transient transfection assays, cells were transferred into 6-well plates in phenol red-free medium containing charcoal-stripped FBS at 30–40% confluency. The following day, fresh medium was added and the cells were transfected using the FuGENE 6 reagent according to the manufacturer's instructions (Roche Molecular Biology, Indianapolis, IN, USA). A DNA mixture consisting of 500 ng reporter plasmids, 100 ng pCH110, 100 ng human ER α expression plasmid and carrier DNA up to a total of 750 ng/well was prepared and added to 2.25 μ l FuGENE 6 diluted in 100 μ l base media (3:1 ratio FuGENE 6 to DNA). For coactivator studies, 200 ng coactivator expression plasmids, 300 ng reporter plasmids, 100 ng pCH110, 50 ng human ER α expression plasmid and carrier DNA up to a total of 750 ng/well was prepared. After a 1-h incubation of DNA and FuGENE 6, the complex was added drop-wise to the cells in 2 ml charcoal-stripped serum media. Sixteen hours after transfection, 10 nM DES were added for an additional 24 h. CAT reporter activities were measured and normalized with the β -galactosidase (pCH110) activities as previously described (Yang *et al.* 1996).

In vitro translation and nuclear protein preparation

The human wild-type ER α cDNA subcloned into the pSG5 vector was transcribed and translated *in vitro* using a coupled rabbit reticulocyte system

according to the manufacturer's instructions (TNT, Promega, Madison, WI, USA). To prepare nuclear protein extract from MCF-7 cells, the HEGO expression plasmids (P Chambon, University of Pasteur, Strasbourg, France) were transiently transfected into the cells and treated with DES as described above. After collecting the cells, nuclear protein extract was prepared and used in EMSA study as previously described (Liu & Teng 1992).

Electrophoretic mobility shift assay (EMSA)

Complementary oligonucleotide DNA sequences containing the estrogen-responsive regions in the lactoferrin and vitellogenin A2 genes were synthesized by Sigma Genosys as described in the Plasmids and oligonucleotides section. The single-stranded oligonucleotides (21 μ g in 50 μ l 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 200 mM NaCl) were annealed by boiling for 5 min and then slowly cooled to room temperature over a period of 3 h. The double-stranded probes (1.25 μ g, \sim 1 μ l) were labeled with [α - 32 P]dCTP (\sim 50 μ Ci) in 25 μ l reactions for 30 min at room temperature. The labeled probes were purified through G-25 spin columns (Amersham Biosciences) and then 2 μ l samples were counted. The specific activity of the probes was \sim 0.8 to 2×10^8 c.p.m./ μ g DNA. The probes were then purified through a preparative 5% non-denaturing acrylamide gel at 170 V for 1 h and then exposed to Kodak Bio-Max film for 5 min to detect the labeled probes. Only the double-stranded bands were cut out and the DNA was eluted from the crushed gel overnight at 4 $^{\circ}$ C in buffer (10 mM Tris-HCl pH 8.0, 10 mM NaCl). The next morning, the eluted DNA was removed and then passed through a G-25 column for purification. Approximately 0.2 to 0.5 ng DNA at 2×10^4 c.p.m. were used for EMSA.

The binding reactions (10 μ l) included the 32 P-labeled probes (2×10^4 c.p.m., approximately 0.2 to 0.5 ng DNA) and binding buffer (4 μ g poly dI-dC (Amersham Biosciences), 8 mM Hepes pH 7.9, 8% glycerol, 2% Ficoll-400, 50 mM KCl, 10.8 mM dithiothreitol, 1 μ g bovine serum albumin, 80 μ M EDTA, 2 mM MgCl $_2$), 10^{-6} M 17 β -estradiol, ER α and either protease or antibody. For limited proteolysis, liganded ER α was incubated with the various 32 P-labeled DNA oligonucleotides and various amounts of proteinase K as indicated in the Figure legends. For antibody supershift

experiments, hER α antibody (H222, ER Ab-10, or H184), ERR α peptide antibody (P3), COUP antibody (gift from MJ Tsai, Cell Biology Department, Baylor College of Medicine, Houston, TX, USA), a polyclonal mouse lactoferrin antibody (LF, Teng *et al.* 2002b) or pre-immune serum (PS) was pre-incubated with liganded ER α on ice for 30 min. Reactions were resolved on a 5% non-denaturing polyacrylamide gel and visualized by autoradiograph on Kodak Bio-Max MR film with an intensifying screen at -70° C overnight.

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test (GraphPad Prism, San Diego, CA, USA).

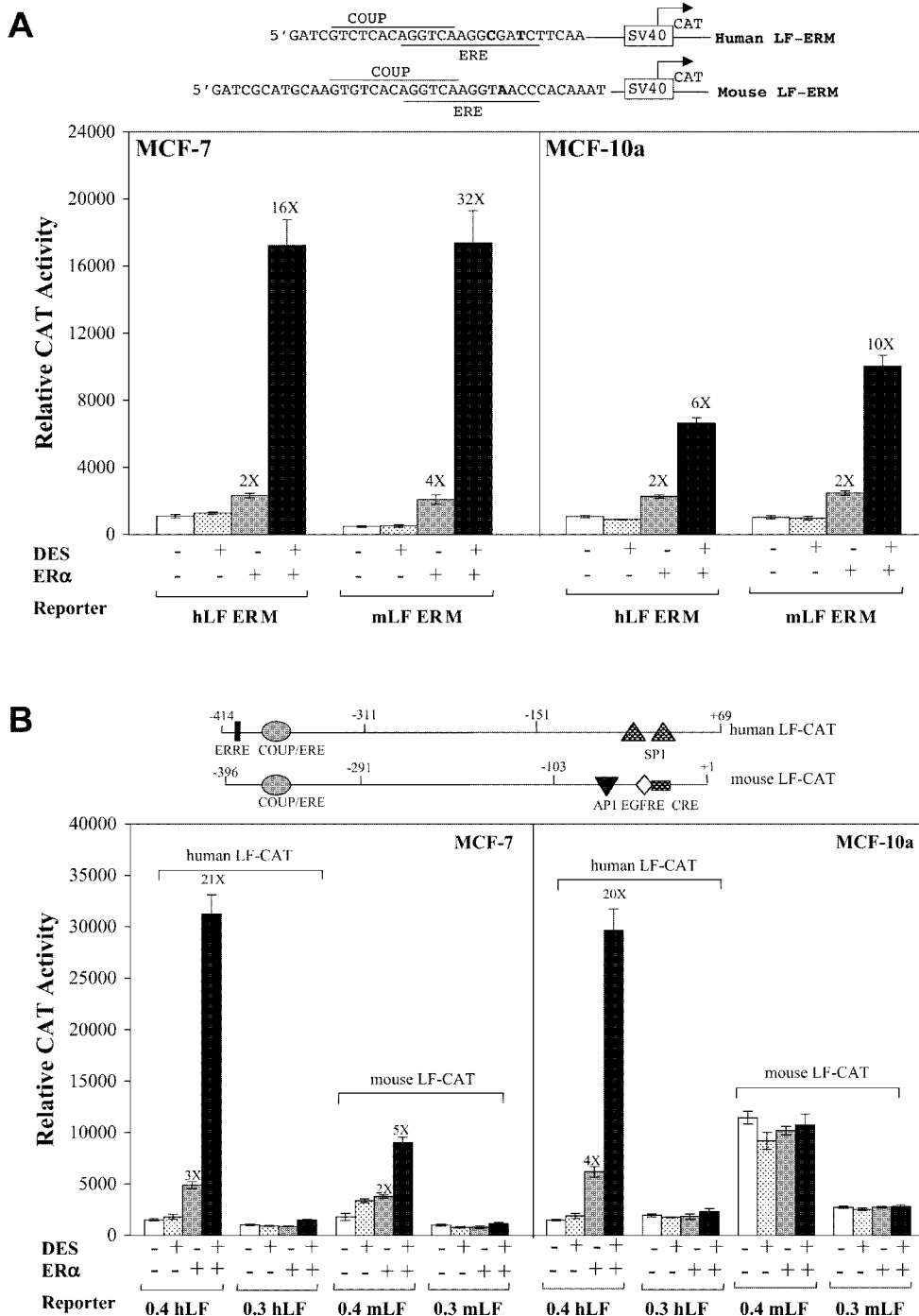
Results

ERRE contributes to the estrogen response of the lactoferrin gene containing an imperfect ERE in the context of its own promoter

The ER exists in two genetically distinct subtypes, ER α and ER β that have distinct expression patterns and functions on target genes (Walter *et al.* 1985, Green *et al.* 1986, Kuiper *et al.* 1996, Mosselman *et al.* 1996, Tremblay *et al.* 1997). In the present study, we focus on ER α because this subtype mediates the major biological functions of estrogen during mammary gland development (Bocchinfuso & Korach 1997, Kregel *et al.* 1998). In the presence of the potent, synthetic estrogen ligand DES, ER α strongly transactivates uterine lactoferrin gene in cell culture systems (Liu & Teng 1992, Teng *et al.* 1992). To characterize the estrogen response of the lactoferrin genes in mammary gland cells, the human (hLF ERM) and mouse (mLF ERM) reporters (Table 1) were transiently transfected into normal (MCF-10a/ER α negative) and tumorigenic (MCF-7/ER α positive) human mammary epithelial cells that express detectable levels of the endogenous lactoferrin gene by RT-PCR (data not shown). Figure 1A demonstrates that one copy of either the human or mouse lactoferrin ERM functions as a transcriptional enhancer in transiently transfected MCF-7 cells and MCF-10a cell lines. There is a

32-fold increase with the mLF ERM versus a 16-fold increase with the hLF ERM relative to untreated MCF-7 cells (left panel) and a 10-fold increase with the mLF ERM versus a 6-fold increase with the hLF ERM relative to untreated MCF-10a cells (right panel).

Next, we examined the 400 bp region of the lactoferrin gene promoter, which includes the ERM and the natural minimal lactoferrin promoter. As depicted in Fig. 1B, the human lactoferrin gene promoter contains an ERRE upstream of the ERM while the mouse gene contains the ERM at



the same position as the human lactoferrin promoter, but lacks the ERRE. Data from cells transiently transfected with the 400 bp regions of the human (0.4 hLF-CAT) and mouse (0.4 mLF-CAT) lactoferrin promoters and ER α showed that estrogen-induced transactivation is dependent upon the ERE in the promoter of the lactoferrin gene. Overexpression of ER α and the 0.4 hLF-CAT in MCF-7 and MCF-10a cells conferred a 20-fold increase in CAT activity in response to estrogen, while deletion of both the ERE and ERRE in this construct (0.3 hLF-CAT) abolished the estrogen response in both MCF-7 and MCF-10a cells (Fig. 1B, compare 0.4 hLF and 0.3 hLF CAT activities). In contrast, the 0.4 mLF-CAT reporter, which contains only the ERM, had a fivefold increase in estrogen-induced CAT activity in MCF-7 cells, which is fourfold lower than the response of the 0.4 hLF-CAT construct (Fig. 1B left panel, compare 0.4 hLF and 0.4 mLF CAT activities). MCF-10a cells overexpressing ER α and the mLF-CAT reporter with or without the ERM were unresponsive to estrogen (Fig. 1B right panel, compare 0.4 mLF and 0.3 mLF CAT activities), although the baseline activity of the 0.4 mLF-CAT reporters was higher in MCF-10a cells. Since the basal activity of the 0.4 mLF-CAT reporter is sixfold higher compared with the 0.4 hLF-CAT construct in the same cellular environment (MCF-10a cells), we investigated whether this elevated basal activity masked the estrogen response of this reporter in MCF-10a cells (compare CAT activities of 0.4 hLF and 0.4 mLF reporters alone in MCF-10a cells). We found that lowering the amount of the 0.4 mLF-CAT reporters transfected into the cells from 500 ng to 200 ng did not produce a strong estrogen response even in MCF-10a cells overexpressing ER α (twofold increase, data not shown). Human endometrial carcinoma (HEC-1B) and mouse mammary epithelial (C57 MG) cells overexpressing

ER α and the 400 bp mLF-CAT were also unresponsive to estrogen (data not shown), hence, in its natural promoter context in the tested cell lines, the mLF ERM was unresponsive to estrogen in the tested cell lines. Taken together, these data suggest that the mouse lactoferrin ERM is functional, but in its natural promoter context, additional positive regulatory elements that may work in tandem with the ERE to increase transactivation may be absent, or negative regulatory elements that may block estrogen-induced transcription may be present within the 400 bp promoter region.

The previous data set led us to directly examine the contribution of the ERRE in estrogen action on the lactoferrin genes. Since we already knew that the ERRE is present in the human but not the mouse lactoferrin gene promoter and that it plays a role in the estrogen response in human endometrial carcinoma RL-95 cells (Yang *et al.* 1996), we decided to investigate whether mutation of this element in the human lactoferrin promoter modulates the estrogen response in mammary gland cells. As shown in Fig. 2A, mutation of the ERRE in the 0.4 hLF-CAT construct (m1) reduces the estrogen response in MCF-7 cells by 50% and in MCF-10a cells by 25%. Mutating the ERE alone (m6) or in combination with the ERRE (m1/m6) further reduced the estrogen response in both cell lines to $\sim 10\%$ of the wild-type human lactoferrin reporter. Having shown that the ERRE played a role in the estrogen response of the human lactoferrin gene promoter, we then inserted the 12 bp ERRE sequence located 18 bp upstream of the ERM into the natural 0.4 mLF promoter (Fig. 2B). MCF-7 cells transfected with the 0.4 mLF plus-CAT construct had an extremely robust response to estrogen, which was significantly greater than either of the responses of the 0.4 mLF-CAT and 0.4 hLF-CAT reporters (compare Figs 2B and 1B, left panels). As further support of

Figure 1 Human and mouse lactoferrin ERMs function as enhancers to heterologous and homologous promoters. (A) Top: schematic presentation of the human and mouse overlapping ERE/COUP module (ERM). Letters in bold denote the nucleotides that are mismatched from the consensus ERE. Bottom: relative CAT activity normalized to β -galactosidase (β -gal) activity is the value expressed as the mean \pm S.E.M. of three independent assays in duplicate. Fold activation in reference to control (transfection of reporter alone) is indicated above the error bars. (B) Top: schematic presentation of the 400 bp region of native human and mouse lactoferrin gene promoters indicating the relative positions of the ERRE, COUP/ERE, AP1, EGF response element (EGFRE), SP1 and CRE elements. Bottom: relative CAT activity normalized to β -gal activity is the value expressed as the mean \pm S.E.M. of four independent assays in duplicate. Fold activation in reference to control (transfection of reporter alone) is indicated above the error bars.

this enhanced sensitivity to estrogen, the limited amount of endogenous ER α in MCF-7 cells activated the 0.4 mLF plus-CAT reporter fourfold compared with a twofold induction reported with the 0.4 mLF-CAT reporter in Fig. 1B, and overexpression of ER α conferred a 60-fold increase in estrogen-induced reporter activity compared

with untreated cells transfected with reporter alone (Fig. 2B, left panel). In fact, the estrogen response of this reporter was elevated in MCF-10a cells co-transfected with ER α compared with the native mouse lactoferrin reporter lacking the ERRE (compare Figs 2B and 1B, right panels). Also, the relative estrogen-induced CAT activity of the 0.4

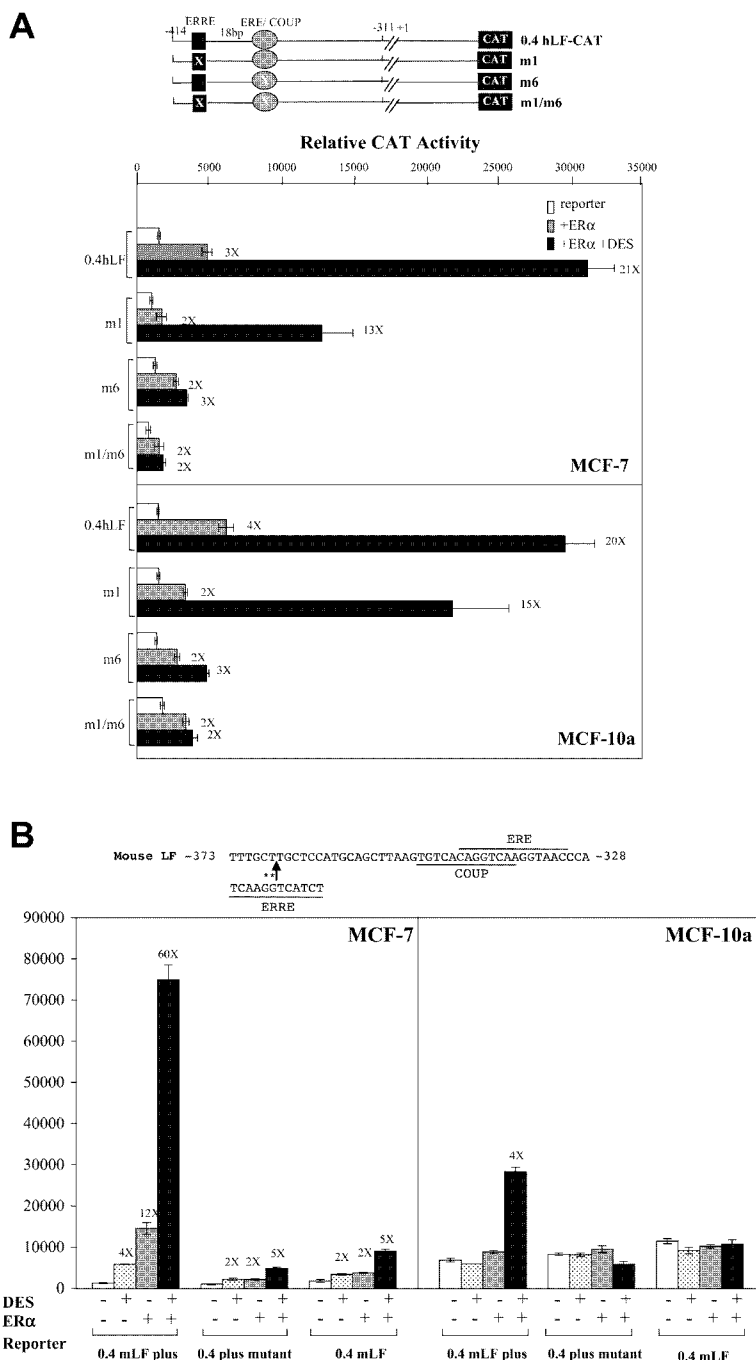


Table 2 Partial nucleotide sequences of ^{32}P -labeled oligonucleotides used in EMSA and limited protease digestion experiments. The lengths of the filled-in double-stranded probes are indicated. Only the ERRE and ERE sequences are noted and nucleotides deviating from the consensus ERE are in bold. The relative intensity of each ER α -shifted and free DNA band was quantitated by pixel histogram analysis using Adobe Photoshop and the sum of the shifted bands was divided by the sum of all bands to determine percent shifted. Range of three independent assays are presented

ERM sequence	% shifted
Double-stranded oligonucleotides	
VitA2 (33 bp)	39–40
Human LF (78 bp)	7–13
Mouse LF (46 bp)	20–32
Mouse LF plus (82 bp)	21–35

mLF plus-CAT was comparable to the 0.4 hLF CAT reporter in MCF-10a cells.

It is important to clarify whether the estrogen response of the 0.4 mLF plus-CAT reporter is due to the ERRE sequence itself or to disruption of negative regulatory elements located just upstream of the ERE that may interfere with the enhancer activity of the mouse lactoferrin ERE. Accordingly, we mutated the guanine dinucleotide sequence of the ERRE to adenines in the 0.4 mLF plus construct (Fig. 2B top panel, an asterisk represents mutated nucleotides). Interestingly, destroying the added ERRE in 0.4 mLF plus-CAT showed a loss of strong estrogen response and the estrogen-stimulated activity was reduced to nearly identical levels as the wild-type 0.4 mLF in either MCF-7 or MCF-10a cells. If any potential inhibitory sequences were disrupted, then the ER α -mediated estrogen response of the 0.4 mLF plus mutant reporter would have remained. This result suggested that the strong estrogen response of the 0.4 mLF plus reporter resulted from synergy between the ERRE and ERE. Thus, the apparent differences in estrogen response of the human and

mouse lactoferrin EREs in the natural promoter context were due to the presence of the ERRE sequence in the human lactoferrin promoter.

Differential ER α binding to ERE sequences

To investigate the mechanism of ERRE contribution in estrogen action, we first examined ER α binding to the double-stranded oligos of human (hLF), mouse (mLF) and mouse plus (mLF plus) and vitA2 (Table 2). By EMSA studies (Fig. 3), liganded ER α was pre-incubated with no antibody, ER α antibody (H222), pre-immune serum (PS) or a mouse lactoferrin antibody (LF) for 30 min on ice prior to the addition of the various ^{32}P -labeled oligos. ER α formed two specific complexes with all four oligos and both complexes were supershifted with the ER α -specific antibody (H222, Fig. 3A, lanes 3, 8 and 13; Fig. 3B, lane 3) but not with non-relevant lactoferrin antibody (LF, Fig. 3A, lanes 5, 10 and 15; Fig. 3B, lane 5) or pre-immune serum (PS, Fig. 3A, lanes 4, 9 and 14; Fig. 3B, 4). We tested two other ER α antibodies (Ab-10 and H184) and they were able to shift the complexes

Figure 2 ERRE synergizes with the ERM to enhance ER α -mediated estrogen responses of the lactoferrin genes. (A) Top: schematic presentation of the human lactoferrin gene reporter constructs 0.4 hLF containing wild-type, mutated ERRE (m1), mutated ERE (m6) and double mutated (m1/m6) sequences. Nucleotide location of the 0.4 hLF-CAT and 0.3 hLF-CAT reporters are indicated. There are 18 bp separating the ERRE and ERM elements in the 0.4 hLF construct and an X in a specific element denotes mutation of the guanine dinucleotide critical for receptor binding. Bottom: relative CAT activity normalized to β -gal activity is the value expressed as the mean \pm S.E.M. of four independent assays in duplicate. Fold activation in reference to control (transfection of reporter alone) is indicated above the error bars. (B) Top: schematic presentation of the mouse lactoferrin gene construct containing wild-type, insertion of ERRE denoted by the arrow (mLF plus) and mutated ERRE denoted by the asterisks (mLF plus mutant). Bottom: relative CAT activity normalized to β -gal activity is the value expressed as the mean \pm S.E.M. of at least three independent assays in duplicate. Fold activation in reference to control (transfection of reporter alone) is indicated above the error bars.

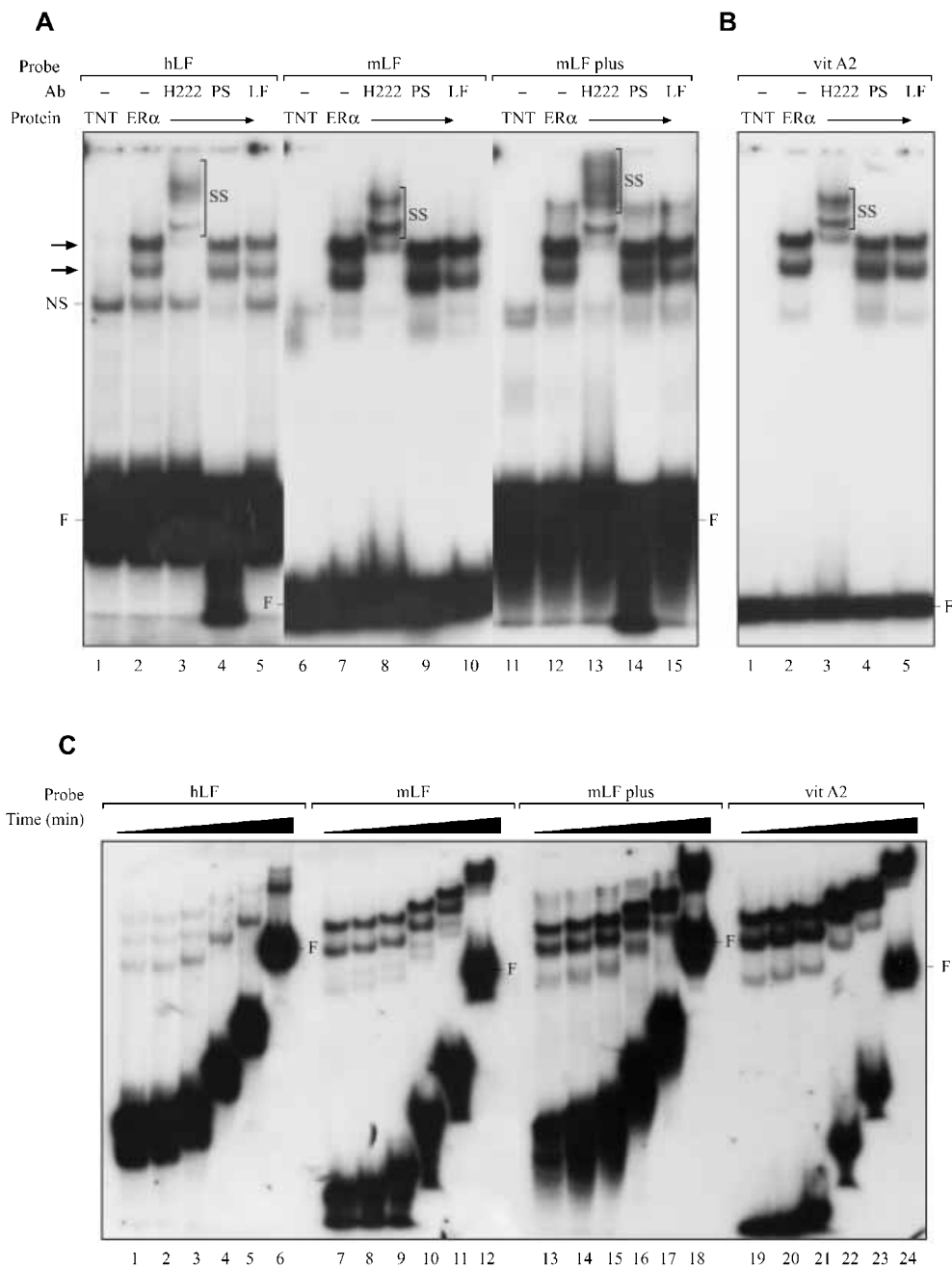


Figure 3 Electrophoretic mobility shift assay (EMSA) detection of ER α specifically binding to the various lactoferrin ERMs and the consensus vitA2 ERE. The *in vitro* translated ER α was used in the binding reaction as described in Materials and methods. (A) 32 P-labeled oligos of human lactoferrin (hLF), mouse lactoferrin (mLF) and mouse lactoferrin plus (mLF plus). The x-ray film was developed for 2 days. (B) 32 P-labeled oligos of vitA2. The x-ray film was developed overnight. Antibodies to human estrogen receptor (H222), human lactoferrin (LF) or pre-immune serum (PS) are indicated. Double arrows indicate the ER α -DNA complexes; SS, supershifted bands; NS, non-specific bands. The free probe (F) is present in every lane at the bottom. (C) Time course. The binding reaction was carried out at room temperature for 5, 10, 20, 40, 60 and 90 min before loading on to the gel. The gel was run at 200 volts until the free probe of the 5-min reaction reached the bottom of the gel (approx. 2 h). The x-ray film was developed for one day. The free probes are marked (F).

(data not shown). The results indicated that the *in vitro* translated receptor binds specifically and effectively to the four EREs because the receptor bands are not detected with the *in vitro* translation mixture (TNT, Fig. 3A, lanes 1, 6 and 11; Fig. 3B, lane 1), which produced non-specific bands (NS). In addition, positions of the shifted complexes (arrows) were identical in all four EREs regardless of the oligo lengths, suggesting that the DNA-bound ER α determines the mobility of the complexes in EMSA. Interestingly, the amount of receptor complexes formed with the four EREs was different even though the receptor was from the same translation reaction and equal amounts were applied. In time course and competition studies, ER α -DNA complexes were detected after a 5-min incubation period and remained constant for more than 60 min for all four probes (Fig. 3C), whereas the competition studies revealed that the binding of receptors to the hLF and mLF probes were competed effectively with lower levels of competitors than with the mLF plus and vitA2 probes (data not shown). The results reflected less binding or unstable interaction of the receptor to the hLF and mLF oligos. To determine the percentage of free probe shifted by ER α relative to the total input, the relative intensity of each band (hLF, mLF, mLF plus, and vitA2) was quantitated by pixel histogram analysis using Adobe Photoshop and the sum of the ER α bands (ER α -shifted) was divided by the sum of the total bands (ER α -shifted, non-specific, and free DNA). From the averages of three independent assays and calculations, the percentage of labeled DNA that was shifted by the receptor was 40% for vitA2, 32% for mLF plus, 25% for mLF and 10% for hLF (Table 2). The relative percentage of binding varied among experiments but the relationship of the binding intensity of the four probes was consistent. These EMSA data were reflected in the lower percentage of receptor-hLF complexes (10%) compared with the receptor-mLF complexes (25%) (Table 2) and is in agreement with our previous study of the human and mouse lactoferrin EREs (Teng *et al.* 1992). However, the binding studies are inconsistent with the functional studies in that the ER α -mediated transactivation from the natural 400 bp human was much more robust than the mouse lactoferrin gene promoter (Fig. 1B), suggesting that other cis-acting elements may be involved.

Next, we investigated whether ER α conformation differed when bound to the various estrogen

responsive modules by using the protease sensitivity assays. Representative limited proteinase K digestion EMSAs of ER α bound to ³²P-labeled hLF, mLF, mLF plus, or vitA2 oligos are shown (Fig. 4). Proteinase K cleaves at peptide bonds adjacent to the carboxyl groups of aliphatic and aromatic amino acids and digestion of ER α bound to the various DNA elements produced proteolytic complexes having distinct migration patterns (Fig. 4). Under identical binding conditions where the only difference is the sequence of the oligos, we observed minor differences in ER α sensitivity to proteinase K. Incubation of the DNA-bound receptor with as little as 1.25 ng proteinase K was sufficient to produce some proteolytic cleavage fragments. Digestion of ER α bound to the four oligos produced proteolytic complexes of similar mobility, but showed different protease sensitivities at lower concentrations (compare the hLF and mLF with mLF plus and vitA2 probes). The difference could result from the initial quantity of ER α bound to the probes. Two other proteases, trypsin and α -chymotrypsin, were also utilized to examine the accessibility of ER α to proteolysis when bound to the four oligos and the same conclusion was drawn from these data (data not shown). Taken together, the ER α binding (Fig. 3) and protease digestion (Fig. 4) studies demonstrated that the addition of ERRE element to the mouse ERE enhances the receptor binding with minor changes of the receptor sensitivity to protease digestion. Nonetheless, these differences may not exclusively account for the dramatic response of mLF plus to ER α -mediated transactivation in the MCF-7 cells.

Since the ERRE and the lactoferrin EREs of human and mouse can be recognized by nuclear receptors COUP-TF and ERR α , in addition to ER α (Liu & Teng 1992, Teng *et al.* 1992, Yang & Teng 1994, Yang *et al.* 1996, Johnston *et al.* 1997, Sladek *et al.* 1997), it is possible that the COUP-TF and ERR α are involved in interaction and transactivation of the EREs in the context of lactoferrin promoters in MCF-7 cells. To explore this possibility, EMSA were performed with nuclear protein extract of the MCF-7 cells overexpressing ER α (Fig. 5). As expected, nuclear protein of the MCF-7 cells binds all four probes and shifted the free probes to a similar position. However, the amount of nuclear protein interacting with the vitA2 probe was reduced as opposed to

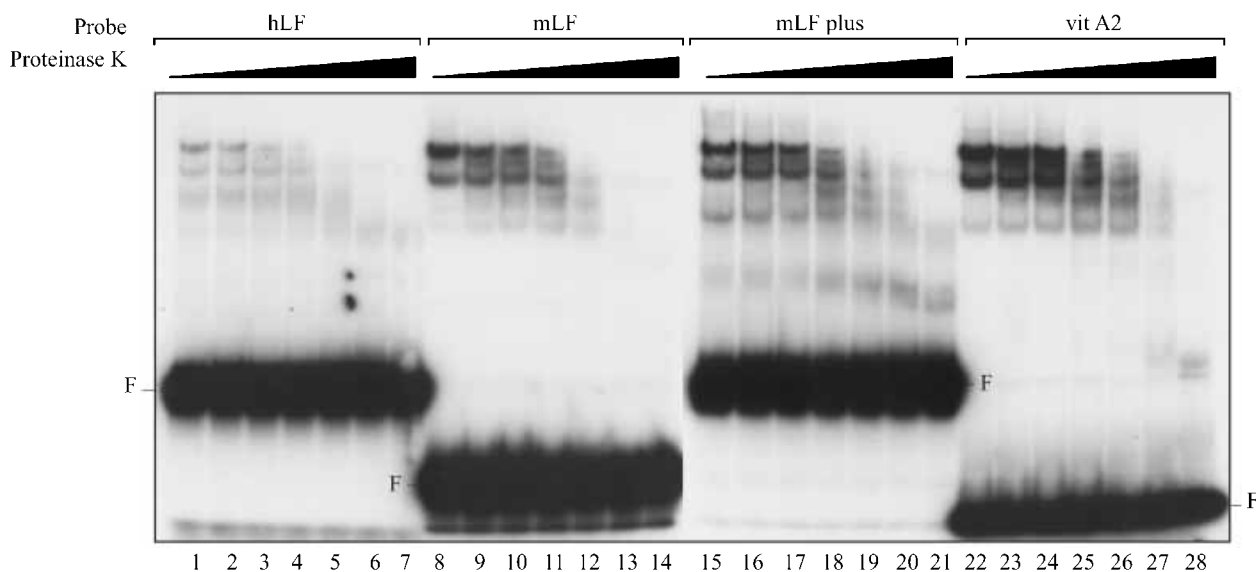


Figure 4 Limited protease digestion of ERE-bound ER α shows sensitivity differences in the receptor bound to the different ERMs. 32 P-labeled DNA fragments containing hLF, mLF, mLF-plus or vitA2 ERE were incubated with *in vitro* translated ER α for 30 min at room temperature. Limited protease digestion of DNA-bound receptor occurred for 10 min at room temperature before EMSA using proteinase K, 0, 1-25, 2-5, 5, 10, 20, or 40 ng. The free probes are marked (F).

binding of the *in vitro* translated ER α (Figs 3 and 4). On the contrary, the intensities of the bands shifted with hLF, mLF or mLF plus probes were stronger and the number of complexes increased (Fig. 5, compare lanes 1–16 with lanes 17–22). To detect the presence of ER α , COUP-TF and ERR α in these complexes, we applied specific antibody to the reaction and examined whether the antibody could disrupt or supershift the complexes in EMSA. The presence of COUP-TF in the nuclear protein complexes of hLF, mLF or mLF plus probes was clearly demonstrated by supershifting the band and simultaneously disrupting the complexes with specific COUP-TF antibody (lanes 4, 10 and 15) while vitA2 probe showed minimal binding of COUP-TF (lane 21). Binding of ERR α was mainly detected with hLF and mLF plus probes which contain the ERRE element (lanes 3 and 14, supershifted band). The mLF probe which lacks the ERRE did not bind ERR α (lane 9) while the vitA2 with typical ER α binding element binds ERR α weakly (lane 19, supershifted and disruption of the band) and this result is in agreement with the previous reports (Johnston *et al.* 1997, Sledak *et al.* 1997). Interestingly, ER α was not detected in the complexes formed with mLF probes (lane 8) but with mLF plus and vitA2 probes (lanes 13 and 19

respectively, disruption of the band), suggesting that the ER α binding to the mLF plus probe is enhanced in the presence of ERR α . Detection of ER α in the complexes of hLF was variable. Whether COUP-TF plays any role in ER α binding and transactivation activity of the lactoferrin promoter in MCF-7 cells is not clear. Nonetheless, the dramatic estrogen response of mLF plus in the context of mouse lactoferrin promoter may result from the cooperation of multiple nuclear receptors binding and transactivation.

ERE sequences influence the recruitment of coactivators to ER α

It is well documented that ligand binding to ER α induces conformational changes affecting the position of α -helices within the ligand binding domain (LBD) allowing recruitment of multiple cofactor complexes to the target gene promoter (Tsai & O'Malley 1994, Brzozowski *et al.* 1997, Shiau *et al.* 1998). Among the coactivators that are known to specifically interact with the estrogen receptor to enhance transactivation are steroid receptor coactivators (SRC-1, SRC-2, SRC-3) and peroxisome proliferator activated receptor- γ coactivator-1 (PGC-1 α) and PGC-1-related estrogen receptor

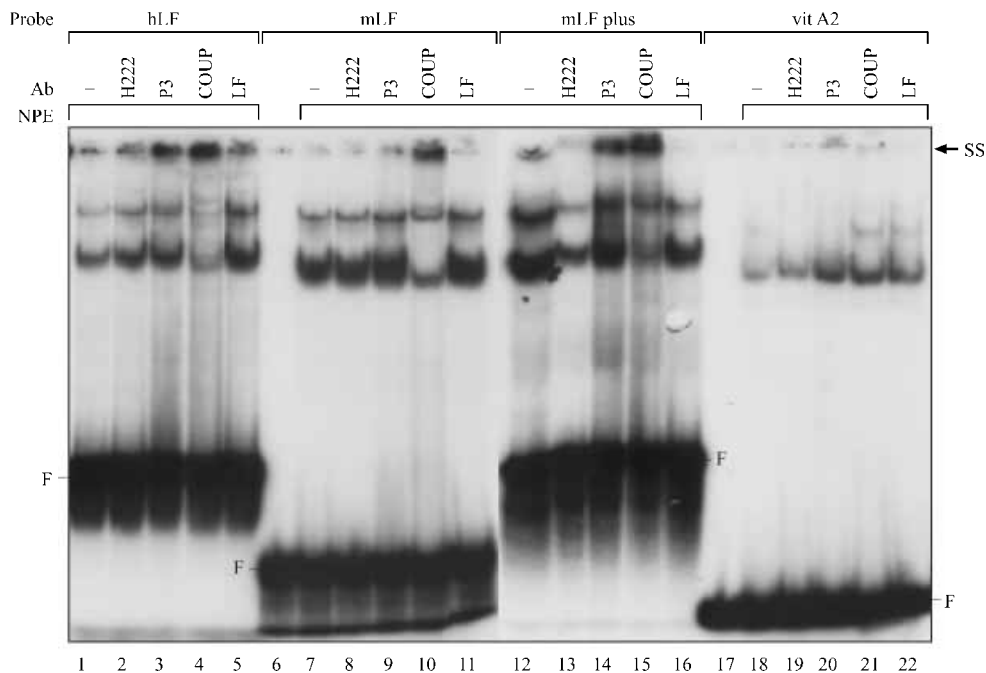


Figure 5 Differential binding of nuclear receptors to various lactoferrin ERMs and the consensus vitA2 ERE in the context of MCF-7 nuclear protein extract. Nuclear protein extract (NPE) from ER α overexpressed MCF-7 cells was prepared and used in EMSA as described in Materials and methods. Specific antibodies to ER α (H222), ERR α (P3), COUP-TF (COUP) and mouse lactoferrin (LF) are indicated. Supershifted band is indicated by arrow and SS. The free probes are marked (F).

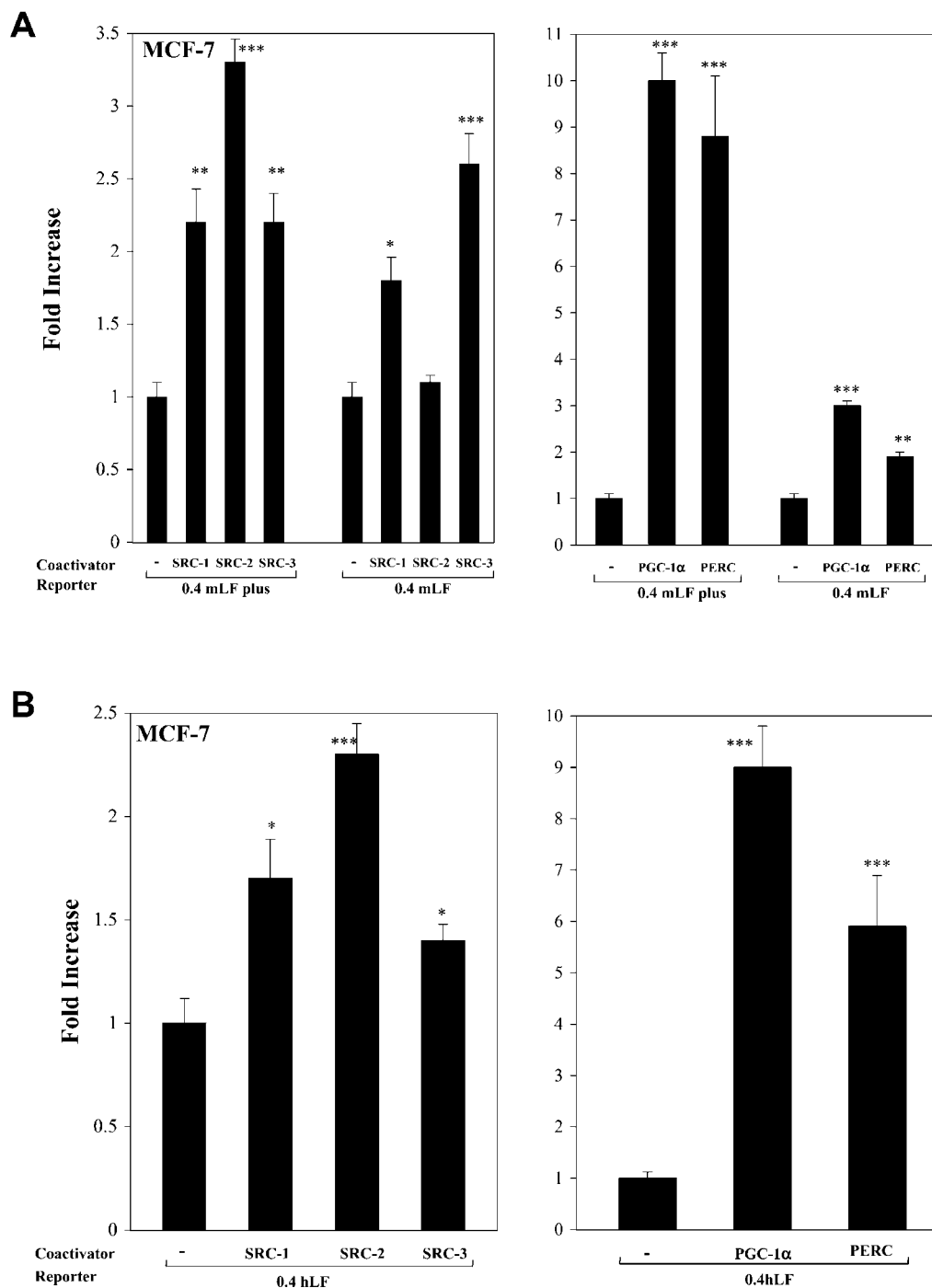
coactivator (PERC) (reviewed in Robyr *et al.* 2000).

Recruitment of coactivator is a major step in ER α -mediated transactivation function. To investigate the pattern of coactivator enhancement of the ERE-bound receptor in the presence or absence of the ERRE in the 400 bp lactoferrin gene promoters, we conducted transient transfection assays in MCF-7 cells in the presence of 10 nM DES. The cells were transfected with the reporter, ER α expression vector and with or without a single p160 family or PGC family coactivator (Fig. 6). All of the p160 family members enhanced the reporter activity of the mouse lactoferrin promoter reporter containing the ERRE (0.4 mLF plus-CAT), but SRC-1 and SRC-3 enhanced ER α -mediated transcription at significantly lower levels ($P < 0.01$) than SRC-2. In contrast, SRC-1 ($P < 0.05$) and SRC-3 ($P < 0.001$) greatly enhanced the transcription of the mouse lactoferrin reporter lacking the ERRE (0.4 mLF-CAT), while SRC-2 did not affect ER α -mediated transcription of this reporter ($P < 0.001$) (Fig. 6A, left panel). The recently

discovered inducible coactivator PGC-1 α and its related family member PERC potently stimulated the hormone-dependent activity of several nuclear receptors including ER α (Knutti *et al.* 2000, Kressler *et al.* 2002). Both members of the PGC family strongly stimulated the transcriptional activity of 0.4 mLF plus-CAT and much less of 0.4 mLF-CAT (Fig. 6A, right panel). Thus, it appeared that the pattern of coactivator recruitment by liganded ER α bound to the lactoferrin ERM differed when the ERRE was present. The human lactoferrin promoter reporter containing the ERRE (0.4 hLF-CAT) showed a similar pattern of p160 coactivator enhancement seen with 0.4 mLF plus (compare left panels of Fig. 6A and B) – SRC-2 ($P < 0.001$) was more efficient than SRC-1 and SRC-3 ($P < 0.05$). The PGC-1 α and PERC coactivators stimulated CAT activity of 0.4 hLF and PGC-1 α or PERC comparable to that observed from cells transfected with 0.4 mLF plus (compare right panels of Fig. 6A and B). Collectively, these experiments showed that the presence of the ERRE in the natural human and

mouse lactoferrin gene promoters conferred preferential enhancement of liganded ER α by SRC-2, PGC-1 α and PERC coactivators to the estrogen receptor, whereas SRC-3, SRC-1 and PGC-1 α

selectively enhanced liganded ER α -mediated transcription of the natural mouse lactoferrin gene promoter that does not contain the ERRE more efficiently.



Discussion

Several studies focusing on the effects of the minimal ERE sequence on ER-mediated transactivation have demonstrated three major points: first, variations in the consensus ERE sequence occur naturally and may elicit unpredictable transcriptional activity; secondly, ER binding to ERE does not always result in a corresponding level of transcriptional activity; and thirdly, the amount of transcriptional activation detected from the same ERE depends on cell-specific factors and surrounding promoter elements (reviewed Klinge 2001). Here, we showed that an imperfect ERE and an adjacent ERRE in the context of natural lactoferrin gene promoters govern ER α -mediated transactivation by altering receptor binding and receptor interactions with cofactors in mammary gland cells.

Most naturally occurring EREs are imperfect palindromes that deviate from the 13 bp consensus sequence 5'GGTCAnnnTGACC3' by an average of 1 bp change in each half-site arm. It is well documented that these imperfect EREs act as enhancers on heterologous promoters, albeit the promoter activities are lower than the perfect palindrome sequences (reviewed in Klinge 2001). Based on these results, one would predict that both the human and mouse lactoferrin imperfect EREs would enhance transcription of a heterologous promoter and our transfection data from MCF-7 and MCF-10a mammary epithelial cell lines confirmed this (Fig. 1A). As expected, the mLF ERM, which has a one nucleotide mismatch (G to A in the 3' arm) from the consensus palindrome ERE, is a more potent activator of the SV40 promoter compared with the hLF ERE that has two base-pair changes (T to C and C to T in the 3' arm) from the consensus sequence (Klein-Hitpass *et al.* 1988). This finding was also supported by the EMSA study with *in vitro* translated ER α binding to

mouse and human EREs (Figs 3 and 4). Surprisingly, the mouse lactoferrin ERM in the context of its natural promoter did not efficiently enhance reporter activity in response to estrogen in the transiently transfected mammary epithelial cell lines (Fig. 1B). The addition of the ERRE in the 0.4 mLF plus reporter substantially increased the AF-2 activity of ER α in response to estrogen in mammary gland cells (Fig. 2B, left panel). In an attempt to determine whether the observed differences in ER α transcription from the human and mouse lactoferrin ERMs in human mammary gland cells result from the cell context, we transfected the reporters in human endometrial and mouse mammary gland cells, and found that they behaved in a similar manner in the different cell types. Since EREs are usually located in the gene promoters containing multiple cis-acting elements, we reasoned that the complexity of the natural 400 bp region of the lactoferrin gene promoters influence ER α -mediated transactivation activity in mammary gland cells.

We then examined the role of the ERRE in the estrogen response of the lactoferrin gene promoters. The human lactoferrin gene promoter naturally contains the ERRE (Yang & Teng 1994), which was initially characterized as a steroidogenic factor-1 binding element (Rice *et al.* 1991, Lala *et al.* 1992) and later as an estrogen-related receptor binding element (ERRE, Yang & Teng 1994, Yang *et al.* 1996, Johnston *et al.* 1997, Sladek *et al.* 1997). The ERR family (Giguere *et al.* 1988, Laudet 1997) was found as constitutive active nuclear receptors closely related to estrogen receptors (Xie *et al.* 1999, Zhang & Teng 2000), but other reports demonstrated that serum components (Vanacker *et al.* 1999b) can activate while DES (Lu *et al.* 2001) and 4-hydroxytamoxifen (Coward *et al.* 2001, Tremblay *et al.* 2001) at a high level (pharmacological levels, 10^{-4} and 10^{-5} M) repress receptor activity. ERR α shares many target genes with the estrogen

Figure 6 Effect of p160 and PGC families of coactivators interacting with ER α bound to mouse lactoferrin, mouse lactoferrin plus, and human lactoferrin gene promoters on reporter activity. (A) Effect of p160 family (left panel) and PGC family (right panel) coactivators on ER α -mediated transactivation of the mouse lactoferrin reporters. (B) Effect of p160 family (left panel) and PGC family (right panel) coactivators on ER α -mediated transactivation of the human lactoferrin reporter. The fold CAT activity is reported as the relative CAT activity of co-transfections with reporter, liganded hER α and coactivator divided by the relative CAT activity of co-transfections with the reporter and liganded hER α . Values are expressed as the means \pm S.E.M. of three independent assays in duplicate. Fold activation in reference to control (transfection of reporter alone) is indicated above the error bars: *** P < 0.001, ** P < 0.01, * P < 0.05.

receptors (Yang *et al.* 1996, Johnston *et al.* 1997, Vanacker *et al.* 1999a,b, Zhang & Teng 2000, Lu *et al.* 2001, reviewed in Giguere 2002), and DNase I footprint protection analysis and EMSA revealed that ER α could bind the ERRE of the human lactoferrin gene, however less efficiently than ERR α (Zhang & Teng 2000). COUP-TF is another nuclear receptor family member and has been shown to act both as activator and repressor in transcription (see review and references therein by Park *et al.* 2003). MCF-7 cells express endogenous ER α , ERR α and COUP-TF mRNA and protein (Green *et al.* 1986, Lu *et al.* 2001, C Teng, unpublished data) and our EMSA data did show differential presence of these nuclear receptors in the protein-DNA complexes with or without ERRE present (Fig. 5). Mutation of the ERE in the 0.4 hLF-CAT reporter alone (m6) or with the ERRE (m1/m6) reduces the ligand-dependent receptor activity in transfected cells (Fig. 2A, compare both gray and black bars of m6 and m1/m6). A slight increase in reporter activity resulted from the ligand-independent receptor function. Furthermore, overexpression of ERR α in MCF-7 cells mainly influenced estrogen-independent reporter activity (data not shown). Thus, the effect of endogenous ER α , ERR α or COUP-TF on the reporter activity influenced the outcome of transient transfection experiments (compare Fig. 1B left panel, 0.4 hLF and Fig. 2B left panel, 0.4 mLF plus with Fig. 2B, left panel 0.4 mLF and 0.4 mLF plus mutant). Several other natural genes contain neighboring ERE and AGGTCA half-site motifs in their promoter regions including the human pS2 gene (Lu *et al.* 2001), rainbow trout estrogen receptor gene (Petit *et al.* 1999), human estrogen receptor- β gene (Li *et al.* 2000), and mouse osteopontin gene (Vanacker *et al.* 1999a). Studies of ER α -dependent transactivation have demonstrated synergism between the ERE and AGGTCA half-site motifs in these genes, supporting our data showing synergy between an imperfect lactoferrin ERE and ERRE and achieving maximum estrogen response in the context of the human and mouse natural lactoferrin promoters.

Combinatorial gene regulation by nucleoprotein complexes consisting of multiple transcription factors and DNA elements has been thoroughly described for interferon- β and T-cell receptor- α genes (Kim & Maniatis 1997, reviewed in

Grosschedl 1995 and Carey 1998). Other than the estrogen response elements, SP1 and AP1 binding elements of estrogen responsive genes also regulate hormone-induced gene expression through physical interactions between SP1 or AP1 and ER α . In ER α positive human mammary gland cell lines, the cAMP response element in the cyclin D1 gene and a GC-rich motif in the E2F-1 gene mediate ligand-dependent transactivation by ER α (Castro-Rivera *et al.* 2001, Ngwenya & Safe 2003). Both the ERE and an AP1 site located 52 bp downstream contribute to the estrogen response of the pS2 gene through the formation of a complex stabilized by SRC-1 (Barkhem *et al.* 2002). In addition to identifying the ERRE in the human lactoferrin gene promoter, we have previously identified an AP1/CRE protein binding site (-56 to -36) in the minimal mouse lactoferrin gene promoter that conferred the basal activity of the gene and the minimal promoter regions (Shi & Teng 1994) and have speculated that the hormone responsive units may cooperatively regulate the estrogen response of the lactoferrin genes.

Several recent studies have examined the conformation of the liganded receptor bound to perfect and imperfect EREs. Using the vitA2, pS2, vitB1 and oxytocin EREs, Loven and colleagues showed that ER α and ER β structural changes were mediated by the DNA elements (Loven *et al.* 2001a,b, Wood *et al.* 2001). Our limited protease digestion experiments were not able to demonstrate that ER α assumed distinct conformations when bound to different ERE sequences (Fig. 4). It is possible that the current approach has limited sensitivity and the conformation change could be detected with other methods. The receptor bound to EREs with an upstream ERRE could form a more stable complex especially with the help of other receptors (Fig. 5). The addition of the ERRE induced a cooperative binding of multiple nuclear receptors, which may explain the differences in coactivator recruitment patterns seen with these two reporters (Fig. 6A).

Initially, it was believed that the relative abundance of coactivators in specific tissues could explain tissue-specific gene expression (Anzick *et al.* 1997, Tikkanen *et al.* 2000, Xu *et al.* 2000). However, the majority of cofactors are widely expressed in similar amounts in most cells (Kurebayashi *et al.* 2000, Viononen *et al.* 2003) and the phenotype of p160 family knock-out mice

showed that these coactivators exhibit redundant biological functions (Xu *et al.* 1998, 2000, Gehin *et al.* 2002). Now, a growing number of studies have indicated the importance of the sequence of the DNA element in regulating ER-mediated transcription (Loven *et al.* 2001a, Wood *et al.* 2001, Hall *et al.* 2002, Yi *et al.* 2002). In our studies, we demonstrated that in the context of the 400 bp natural lactoferrin gene promoters, SRC-2 preferentially enhanced the ER α -mediated estrogen-induced transcriptional activity of the EREs together with an upstream ERRE, while the activity of the receptor bound to only an imperfect mouse lactoferrin ERE was selectively enhanced by SRC-3 and SRC-1 (Fig. 6A and B). Although both members of the PGC family of coactivators were recruited to the receptor bound to the lactoferrin ERE alone or with an adjacent ERRE, the presence of the ERRE enhanced the efficiency of ER α -mediated estrogen action when these coactivators were overexpressed (Fig. 6A and B). Accordingly, our data are in agreement with the premise that the ERE sequence and surrounding elements ultimately determine receptor binding, receptor conformation, and transcription. Hall *et al.* (2002) used an ELISA-based assay to detect differential interactions of ER α bound to the mLF, pS2, vitA2 and complement 3 EREs with a single LXXLL peptide motif from the SRC family coactivators. Although these experiments were not performed with the natural promoter regions or the full-length coactivator, they specifically demonstrated that structural changes in the cofactor recognition surface of the receptor LBD were influenced by the DNA element as different ligands and different coactivator LXXLL motifs did not alter receptor–DNA interactions.

Interestingly, our laboratory has recently shown that ERR α gene expression is induced by estrogen in the mouse uterus and heart (Liu *et al.* 2003). Promoter analysis of the ERR α gene revealed the presence of a multiple hormone response element (MHRE), which is composed of three hormone response elements in tandem, and chromatin immunoprecipitation assays demonstrated the interaction of the ER α with MHRE of the endogenous ERR α gene in estrogen-treated MCF-7 cells. In addition, overexpression of PGC-1 α alone in MCF-7 cells strongly stimulated the activity of the ERR α -CAT reporter containing the MHRE (C Teng, unpublished

data). Given that the spatial organization of the three hormone responsive elements in the ERR α gene is comparable to the three estrogen response half-sites of the lactoferrin genes (ERRE plus the ERE), it is likely that ER α functions similarly on the lactoferrin gene promoters containing the ERRE to influence the selectivity of coactivators in liganded ER α -mediated transcriptional activity.

In addition to the sequence of the ERE itself, chromatin is another level of transcriptional control of estrogen responsive genes. In a closed conformation, chromatin is tightly packaged and many promoters are transcriptionally repressed because access to the steroid nuclear receptor promoter binding sites is blocked (reviewed in Orphanides & Reinberg 2000). Upon proper epigenetic changes and external stimuli, the chromatin assumes an open conformation allowing the receptors access to the promoter. This is true of the human lactoferrin gene in MCF-7 cells. The gene is under-methylated as determined by restriction digestion at potential methylation sites (Panella *et al.* 1991). Additionally, a 48-h treatment with DES induces endogenous lactoferrin mRNA expression in MCF-7 cells cultured in charcoal-stripped media for three days prior to hormone stimulation (data not shown). It should be noted that models developed from chromatin immunoprecipitation assays have proposed dynamic association and dissociation of estrogen receptor and basal transcriptional cofactors during estrogen signaling (Shang *et al.* 2000, Reid *et al.* 2003). Although our transient transfection experiments were performed on non-chromatin templates, the complexity of the model of ER α action may be similar in that the precise combination of nuclear receptors and cofactors, stability of nuclear receptor binding, and time of this cooperative association are dependent upon the ERE sequence and additional flanking elements within the promoter context.

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