Abstract

STOKES, KENYA. 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. (Under the direction of Brenda Alston-Mills and Christina T. Teng.)

The purpose of this research has been to determine whether an extended estrogen response element half-site (ERRE) contributes to the differential estrogen responses of the human and mouse lactoferrin estrogen response element (ERE) in the context of their natural promoters. This research utilized molecular biology techniques to evaluate gene activation. Transfections of MCF-7 cells showed that liganded ERα activates transcription of the human lactoferrin ERE 4-fold higher than the mouse lactoferrin ERE in the context of their natural promoters. Since the ERRE of the human lactoferrin gene naturally occurs 18 bp upstream from the ERE 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. We also demonstrated that the conformation of the estrogen receptor bound to the ERE alone or in the presence of ERRE differed and that the ERRE influenced the selectivity of coactivators in liganded ERα-mediated transcriptional activity. Like the lactoferrin gene ERE, most known natural estrogen response elements are imperfect palindromes that differ from the consensus by at least a 1 base pair change and confer different levels of ER transcriptional activation compared to the consensus ERE. In contrast to our transient transfection data showing a lower estrogen response of the mouse lactoferrin ERE compared to the human lactoferrin ERE in the context of their natural promoters, *in vivo* data showed that the gene is robustly

transcribed in response of estrogen in both species. Therefore, this research model can be applied to studies of genes that have different hormone responses *in vivo* versus *in vitro* in an attempt to identify non-typical estrogen response elements that influence ERα-mediated transactivation.

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

by

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Biography

I attended primary school in Baltimore, Maryland and developed an interest in science at a young age. I was a very curious child and my mother encouraged me to participate in several science summer programs through middle and high school. After graduating from Western Senior High School, I began my studies in Applied Biology at Georgia Institute of Technology in Atlanta, Georgia. While attending Georgia Tech, I volunteered at Grady Memorial Hospital and mentored elementary school girls in reading and math with the Nexus Art Project. Later as a graduate student in the Physiology Program at North Carolina State University, I continued to mentor girls as a part of the Women in Math and Science Program.

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Chapter 1

Review of Literature on Estrogen Actions

I. Introduction

Lactoferrin, a major milk whey protein, was first isolated from human milk in 1960 (Johansson 1960). Based on its gene structure, lactoferrin was classified as a non-heme member of the transferrin gene family of iron-binding glycoproteins. Subsequent studies showed that this protein was also expressed in polymorphonuclear leukocytes, mucosal secretions, and a variety of tissues including the mammary gland, uterus, vagina, prostate, seminal vesicle fluid, bone marrow, stomach, intestine, and colon (reviewed in Teng 2002). Lactoferrin gene expression is constitutive in wet surface mucosal epithelia, but regulated by developmental factors during embryogenesis and in other tissues including blood cells, the testis, the uterus and the mammary gland postnatally.

In human milk, lactoferrin expression is contrary to other milk proteins; lactoferrin concentration is highest during involution and in colostrum while the secretion of other milk proteins peaks during lactation (Schanbacher *et al.* 1993). Initially, prolactin was proposed to regulate lactoferrin gene expression during lactogenesis, as it was known to induce expression of other milk protein genes. Using cultured mammary explants from mid-pregnant mice, Green and Pastewka (1978) demonstrated that the amount of lactoferrin protein secreted into the medium increased as the concentration of exogenous prolactin increased during cell culture. However, this result remains controversial as recent experiments using porcine mammary epithelial cells cultured on collagen suggested that

lactoferrin gene expression, analyzed by RT-PCR, occurred by prolactin-independent mechanisms (Kumura *et al.* 2001). Endogenous expression of the β-casein gene was induced by the lactogenic hormones insulin, hydrocortisone, and prolactin, whereas lactoferrin transcripts were observed regardless of the presence of prolactin and hydrocortisone in the culture medium. Additionally, components of the extracellular matrix elicited cell shape changes that induced lactoferrin gene expression in cultured mouse mammary epithelial CID-9 cells (Close *et al.* 1997). In fact, the actual lactoferrin content in the human, murine and bovine milks varies, but colostrum consistently contains higher concentrations of lactoferrin compared to mature milk (Table 1.1). Given that the estrogen levels in bovine colostrum and mature milk (Table 1.2) parallel lactoferrin concentrations in these glandular secretions, it can be postulated that estrogen may participate in the regulation of stage-specific lactoferrin gene expression in the mammary gland. This is true in the mouse uterus, where lactoferrin protein expression parallels cycling estrogen levels during the estrous cycle (Fig 1.1). Lactoferrin was highly expressed during proestrus and estrus, when estrogen levels were highest, and lactoferrin protein levels declined during metestrus and diestrus, characterized by lower circulating estrogen levels.

Alignment of the human and mouse lactoferrin gene promoters revealed overlapping COUP-TF binding element (COUP) and estrogen response element (ERE) in the same positions in both promoters (Liu and Teng 1991, Teng *et al.* 1992). Based on the presence of an ERE in the natural lactoferrin gene promoters and the correlation between high estrogen and lactoferrin levels in colostrums, we proposed to study the mechanisms of estrogen action on the human and mouse lactoferrin gene promoters in mammary gland cells. In this research, we analyzed the mechanism by which the ERE sequence in the context of its

natural promoter environment affects receptor conformation and receptor interactions with transcriptional cofactors. Therefore, in this review, we will summarize the mechanisms of estrogen action on hormone responsive genes by specifically examining the contributions of the nuclear receptor, ligand, hormone response element, and cofactors to coordinated estrogen-induced gene expression. A brief description of the nuclear receptor superfamily members and a more detailed account of the estrogen receptors and their classical mechanisms of transcriptional activation and repression involving complex interactions with agonists, antagonists, various EREs, and large coregulatory protein complexes will be reported. We will also address non-classical estrogen responses mediated by either novel membrane-associated estrogen receptors or nuclear estrogen receptor interactions with other DNA-dependent transcription factors at non-estrogen responsive elements.

II. Nuclear Receptor Superfamily

The nuclear hormone receptor (NR) superfamily consists of a group of transcription factors that share a common structural organization. Their ligands and the mechanisms by which they bind DNA enhancer elements located in the promoter regions of target genes to regulate transcription are intensely studied to date. According to the tripartite model of steroid hormone action, the function of each individual nuclear receptor is defined by its ligand, but other factors including the DNA binding element and surrounding sequences and receptor interactions with cofactors that link it to the basal transcription machinery determine receptor effectiveness (Katzenellenbogen *et al*. 1996).

The nuclear hormone receptor (NR) superfamily is divided into six subfamilies grouped according to evolutionary analysis of two well-conserved NR domains, the DNA-

binding domain and the ligand-binding domain (Nuclear Receptor Nomenclature Committee 1999). Type I nuclear receptors include thyroid hormone receptor (TR), vitamin D receptor (VDR), retinoic acid receptor (RAR), constitutive activated receptor (CAR), and peroxisome proliferator activated receptor (PPAR). The ligands for this subfamily of NRs are very diverse, ranging from peptide hormones to fatty acids to xenobiotics to leukotrienes and prostaglandins. Ligand binding prompts these NRs to bind their individual direct repeat DNA sequences as heterodimeric complexes with their binding partner retinoid X receptor (RXR), a member of the type II subfamily of NRs (Fig. 1.2A). Other notable members of the type II subfamily include chicken ovalbumin upstream promoter transcription factor (COUP-TF), hepatocyte nuclear receptor 4 (HNF4), and testis receptors (TR2), and these transcription factors bind their specific direct repeat DNA sequences as dimers (Fig. 1.2B). Ligands for some of the type II subfamily of NRs have not been identified, thus, these nuclear receptors are referred to as orphan nuclear, whereas fatty acids are endogenous HNF4 ligands and 9-cis-retinoic acid is the RXR ligand. Type III receptors include the classical steroid receptors (estrogen receptor ER, progesterone receptor PR, androgen receptor AR, glucocorticoid receptor GR, and mineralcorticoid receptor MR), which are sequestered in chaperone complexes with heat shock proteins and immunophilins in the absence of their specific sex and adrenal steroid ligands (reviewed in Tsai and O'Malley 1994). Upon ligand binding, the steroid receptors are released from inhibitory complexes and subsequently bind as homodimers to their cognate inverted repeat DNA sequences separated by three nucleotides in the promoters of hormone responsive genes (Klein-Hitpass *et al*. 1989, Fig. 1.2C). Another member of the type III subfamily of NRs is the estrogen-related receptor (ERR), which is closely related to ER, but whose natural ligand has not been identified to

date. The fourth, fifth, and sixth NR subfamilies contain members of the NGF-induced clone B orphan receptors (NGFIB), members of the steroidogenic factor 1/ Fushi Tarazu factor 1 receptors (SF-1/ FTZ-F1), and the germ cell nuclear factor orphan receptor (GCNF1), respectively, which bind their hormone response elements as monomers, homodimers, and heterodimers.

III. Estrogen Receptor-Mediated Estrogen Action

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 and Korach 1999). The biological actions of this steroid are relatively understood in that several main players including the estrogen receptor, the cis-regulatory DNA element and the availability of cofactors in the cellular environment collectively influence transcription through multiple physical interactions. The physiological actions of estrogens in the mammary gland and other target tissues are mediated by two estrogen receptor subtypes, ERα and ERβ (Green *et al.* 1986, Kuiper *et al*. 1996, Mosselman *et al*. 1996, Tremblay *et al*. 1997, Walter *et al*. 1985). These subtypes are not isoforms, but rather genetically different ligand-activated receptors encoded on different chromosomes in both humans and mice. The human ERα gene is localized on chromosome 6 (Menasce *et al*. 1993) and the human ERβ gene is localized on chromosome 14 (Enmark *et al*. 1997).

a. Structural Organization

Estrogen receptors -α and -β transcripts consist of eight exons in humans or nine exons in mice (Fig. 1.3A) and encode proteins of an approximate molecular mass of 66 kDa (ERα) and 50 kDa (ERβ) (Green *et al.* 1986, Kuiper *et al*. 1996, Mosselman *et al*. 1996, Tremblay *et al*. 1997, Walter *et al*. 1985). The N-terminus of ERβ is shorter than ERα, contributing to most of the size difference between the two receptors. A typical NR has five distinct functional domains that regulate receptor function through various mechanisms and the general structural organization of the ERs is depicted in Figure 1.3B. The N-terminus, defined as the (A/B) domain, shares only 17% overall amino acid sequence identity of ERα and ERβ. This domain harbors an intrinsic ligand-independent transactivation function, which is constitutively active in a cell-type and promoter-specific manner (Metzger *et al*. 1995) and has variable transcriptional potency between the two ERs (Cowley and Parker 1999, McInerney *et al*. 1998b). The DNA binding domain (DBD, domain C) is the most conserved region between the ER subtypes bearing 94% amino acid sequence identity. The DBD directs receptor binding to the major groove of cis-regulatory DNA sequences in the promoters of estrogen responsive genes through two zinc-finger DNA-binding motifs (Kumar *et al*. 1987, Fig. 1.3C). Additionally, the DBD confers DNA binding specificity through a group of six amino acids within the first zinc finger of the DNA binding domain termed P box (Schwabe *et al.* 1995, Fig. 1.3C). The ERα and ERβ P-box sequences are the same, bearing the residues CEGCKA, which are different from those of the other steroid nuclear receptors PR, GR, MR and AR (CGSCKV), allowing the estrogen receptor to distinguish an ERE from another hormone response elements (Schwabe *et al.* 1993). Although the P box is not the sole determinant of selective DNA sequence recognition,

mutation of the ERα P box sequence to the corresponding GR sequence directed ER binding to glucocorticoid responsive elements instead of the consensus ERE (Mader *et al*. 1989). The D box region of the DBD has been shown to participate in receptor dimerization, although the major site for receptor-receptor interaction is located in the E domain (Fig. 1.3C). The hinge domain (D) is the most poorly conserved region between the two estrogen receptors, which share only 10% amino acid sequence identity. Inhibitory complexes composed of heat shock proteins and other factors bind this domain in the absence of ligand and these complexes are displaced once the ligand binds the receptor (reviewed in Tsai and O'Malley 1994). The hinge domain also contains the nuclear localization sequence. The ligand binding domain (LBD, domain E) of the estrogen receptors shares 55% amino acid sequence identity and this multifunctional domain mediates receptor interactions with its ligand (Kumar *et al*. 1986 and 1987), transcriptional cofactors (Mak *et al*. 1999), and other ER molecules to form homodimers (Kumar and Chambon 1988). Despite a relatively low homology in the LBD between the ERα and ERβ, their three-dimensional LBD structures are quite similar (Brzozowski *et al*. 1997, Pike *et al*. 1999, Shiau *et al*. 1998). Crystallographic analyses have revealed that the ER LBD is composed of twelve anti-parallel α-helices arranged to facilitate contact with agonists, antagonists and cofactors. The C-terminus of the ER constitutes the F domain, which is not well conserved between the ER subtypes that share only 18% amino acid homology in this region. Mutational studies of ERs lacking this domain showed that this region was also involved in receptor dimerization and interaction with coactivators.

The coexpression of ERα and ERβ mRNA in the ovary and uterus (Couse *et al*. 1997, Kuiper *et* al. 1997) and the fact that the proteins share a high degree of homology of their DBDs raised the possibility that the receptors formed heterodimers *in vivo* in tissues expressing both receptor subtypes. Experiments designed to answer this hypothesis showed that when ERα and ERβ were simultaneously transfected into the same cell line, the reporter activity from multiple copies of the consensus ERE did not change significantly compared with the activity observed with transfection of ERα alone (Pettersson *et al*. 1997). Moreover, incubation of *in vitro* translated mouse ERβ and human ERα proteins with 17β-estradiol and a radiolabeled double-stranded ERE oligonucleotide produced DNA-protein complexes consisting of both ERα and ERβ, as detected by antibodies specific for each subtype. Additional experiments designed to detect protein-protein interactions also suggested that ERα and ERβ were able to form heterodimers *in vitro*, however the existence of ER heterodimers *in vivo* has not been substantiated.

In addition to these five major functional domains, two inherent activation functions located within the A/B and E domains activate ERα and ERβ (Fig 1.3B). The AF-1 domain, located in the amino terminus of the receptor, functions in the absence of ligand, while the AF-2 domain, located helix 12 of the LBD, has a ligand-dependent transactivation function (Tasset *et al*. 1990). Functional interactions between both AF-1 and AF-2 domains are required for full transcriptional activity of the estrogen receptors. (Kumar *et al*. 1987, Cowley and Parker 1999, Tora *et al*. 1989). Subsequent sections of this literature review will summarize how the various NR functional domains and activation domains function to stimulate optimal ER-mediated estrogen responses.

b. Tissue Distribution and Predicted Functions

The identification of NR gene and protein expression patterns has broadened our knowledge of their biological functions in a plethora of tissues under physiological conditions. This abundance of data has been generated predominantly by the use of gene-targeting technology to disrupt the genes encoding the various nuclear receptors, reverse transcription polymerase chain reaction technology, Northern and Western blot analyses, and immunohistological studies and has affirmed noteworthy differences in tissue distribution between ERα and ERβ. Whereas ERα mRNA and protein are mainly expressed in female reproductive organs including thecal cells of the ovaries, uterine epithelial cells, the vagina and mammary gland and in some non-reproductive tissues including the bones and central nervous system, ERβ mRNA and protein are expressed abundantly in male reproductive organs including prostate and testis and to a lesser extent in the ovaries, uteri, and mammary glands of females (Couse *et al*. 1997, Kuiper *et al*. 1997).

In addition to displaying distinct protein expression profiles, ERα and ERβ also function independently when expressed in identical reproductive tissues including the ovary, uterus, sperm, prostate, and mammary gland. Targeted disruption of the ERα gene (αERKO) produced infertile females while the males have decreased fertility rates (Lubahn *et al*. 1993). The females did not ovulate because of undeveloped follicles that were devoid of corpora lutea and their serum levels of estrogens, androgens and luteinizing hormone (LH) were higher compared to age-matched wild-type littermates. In contrast, the ovaries and follicles of ERβ knockout mice (βERKO) appeared normal, but their rates of ovulation decreased compared to wild-type littermates (Krege *et al*. 1998). Although the follicles developed at all stages of development, the sera of βERKO mice contained normal levels of

estrogens, androgens, and LH. These studies indicated that the ovarian phenotype of the αERKO mice was a result of hormone imbalance, while that of the βERKO mice was due to loss of ERβ signaling (Krege *et al*. 1998, Lubahn *et* al. 1993, Schomberg *et al.* 1999). Furthermore, estrogen treatments up-regulated expression of well-established estrogen responsive genes in both wild-type and βERKO uterine tissue samples, but not in the uterine tissues of αERKO mice, indicating the critical role of ERα, but not ERβ, in mediating genomic responses in the uterus (Krege *et al*. 1998). ERα has also been implicated in playing a role in spermatogenesis as the motility of sperm cells was severely impaired in αERKO males (Eddy *et al*. 1996, Hess *et al*. 1997). On the contrary, sperm cells in βERKO mice developed normally, but their prostates were overgrown, displaying evidence of hyperplasia as early as three months in age (Krege *et al*. 1998, Rosenfeld *et al*. 1998), suggesting that ERα and ERβ performed different biological functions in the same estrogen-responsive target tissue. In the mammary gland, ERβ protein appeared to be ubiquitously expressed during all stages of mammary gland development, whereas ERα protein distribution pattern varied and proliferating mammary epithelial cells did not express ERα (Saji *et* al. 2000, Speirs *et al*. 2002). Furthermore, stromal-epithelial recombinant graft studies using cells from wild-type and αERKO mice have demonstrated that paracrine factors secreted from ERα positive stromal cells stimulated ERα negative epithelial cells to branch and invade the fat pad in response to estrogen (Cunha *et al*. 1997). Accordingly, the mammary glands of βERKO mice developed normally during puberty (Krege *et al*. 1998), however, ductal development was not observed in pubertal αERKO mice (Bocchinfuso and Korach 1997). Taken together, these results provided evidence of an essential role for ERα

and not ERβ in mammary gland development, although both receptors are expressed in the gland. Although the spatio-temporal expression patterns of the estrogen receptor subtypes helps to determine whether estrogen has a physiological role in that tissue, several other factors probably contribute to the establishment of tissue- and receptor-specific estrogen responses. In subsequent sections of this review, we will address the role of ligands, promoter elements, and coregulatory factors in regulating tissue- and receptor-specific estrogenic actions.

c. Human Disease Models of Estrogen Action

Within the past decade, a man with naturally occurring inactivating mutation in both alleles of ERα (Smith *et al*. 1994) and several patients of both sexes harboring mutations in the aromatase gene (reviewed in Faustini-Fustini *et al*. 1999), whose protein product converts androgens to estrogens, were identified. The female patients with aromatase gene mutations displayed ambiguous external genitalia at birth, although their internal reproductive organs were normal. At puberty, these women failed to develop breasts and menstruate and their ovaries contained multiple cysts. In addition, the clitoris of one patient was enlarged, which is a sign of virilization. These human aromatase mutation cases detailing the function of estrogen in human female reproduction confirmed the phenotypes of αERKO mice, which have undeveloped mammary glands and ovaries and they do not ovulate (Lubahn *et al*. 1993).

A change in the thymine nucleotide to a cytosine at codon 157 of exon 2 created the ERα mutation in the male patient (Smith *et al*. 1994), resulting in a premature stop codon that produced a truncated receptor lacking both DNA and ligand binding ability (Fig. 1.3A).

This male with the ERα mutation (Smith *et al*. 1994) and the male patients bearing mutations in the aromatase gene (reviewed in Faustini-Fustini *et al*. 1999) exhibited normal external genitalia and they underwent puberty, but they continued to grow in height during adulthood. Further examination of their bones showed unfused epiphyseal growth plates, contributing to their unusually tall stature of nearly seven feet. Furthermore, their serum profiles revealed high levels of estrogens, LH and follicle stimulating hormone (FSH) and normal levels of testosterone and growth hormone, indicative of estrogen resistance and similar to the phenotype of αERKO mice (Lubahn *et al*. 1993). These patients also displayed signs of delayed bone age and osteoporosis, demonstrating a role for estrogen in the development and maintenance bones. Similar results defining the role of estrogen in bone development and metabolism were obtained from studies of ovariectomized rats (reviewed in Turner *et al*. 1994). The effects of ovariectomy included decreased bone mineral density and bone strength, whereas increases in height and rates of bone turnover were also observed. These results are in agreement with the effects of estrogen withdrawal in postmenopausal women (reviewed in Vaananen and Harkonen), accordingly estrogen replacement therapy reversed several of the effects caused by ovariectomy in rats. Taken together, these case studies confirmed the complex role of estrogen in the function and maintenance of diverse physiological systems in mammals.

d. Ligands

Unliganded ER subtypes are predominantly localized in the nucleus (Aumais *et al*. 1997, reviewed in Ylikomi *et al*. 1998) and binding of the natural ligand 17β-estradiol induces conformational changes in the receptor LBD (Brzozowski *et al*. 1997, Pike *et al*.

1999, Shiau *et al*. 1998), resulting in receptor departure from an inhibitory complex with heat shock protein, formation of receptor homodimer, and binding of receptor homodimer to estrogen response elements (ERE) in target gene promoters (reviewed in Tsai and O'Malley 1994). Recently, three-dimensional crystallographic structures of ERα and ERβ LBDs alone or complexed with the agonists 17β-estradiol or the synthetic estrogen diethylstilbestrol (DES) and the antagonists raloxifene or tamoxifen have been described (Brzozowski *et al*. 1997, Pike *et al*. 1999, Shiau *et al*. 1998). The x-ray crystal structure of unliganded ERα LBD showed that it was folded into a 3-layered anti-parallel α-helical sandwich comprising a central core layer of 3 helices (H5/6, H9, H10) sandwiched between two additional layers of helices (H1-4 and H7, H8, H11) (Brzozowski *et al.* 1997). Estrogen binding to ERα altered the position of α-helices 3, 4, 5 and 12 of the LBD, specifically, helix 12 (Fig. 1.4A, blue rod) was re-positioned to open the ligand-binding pocket and formed a coactivator recognition surface that allowed recruitment of multiple cofactor complexes to the receptor-occupied target gene promoter (Brzozowski *et al.* 1997, Shiau *et al.* 1998). This same receptor conformation was also reported when diethylstilbestrol, a potent synthetic estrogen, was bound to the ERα LBD; the ligand was completely enclosed in a predominantly hydrophobic cavity and the position of helix 12 in the LBD exposed the coactivator recognition surface within the receptor (Shiau *et al.* 1998). Because point mutations in helix 12 abolished AF-2 activity but did not affect ligand binding or DNA binding (Pike *et al*. 1999), it was predicted that helix 12 contributed to the formation of the cofactor recognition surface in the presence of agonists (Danielian *et al*. 1992, Feng *et al*. 1998, Wrenn and Katzenellenbogen 1993). Taken together, these structural studies along with mutational analysis of the LBD specifically identified helix 12 as an important component of AF-2

ligand-dependent receptor activity. The three-dimensional crystal structure of the agonist bound ERβ LBD is identical to that of ERα (Pike *et al*. 1999). When complexed with estradiol, the ligand-binding pocket is buried within the hydrophobic core of LBD by helices 3, 6, 8, 11, and 12. The similar tertiary structures of the ERα and ERβ LBD bound to agonists may explain why the majority of ligands bind each receptor subtype with comparable affinities (Kuiper *et al*. 1997) and suggests that cofactor selectivity may be a consequence of the shared LBD structure (Hall *et al*. 2002). Since both ER subtypes are expressed in the same tissues and function differently in response to the same ligand (see section III part b), another regulatory level of receptor transcriptional activity must exist.

In addition to agonists, several antagonists complexed to the receptor also modulated receptor conformation as revealed by crystallographic studies. When ERα or ERβ LBDs were complexed with antagonists raloxifene (Brzozowski *et al*. 1997) or tamoxifen (Shiau *et al.* 1998), helix 12 (Fig. 1.4B, green rod) was displaced from its agonist position to a conformation that obstructed cofactor binding. Thus, the LBD of the estrogen receptor assumed different conformations in the presence of agonists and antagonists (compare Figs. 1.4A and 1.4B), which may influence subsequent receptor interactions with DNA binding elements and cofactors that function to enhance the level of receptor-mediated gene transcription.

e. Receptor Phosphorylation

It is generally accepted that phosphorylation plays a role in the transition of a latent nuclear receptor to a transcriptional active state as all NRs, including both ER subtypes, are phosphorylated after binding their respective ligands. In addition to ligand binding, many of

the subsequent steps in nuclear receptor transcriptional activation appear to be influenced by kinase activity, including ligand-dependent and ligand-independent activations functions, receptor dimerization, and receptor interactions with coregulatory proteins. Although NR phosphorylation traditionally occurs after ligand binding to help facilitate binding to DNA, the role of phosphorylation in the absence of ligand is beginning to emerge (Denton and Notides 1992).

The AF-1 ligand-independent activation function of ERα is modulated by mitogen activated protein kinase (MAPK)-mediated phosphorylation of the A/B domain at serine 118 in response to epidermal growth factor (EGF) (Kato *et al*. 1995, White *et al*. 1997). In fact, αERKO mice lacked uterotropic responses to EGF stimulation (Curtis *et al*. 1996), confirming the involvement of ERα in mediating EGF action. Phosphorylation also increased the interaction of unliganded estrogen receptors with coregulators. As in the case of ERβ, ligand-independent phosphorylation of serines 106 and 124 in the AF-1 region occurred via MAPK activity and resulted in the recruitment of coregulators that enhanced receptor-mediated transcriptional activation (Tremblay *et al*. 1999). Another kinase target in the ERα is serine 236, located in the DBD. Phosphorylation of this residue by protein kinase A in response to cAMP enhanced receptor dimerization in the absence of ligand (Chen *et al*. 1999b). Phosphorylation of residues within the AF-2 region of ERα could also stimualte transcriptional activity through the recruitment of cofactors in the absence of estrogen (White *et al*. 1997). Upon substitution of tyrosine 537, located in the C-terminus of helix 11 of the LBD of ERα, to a serine residue, the serine residue was subsequently phosphorylated and the receptor was then able to recruit positive coregulators that enhanced receptor-mediated transcription in the absence of ligand (Weis *et al*. 1996). Thus, the effects of estrogen to

produce a transcriptionally active receptor and alter receptor conformation were mimicked by the amino acid substitution and a subsequent phosphorylation event. Any knowledge gained from such phosphorylation studies may help disclose the mechanism of transactivation of the so-called constitutive active orphan nuclear receptors whose natural ligands are unidentified to date.

IV. Promoter-Mediated Regulation of Estrogen Responsive Gene Expression

A critical step in estrogen action is the recognition of the ERE by liganded ERs and the role of the ERE in estrogen action is under intensive investigation currently. Several studies focusing on effects of estrogen response elements on classical ER-mediated transactivation have demonstrated the following three major points: 1) variations in the consensus ERE sequence occur naturally and may result in different ER transcriptional activity, 2) ER binding to ERE does not always result in a corresponding level of transcriptional activity, and 3) the amount of transcriptional activation detected from the same ERE depends on cell-specific factors and surrounding promoter elements (reviewed in Klinge 2001). With an increasing number of characterized estrogen responsive genes encoding natural EREs and the variable DNA binding affinities and transcriptional activities of the ER subtypes bound to these enhancer regions, the complexity of understanding estrogen responses in various tissues grows continuously. Here, we will discuss how variations in the DNA sequence itself impacts both ERα and ERβ binding affinities and transcriptional activities at estrogen responsive DNA-binding elements.

The consensus ERE was determined by aligning the promoter regions of the *Xenopus laevis* vitellogenin (vit) genes A1, A2, B1, B2 and the chicken apo-VLVLII gene (Walker *et*

al. 1984), yielding a minimal 13 bp palindromic sequence 5'GGTCAnnnTGACC3' (n, any nucleotide) (Klein-Hitpass *et al.* 1988). To date, approximately twenty-five estrogen responsive genes have been identified and their estrogen responses in transfected cells characterized (reviewed in Klinge 2001). Of these genes, only one, the vitA2 gene, encodes the consensus palindromic ERE. All other known natural estrogen response elements are imperfect palindromes that differ from the consensus by at least one base pair (bp) change (Table 1.3). The vitA2 gene is the most widely studied estrogen responsive gene, therefore it is the only gene for which both ERα and ERβ binding affinities have been determined (reviewed in Klinge 2001).

Numerous studies have reported that both consensus and imperfect EREs act as enhancers to heterologous promoters, albeit the ERα- and ERβ-mediated transcriptional activities from these enhancers are different from one another. For example, ERα activated transcription of the consensus ERE sequence linked upstream of the thymidine kinase-luciferase reporter construct at a much higher rate compared to ERβ in various cells and promoter contexts (Cowley and Parker 1999). In this report, the authors delineated that the weaker ligand-independent AF-1 function of ERβ located in the A/B domain was the basis for the lower transcriptional activity of ERβ relative to ERα. Furthermore, replacement of the A/B domain of ERβ with the A/B domain of ERα produced an ER chimera that now exhibited an improved transcriptional response to estrogens in three different cell lines transfected with the pS2 ERE-CAT reporter and the chimeric ER expression vector (McInerney *et al*. 1998b).

Additional studies of reporter genes containing simplified imperfect EREs that lack the surrounding sequences in the natural promoter have also demonstrated lower

transcriptional activity of ERβ compared with ERα (Barkhem *et al*. 1998, Hall and Korach 2002, Hall and McDonnell 1999, Loven *et al*. 2001a and 2001b). An important realization from these studies with imperfect EREs was that these natural ERE variants conferred different transcriptional responses to the same receptor and the same ligand. For example, transient transfection studies performed in HepG2 cells overexpressing ERα and the human complement 3, mouse lactoferrin or human pS2 EREs linked to the minimal TATA-luciferase reporter showed that the highest level of ERα-mediated estrogen-induced reporter activity occurred with the complement 3 ERE, followed by the mouse lactoferrin ERE, and lastly the pS2 ERE (Hall *et al*. 2002). The complement 3 gene ERE has two nucleotide substitutions in its 5' half-site arm compared to the consensus sequence, while the mouse lactoferrin and human pS2 EREs have only one bp change each (Table 1.3), suggesting that the number of bp substitutions in the ERE may not be the major determinant of ERα-mediated transactivation of a given gene. Rather, specific nucleotides within the ERE may play more critical roles in mediating contact with the receptor. Given that the ER subtypes share an extremely high degree of homology in their DBDs, it is highly possible that the nature of the ligand, cell-context, and promoter-specific sequences, particularly the ERE, contributes to the receptor binding affinity and transcriptional responses to ligand (reviewed in Klinge 2000). This point is substantiated by receptor binding studies showing that the ERE sequence itself modulates receptor activity. The human pS2, *Xenopus* vitellogenin B1 (vitB1) and human oxytocin genes encode naturally diverse imperfect EREs that were differentially protected by MCF-7 nuclear protein extracts in DNase I footprint assays (Wood *et al*. 2001). Table 1.4 shows the ERE sequence of these three genes differs from the perfect palindrome by a one bp change (bold nucleotides) and highlighted by asterisks are the

consensus ERE nucleotides that the ERα homodimer makes important contacts. It is clear that the nucleotide change in the oxytocin 5' arm of the ERE does not affect a critical nucleotide, while nucleotides critical receptor binding are altered from the consensus sequence in the vitB1 and pS2 genes. Accordingly, there was enhanced protection of the oxytocin ERE compared to the pS2 and vitB1 EREs by MCF-7 nuclear protein extracts (Wood *et al*. 2001).

Electrophoretic mobility shift assays (EMSAs) performed using ^{32}P-labeled DNA fragments containing the consensus of the human pS2, human oxytocin, or vitB1 EREs also indicated that both ER subtypes bound effectively to the various DNA fragments (Loven *et al*. 2001a and 2001b). The only apparent differences in receptor-DNA complexes were noted among the EREs themselves. The regulatory role of the ERE in ERα transcriptional activity was also confirmed by EMSA studies using a single or multiple copies of a 38 bp consensus ERE. Both ER subtypes bound the various tandem DNA fragments with almost identical binding affinities (Tyulmenkov et al. 2000) and limited protease digestion EMSAs of ERα and ERβ bound the various synthetic EREs alone or complexed with agonists or antagonists did not reveal differences in receptor conformation due to the various ligands, although the ligands imparted variable transcriptional activities to the two estrogen receptors (Yi *et al*. 2002). Taken together, these data showed a clear discord between ERα binding affinity and transcriptional activity, suggesting that the ERE sequence itself is a major determinant of receptor binding affinity. In summary, with regard to transcriptional activity, ERα generally, but not exclusively is a more potent transcriptional activator than ERβ in cell based assays utilizing the same estrogen responsive gene and identical ligand.

V. Alternative Mechanisms of Estrogen Action

Estrogen actions are traditionally mediated by the ER subtypes that function in the nucleus by directly contacting enhancer regions in promoters of target genes to activate or repress transcription. Now, other potentially important pathways of estrogen action are becoming apparent. One alternative mechanism of estrogen action occurs at the genomic level through protein-protein interactions at non-estrogen responsive elements. Another mechanism of estrogen action does not directly affect the genome, but rather involves rapid signaling pathways mediated by putative plasma membrane-associated estrogen receptors.

a. Non-Estrogen Response Elements

Most of the studies of estrogen responsive genes have focused on the ER-mediated transcriptional activity from a simplified ERE sequence and have not examined the activity of the receptor bound to the ERE in the context of its natural promoter elements. The significance of surrounding regulatory elements in mediating estrogen responses is illustrated in studies of estrogen responses from genes lacking perfect of imperfect ERE sequences. For decades it was thought that direct binding of ER to an ERE was the only mechanism of estrogen action, but over the past several years, researchers have also reported estrogenic responses from genes lacking predicted EREs in their promoters. Now we know that ERs can influence expression of genes without directly binding to ERE sequences in DNA. These non-classical mechanisms of estrogen action are mediated by physical interactions between liganded ER and AP1 or SP1 transcription factors at their respective DNA binding elements.

It is well documented that the cyclin D1 is an estrogen inducible gene, but promoter analysis did not reveal the presence a classical estrogen responsive sequence. Subsequent

transient transfection studies with reporter constructs containing sequential deletions of the cyclin D1 promoter region showed that an upstream AP1 site was responsible for the ER-mediated estrogen response of the cyclin D1 gene (Liu *et al*. 2002). Furthermore, ERα and ERβ displayed opposite actions in the presence of both estrogens and antogonists at the AP1 site in the cyclin D1 gene. Lysates from HeLa cells transfected with ERα and treated with 17β-estradiol or diethylstilbestrol contained higher levels of cyclin D1 mRNA, while transfection of ERβ in these cells inhibited the induction of cyclin D1 gene expression in response to the same ligands (Liu *et al*. 2002). In contrast, anti-estrogens stimulated endogenous cyclin D1 expression in HeLa cells overexpressing ERβ, but transcription of this gene was repressed when an ERα expression vector was transfected into the cells. Differential activation of ERα and ERβ at AP1 sites in response to the same ligand has also been noted in transfection experiments using a synthetic AP1 reporter gene (Paech *et al*. 1997). Again, 17β-estradiol activated transcriptional activity of ERα and inhibited ERβ-mediated transcription. Moreover, the ER antagonists raloxifene and tamoxifen activated ERβ-mediated transcription at AP1 sites, but not ERα. The mechanism of liganded ER enhancement of AP1 activity involved both AF-1 and AF-2 receptor functions that interacted with both Fos and Jun subunits of the AP1 protein complex and then recruited distinct members of the p160 family of coactivators to the liganded estrogen receptor (Webb *et al*. 1999).

The cAMP responsive element (CRE) located in the proximal promoter region of the cyclin D1 gene was also shown to participate in the estrogen response of the gene as chromatin immunoprecipitation assays showed that both SP1 and ERα interacted at the CRE to mediate estrogen-induced cyclin D1 gene expression (Castro-Rivera *et al*. 2001). ERα

also interacted with SP1 bound to GC-rich regions in the distal promoter region of the E2F-1 gene in MCF-7 cells stimulated with 17β-estradiol (Ngwenya and Safe 2003). It is unknown at this time whether ERα and ERβ interactions with SP1 result in different transcriptional activities in response agonists and antagonists, but the observed opposites actions of the ER subtypes at AP1 sites may explain the dissimilar roles of the receptors in mammary gland development.

b. Membrane Bound Actions

Rapid biological responses to steroid hormones that occur within 2-10 minutes of hormone treatment have been documented for years and examples of rapid responses to estrogen include the proliferation of breast cancer cell lines, prolactin gene expression via MAPK signaling, blood vessel dilation, and the uptake of fluid in the mouse uterus. Estrogen responses in the mouse uterus have been traditionally divided into two phase; rapid events that occur within the first hr of estrogen stimulation include fluid uptake (Tchernitchin 1972), while late events generally occur 24 hr after estrogen treatment and include the expression of the lactoferrin gene (Pentecost and Teng 1987). Emerging evidence from studies using antibodies directed against the classical ER epitopes has suggested that rapid estrogen responses are mediated through plasma membrane-associated estrogen receptors (mERs) (Benten *et al*. 2001, Chen *et al*. 1999c, Fiorelli *et al*. 1996, Powell *et al*. 2001, Razandi *et al*. 1999 and 2000). Although the putative mERs have not been physically isolated or sequenced to date, there exists functional and structural data suggesting the existence of mERs of both estrogen receptor subtypes.

In an attempt to study the gene and protein expression patterns of mERs, researchers transiently transfected the cDNAs for ERα or ERβ into Chinese hamster ovary (CHO) cells and detected a single transcript by Northern blot analysis (Razandi *et al*. 1999). The protein products were distributed in both nuclear and membrane subcellular fractions, although plasma membrane-associated ERα constituted only 3% of the nuclear ERα density and mERβ comprised only 2% of its nuclear counterpart density. Nonetheless, these data suggested that both the nuclear and plasma membrane-associated ERs were translated from the same transcript. In addition, competitive binding studies using 17β-estradiol and distinct receptor populations that localized to either cellular compartment demonstrated almost indistinguishable dissociation constants (K_d). For nuclear ERα, the K_d = 0.283 nM, while the K_d = 0.287 nM for mERα and the K_d's for nuclear and plasma membrane-associated ERβ were 1.23 nM and 1.14 nM respectively. Although the previous studies were performed with transiently transfected with ERα and ERβ expression vectors, Western blot analyses of purified plasma membrane extracts from MCF-7 human mammary cancer cells suggested the existence of several endogenous species of mERα that immuno-reacted with several nuclear ERα specific antibodies (Powell *et al*. 2001). The mERα proteins had relative molecular masses of 130 kDa, 110 kDa, 92 kDa and 67 kDa and only the 67 kDa protein localized in the nuclear fraction. But based on the approximate molecular mass of these uncharacterized ERα species, it is highly unlikely that they are GPCR-like proteins, which are usually ~40 kDa in size. In pituitary cell lines, an endogenous mERα that cross-reacted with an antibody specific for the nuclear ERα, was also identified based on its localization in the plasma membrane (Benten *et al*. 2001). However, the size of this protein was not detected since immunochemical labeling experiments and not Western blots were performed to detect ER

localization. Nonetheless, functional studies performed in the presence of physiological concentrations of 17β-estradiol (1nM) resulted in a rapid increase in intracellular Ca^{2+} levels that occurred within 5 min of hormone treatment in these pituitary cells. Moreover, pertussis toxin and a phospholipase C (PLC) specific inhibitor blocked this rise in intracellular Ca^{2+} stores. It is well known that specific GPCR subtypes activate PLC to generate a release of Ca^{2+} from intracellular stores and pertussis toxin can inhibit GPCR-mediated cellular responses. Therefore, these results suggested that this mER, which is localized in the plasma membrane of pituitary cells, might share structural similarity with classic GPCRs. In support of the reported GPCR-like activity of mERα in pituitary cells, physiological concentrations of 17β-estradiol also promptly induced significant increases in intracellular Ca^{2+} and cAMP levels in preosteoclast-like cells (Fiorelli *et al*. 1996). Thus, the role of estrogen in bone development as elucidated from αERKO studies and human ERα and human aromatase gene mutation case studies (Smith *et al*. 1994, reviewed in Faustini-Fustini *et al*. 1999) may be mediated by mERs in addition to nuclear ERs.

Targeted disruption of the ERα gene in mice has also proposed the role of estrogen in cardiovascular physiology (Rubanyi *et al*. 1997) and putative mERs have also been suggested to play a role in estrogen action in the cardiovascular system. In COS-7 cells, overexpression of ERα and subsequent estrogen treatment quickly increased the availability of nitric oxide by enhancing the enzymatic function of endothelial nitric oxide synthase (Chen *et al*. 1999c). This rapid estrogen response required the ERα LBD, was blocked by ER antagonists, and stimulated rapid ER-dependent activation of MAPK. In physiological relevant models, estrogen also induced rapid increases in the bioavailability of nitric oxide in blood vessels. Moreover, male αERKO mice have significantly lower basal levels of

endothelial-derived nitric oxide in their aortic blood vessels compared with wild-type male mice (Rubanyi *et al*. 1997). Based on this *in vivo* study and the *in vitro* study performed in preosteoclast-like cells mentioned above, it has been suggested that the nuclear and membrane-associated ERs may hold complementary functions; kinase signaling may rapidly and transiently activate transcription while nuclear signaling may sustain transcription (Chen *et al*. 1999c). The merits of this hypothesis describing coordinated functions of membrane and nuclear NRs are embodied in classic experiments showing MAPK-mediated phosphorylation of the various domains of unliganded nuclear ERs (Chen *et al*. 1999b, Kato *et al*. 1995, Tremblay *et al*. 1999, Weis *et al*. 1996, White *et al*. 1997). Although EGF signaling has been shown to induce activation of MAPK during MAPK-mediated phosphorylation of serine 118 in the unliganded ERα A/B domain (Kato *et al*. 1995, White *et al*. 1997), the specific signaling pathway that induced kinase phosphorylation of other residues in unliganded ERs have not been identified, thus it is possible that mER may serve to activate such kinases. A more recent study demonstrated that the nuclear receptor coactivator SRC-1 could be rapidly phosphorylated by MAPK in response to cAMP, triggering enhancement of ligand-independent PR transactivation function (Rowan *et al*. 2002).

In addition to estrogen-induced rapid responses in the mammary gland and uterus, another steroid hormone, progesterone, has been reported to mediate rapid responses during sperm acrosomal reaction and oocyte maturation (Zhu *et al*. 2003, reviewed in Revelli *et al*. 1998, reviewed in Watson and Gametchu 1999). According to studies of a recently isolated plasma membrane-associated progesterone receptor (mPR) cDNA cloned from spotted seatrout ovaries and then translated its protein product, there are seven criteria that must all

be fulfilled in order to classify a protein product as a steroid membrane receptor (Zhu *et al*. 2003). These criteria are as follows: 1) the receptor must have a plausible structure similar to G-protein coupled membrane receptors (GPCRs) since it mediates a rapid signal transduction pathway, 2) receptor expression must be tissue specific, 3) the receptor must localize in plasma membrane fractions, 4) the receptor should have a high affinity for its specific steroid and both association and dissociation kinetics should be rapid, 5) hormone binding to the receptor should produce rapid activation of intracellular signaling cascades, 6) both gene and protein expression should be up-regulated by hormone treatment, and 7) the membrane receptor should mediate a biologically relevant phenomenon. Accordingly, the mPR studied in this report satisfied all of these criteria as it was shown to participate in oocyte maturation. Unlike the mPR, collective studies of the mERs have only demonstrated cellular distribution of the putative receptor, steroid binding specificity, signal transduction, and biological relevance. It remains unclear whether mERs are expressed from the same gene as their nuclear counterparts, possess a seven transmembrane domain typical of GPCR, or whether they are up-regulated in response to estrogen in a tissue-specific manner. Nonetheless, the role of plasma membrane-associated ERs in mediating estrogen responses is possible given that scientists originally thought that only one nuclear ER existed for over 40 years and the second receptor subtype, ERβ was cloned only 7 years ago.

VI. Nuclear Receptor Coregulatory Proteins

In the previous sections, we discussed the regulatory role of ligands in ER-mediated estrogen action. Here, we will discuss the role of NR-interacting cofactors in augmenting ER-mediated estrogen action. Based on interactions with either agonists or antagonists and

subsequent engagement with coregulatory proteins, nuclear receptors function as both positive and negative transcriptional regulators. Positive NR-interacting cofactors, coactivators, enhance receptor-mediated transcription by promoting a relaxed chromatin structure and linking the receptor to the basal transcription machinery (Danielian *et al*. 1992, Feng *et al*. 1998, Wrenn and Katzenellenbogen 1993), while negative NR-interacting cofactors, corepressors, augment receptor-mediated transcription by maintaining a condensed chromatin structure that prevents the association of promoter complexes with the basal transcription machinery (Alland *et al*. 1997, Heinzel *et al*. 1997, Nagy *et al*. 1997, Spencer *et al.* 1997).

Repression of NR-mediated transcription involves many mechanisms, but it occurs mainly in the absence of a ligand or when an antagonist is bound to the receptor. All ER antagonists to date function to repress AF-2 activity by preventing positive NR-interacting cofactors from binding to the LBD, while corepressors mainly bind the receptor in absence of agonist. In order to obtain corepressor classification, a protein must meet the following criteria: 1) interact directly with the unliganded receptor to enhance repression of basal transcription repression, 2) interact with components of the basal transcription machinery and 3) possess an autonomous repression domain (reviewed in Robyr *et al*. 2000). Agonist binding to the NR promotes conformational changes in the receptor that support recruitment of coactivators, which leads to the displacement of corepressors. Like their corepressor counterparts, coactivators must also fulfill specific criteria to obtain this classification. These criteria are as follows: 1) interact directly with the activation domain of the NR in an agonist-dependent manner, but not in the presence of antagonists, 2) enhance NR function, 3) interact directly with the basal transcription machinery, and 4) should not enhance the basal

transcriptional activity in the absence of the NR because they should not be recruited to the promoters (reviewed in Robyr *et al*. 2000). Transcriptional activation occurs through ATP-dependent and -independent mechanisms of chromatin remodeling. ATP-dependent chromatin remodeling complexes use the energy of ATP hydrolysis to locally disrupt the association of histones with DNA (reviewed in Deroo and Archer 2001). Here, we will describe the various ATP-independent NR coregulators that chemically modify histones to disrupt chromatin structure, citing specific findings about interactions with the ER subtypes and the various mechanisms of transcriptional enhancement or repression.

a. Nuclear Receptor Coactivating Complexes

Cofactors that enhance receptor-mediated transcription are termed coactivators and they are initially recruited to the liganded receptor once the cofactor recognition surface becomes exposed. These coactivators interact strongly with the NR LBD in ligand- and AF-2-dependent manners. Afterward, larger coactivator complexes consisting of chromatin remodeling cointegrators that possess histone acetyltransferase and methyltransferase activities are recruited to the NR, resulting in chromatin decondensation to facilitate NR access to DNA. Another coactivator complex, termed mediator-like complexes, is subsequently recruited to connect NRs directly to the basal transcriptional machinery, resulting in transcriptional activation of target genes (reviewed in Robyr *et al*. 2000).

Among the best characterized coactivators known to specifically interact with the liganded ERs to enhance transcriptional activity are the p160 family of steroid receptor coactivators (SRC-1, SRC-2, and SRC-3, reviewed in Robyr *et al*. 2000). To date, the p160/ SRC (steroid receptor coactivator) family of coactivators has three distinct members sharing

similar protein structures (Fig. 1.5A). The most highly conserved N-terminal region contains a basic helix-loop-helix motif and serine- and threonine-rich PAS domain. The function of the PAS domain in these coactivators is unknown since coactivators are not recruited to the promoters in the absence of liganded NR bound to DNA response element. Nonetheless, this domain functions as DNA binding surface in many other transcription factors. The nuclear receptor interacting domain or NR box is located in the middle of the protein. This region mediates ligand-dependent interactions with NRs at the cofactor recognition surface through multiple, hydrophobic, highly conserved LXXLL helical motifs, where L is leucine and X is any amino acid (Heery *et al*. 1997, Fig 1.5B). Located in the C-terminus are two activation domains (AD). The general transcription cointegrator p300/CBP/pCIP interacts with AD-1 of the SRC family coactivator (Li *et al*. 2000, Voegel *et al*. 1998) and histone methyltransferases including coactivator-associated arginine methyltransferase 1 (CARM-1) and protein arginine methyltransferase (PRMT) that modify core histones to produce a relaxed chromatin structure interact with AD-2 of the SRC family coactivator (Chen *et al.* 1999a, Koh *et al*. 2001, Lee *et al*. 2002, Li *et al*. 2003). The most C-terminal region of some SRC family members encodes intrinsic acetyltransferase activity, which also modifies local chromatin architecture to facilitate NR access to promoter elements.

SRC-1 was the first human p160 gene family member cloned (Onate *et al*. 1995). Its amino acid sequence was determined and revealed the presence of four LXXLL motifs. SRC-2 (transcription intermediated factor 1, TIF-2 / GR-interacting protein, GRIP-1) was the second p160 gene family member cloned and its protein product has three NR boxes (Hong *et al*. 1996, Voegel *et al*. 1996). SRC-3 (activator of thyroid and retinoic acid receptors, ACTR/ amplified in breast cancer 1, AIB1/ receptor-associated activator 3, RAC3/ thyroid

hormone receptor activator protein 1, TRAM-1/ CBP-interacting protein, p/CIP) was the third p160 gene family member cloned and its protein product also has three centrally located LXXLL motifs (Anzick *et al*. 1997, Chen *et al*. 1997, Li *et al*. 1997, Takeshita *et al*. 1997, Torchia *et al*. 1997). Northern hybridization studies showed that SRC-3 transcripts were amplified over 20-fold in human breast and ovarian cancer cell lines including MCF-7 and in malignant primary mammary epithelial cells (Anzick *et al*. 1997). Furthermore, SRC-3 protein was highly expressed in the ERα positive MCF-7 cell line and an ERα positive endometrial carcinoma (Suen *et al*. 1998).

Ligand binding to the NR induces conformational changes, exposing a coactivator recognition surface in helix 12 of the LBD (Brzozowski *et al*. 1997, Pike *et al*. 1999, Shiau *et al*. 1998). The affinity of the interaction between the nuclear receptor LBD and the coactivator critically depends on the conserved LXXLL motifs present in the coactivator (Heery *et al*. 1997, Fig 1.5B). Each SRC protein contains at least three separate NR boxes and different NR boxes within a given coactivator are preferred by a specific NR for interaction. Coactivator-ERα binding studies and immuno-depletion studies of cells microinjected with an antibody directed against ERα and SRC-1 expression vector demonstrated that SRC-1 NR box 2 (ILHRLL) was the most important motif for mediating ligand-dependent interactions with ERα and subsequent mutation of the SCR-1 NR box 2 sequence prevented the rescue of ERα function in cells immuno-depleted of ERα and overexpressing SRC-1 (McInerney *et al*. 1998a). Likewise, the NR box 2 of SRC-2 (ILHRLL) (Ding *et al*. 1998, Leers *et al*. 1998, Voegel *et al*. 1998) and SRC-3 (ILHKLL) (Wong *et al*. 2001) bound most tightly to the ERα LBD. In addition to serving as a preferential basis for NR interactions with a particular coactivator, the NR boxes also serve

as the basis for NR discrimination among the various coactivators (Darimont *et al.* 1998), adding another level of complexity to the combinatorial control of NR-mediated gene expression. Additional ERα immuno-depletion studies showed that SRC-1 NR boxes 2 and 3 were required for the rescue of RAR and TR activity while PPARγ and PR activity was dependent upon NR boxes 1 and 2 (McInerney *et al*. 1998a). Furthermore, endogenous SRC-3 and ERα co-immunoprecipitated in MCF-7 cells under estrogen stimulation, but physical interactions between SRC-1 and ERα were not detected (Tikkanen *et al*. 2001). The authors attributed these results to the lower concentration of SRC-1 in the cell line and transient transfection experiments showed that in the absence of overexpressed SRC-3, estrogen stimulated ~4-fold increase in ERα-mediated reporter gene activity, while the addition of increasing amounts of SCR-3 enhanced receptor transcriptional activity up to 22-fold. Thus, it appears that NRs interact selectively with particular SRC family members, and with individual NR boxes within a given SRC coactivator during to enhance their transactivation activity.

The primary function of nuclear receptor coactivators is to enhance receptor-mediated transcription by recruiting cointegrators like p300/CBP that have histone acetyltransferase activity to the receptor bound to enhancer regions of target genes to modify (Kamei *at al*. 1996, Lee *et al*. 1993, Nightingale *et al*. 1998, Ogryzko *et al*. 1996, Tse *et al*. 1998, Ura *et al*. 1997). Acetylation of the lysine residues of histone tails promotes a relaxed chromatin conformation that enables transcriptional activity. The SRC family members interact with p300/CBP via its AD-1 region and the resulting complexes were unstable alone, but a stable multi-subunit complex was formed in the presence of a NR (Hanstein *et al.* 1996, Li and Chen 1998). In turn, cointegrators recruited larger coactivator complexes composed of

histone methyltransferases and mediator-like complexes. The histone methyltransferase CARM-1 interacted with SRC-2 and preferentially methylated the tails of core histone H3, which reorganized the chromatin structure to an open conformation and therefore enhanced ERα-mediated transcription (Chen *et al*. 1999a). In addition to recruiting histone methyltransferases, cointegrators also recruited multi-subunit components of mediator-like complexes to the AF-2 domain of liganded receptor. Although it is unknown whether these mediator-like complexes interacted directly or indirectly with SRC coactivators or p300/CBP, they do contain a single LXXLL motif that facilitated its interactions with the NR (Rachez *et al*. 1999, Yuan *et al*. 1998). Furthermore, these mediator-like complexes functioned to connect the NRs directly to the basal transcriptional machinery and RNA polymerase II (Shang *et al*. 2000). Like p300/CBP, SRC-1 and SRC-3 coactivators possess histone acetyltransferase activity to covalently modify core histone proteins, disrupt local higher-order chromatin structure, and facilitate NR access promoter elements, resulting in enhancement of receptor-mediated transcriptional activity). However, the histone acetyltransferase activity of SRC-1 and SRC-3 are much weaker than p300/CBP (Chen *et al*. 1997, Liu *et al.* 2001, Spencer *et al*. 1997).

It was believed initially 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, functional data generated by targeted disruption of the SRC genes have suggested complementary biological activities among this coactivator family (Xu *et al*. 1998, Xu *et al*. 2000, Gehin *et al*. 2002). Support for the hypothesis of functional redundancy among the SRC family coactivators was provided by the observation that the SRC coactivators were widely expressed in similar amounts in most cells (Kurebayashi *et al*.

2000, Vienonen *et al*. 2003) and the viable phenotypes of the various SRC null mice (Xu *et al*. 1998, Xu *et al*. 2000, Gehin *et al*. 2002). In general, there was not a dramatic phenotype in SRC-1 -/- mice as growth and fertility were normal. However, ovariectomized null mice displayed reduced mammary gland ductal branching and alveolar formation in response to estrogen and progesterone treatment (Xu *et al*. 1998). Moreover, SRC-2 was overexpressed in SRC-1 null mice, further supporting the compensatory role of the SRC proteins. Data from SRC-3 -/- mice also linked this coactivator to a regulatory function in female reproduction and mammary gland development (Xu *et al*. 2000). These mice had compromised ability to produce estradiol and were therefore estrogen deficient. Since serum estradiol levels in the SRC-3 null mice reached only ~60% of the levels in wild-type littermates, these mice experienced delayed onset of puberty and retarded mammary gland development. SRC-2 -/- mice exhibited reduced fertility in males and females and the sub-fertile status of the males was shown to be a result of defective maturation of spermatid acrosome (Gehin *et al*. 2002). Other phenotypes of these mice were manifested in the predicted role of SRC-2 in lipid metabolism and energy balance. The mice had increased ability to protect themselves against obesity induced by high-fat diets as they demonstrated higher body temperature under cold conditions and accumulated less fat and lower levels of fasting triglycerides. Although the phenotypes of each SRC null mice were unique, they were not severe, suggesting that these coactivators share similar functions in the same tissue. As in the case of SRC-1 and SRC-3, targeted disruption of these genes produced similar outcomes in the mammary gland, even though the developmental phenotypes of this gland occurred by different mechanism; SCR-1 null mice exhibited reduced estrogenic responses (Xu *et al*. 1998), while SRC-3 were estrogen deficient (Xu *et al*. 2000).

b. Nuclear Receptor Corepressing Complexes

An important aspect of NR-mediated transactivation is the association of the basal transcription machinery and RNA polymerase II with the larger protein complexes that are linked to the receptor bound to the ERE in the promoter. Unliganded ERα cycles on and off the pS2 promoter, but cannot recruit RNA polymerase II to the promoter region (Reid *et al*. 2003) because corepressors bound to the NR in the absence of ligand prevent the association of the promoter complex with the basal transcription machinery. Well-characterized members of the NR corepressor family include silencing mediator retinoic acid and thyroid hormone receptor transcription (SMRT) (Chen and Evans 1995, Nagy *et al*. 1997) and nuclear receptor corepressor (NCoR) (Alland *et al*. 1997, Horlein *et al*. 1995, Heinzel *et al*. 1997, Kurokawa *et al*. 1995) (Fig. 1.6). SMRT and NCoR are components of a corepression complex that contains histone deacetylases, which exert their functions by maintaining local condensation of chromatin, thereby preventing the association of basal transcription machinery with the receptor at the hormone response element (Alland *et al*. 1997, Heinzel *et al*. 1997, Nagy *et al*. 1997, Spencer *et al.* 1997). SMRT and NCoR were shown to interact with TR and RAR when associated with their RXR heterodimeric partner through their receptor interacting domains and the receptor DBD and LBD were critical for this association (Chen and Evans 1995, Horlein *et al*. 1995, Lavinsky *et al*. 1998). In addition to binding NRs in the absence of ligand, SMRT and NCoR have been shown to bind ERα in the presence of antagonists like tamoxifen and RU486 (Laherty *et al*. 1998, Lavinsky *et al*. 1998). However, upon agonist binding, these corepressors dissociate from the receptor, which ultimately undergoes conformational changes and recruits coactivator complexes that enhance transcription (Figure 1.7).

Traditionally, NR corepressors interact with unliganded or antagonist bound receptor at the DBD and LBD to prevent receptor access to DNA and recruitment of coactivators. For example, the anti-estrogen ICI 182,780 blocked SRC-3 enhancement of receptor transcriptional activity (Tikkanen *et al*. 2001) by reducing the cellular content of ERα through proteosome-mediated increase in receptor turnover (Suen *et al*. 1998), hence preventing both the interaction between ERα and SRC-3 and the receptor from binding the DNA (Reid *et al*. 2003). Unlike the traditional mechanism ER repression, two ER corepressors with unique repression functions have been partially characterized. The first, denoted repressor of tamoxifen transcriptional activity (RTA) interacted with the N-terminus of ERα in the presence of antagonists (Norris *et al*. 2002) and the second, termed ligand-dependent corepressor (LCoR), interacted with agonist bound ERα (Fernandes *et al*. 2003).

In general, tamoxifen fully antagonizes ERα in the mammary gland, but functions as a partial agonist in the uterus, bone and cardiovascular system, making it an ideal candidate for breast cancer chemotherapy (Bertelli *et al*. 1988, Kedar *et al*. 1994, Love *et al*. 1992). The mechanism by which RTA represses tamoxifen bound ERα transcriptional activity in tissues besides the mammary gland involves inhibition of receptor ligand-independent function. When bound to tamoxifen, ERα binds DNA, but the constitutive AF-1 activity of ERα was suppressed by RTA and an autonomous repression domain in RTA was found to mediate this unique coregulatory function. The structure of RTA is also dissimilar from the traditional NR corepressors as it contains a consensus RNA recognition motif. Since this corepressor is a fairly new discovery, the precise mechanism of RTA-mediated repression of ERα activity and whether larger corepressor complexes are recruited to the receptor are unknown. Nonetheless, it is known that agonist binding to ERα induced AF-2 activity,

which overcame RTA-mediated repression (Norris *et al*. 2002) and this finding supports the paradigm that agonist binding activates the receptor (reviewed in Tsai and O'Malley 1994) and that both AF-1 and AF-2 receptor activities are necessary for mediating maximal transcription is response to estrogen (Kumar *et al*. 1987).

Ligand-dependent corepressor (LCoR) was the first and only agonist-dependent corepressor described to date (Fernandes *et al*. 2003). Like RTA, LCoR does not structurally resemble the other well-characterized corepressors. But unlike RTA, LCoR bears a single LXXLL NR box motif that is usually characteristic of coactivators. Several *in vitro* and *in vivo* interaction assays did not show an association between LCoR and ERα in the presence of the antagonist (tamoxifen, raloxifene, or ICI) or in the absence of agonist (17β-estradiol). Rather, LCoR co-immunoprecipitated with endogenous ERα and histone deacetylase complexes in an estrogen-dependent manner in MCF-7 cells. The association of LCoR and ERα was mediated by helix 12 of the receptor LBD as mutations within this helix abolished ligand-dependent binding of LCoR. Since LCoR interacted with the receptor at its coactivator recognition surface, it can be hypothesized that competition between coactivator and corepressor binding to this site may exist (Fernandes *et al*. 2003). Thus, future studies are necessary to fully understand the unique mechanism by which LCoR functions to repress liganded ERα transcriptional activity.

VII. Summary

The preceding literature review summarized the scientific knowledge of the mechanism of estrogen action. The role of the estrogen receptor in mediating a classical estrogen response was detailed in terms of its domain structure, expression profile, conformational changes induced by agonists, antagonists, and DNA hormone response element, and the recruitment of coregulatory proteins to augment receptor-mediated transcriptional activity. The expression profiles of ERα and ERβ in various tissue are quite unique but when expressed in the same tissue or acting on the same promoter in transient transfection assays, ERα was in general a more potent transcriptional activator that ERβ. Collectively, ERs were critical for the regulation of male and female fertility, mammary gland development, bone development, and cardiovascular physiology. Based on the reported data, it is well documented that both AF-1 and AF-2 receptor functions are required for optimal ER-mediated estrogen action. Ligand-independent receptor-mediated transactivation functions were influenced by phosphorylation of specific residues in the AF-1 domain. The binding of antagonists to the receptor repressed receptor-mediated basal activity by recruiting corepressor complexes to the receptor that inhibiting receptor access to the DNA and coactivator access to the receptor. In contrast, agonist binding induced a receptor conformation that promoted recruitment of positive coregulatory complexes that rendered the chromatin accessible to receptor binding and linked the receptor with the basal transcriptional machinery. In addition, ER preferentially interacted with specific SRC family members and with individual NR boxes within a given coactivator with the presence of agonists.

From the reported data, we also learned that different ligands did not induce variable receptor conformations when bound to the same ERE, rather the ERE sequence itself determined receptor conformation. Considering that most naturally occurring EREs are imperfect palindromes and the ERE sequence itself affects ER binding affinity and consequently ER transcriptional activity, we chose to ascertain how the ERE sequence regulates ERα-mediated transcriptional activity of the human and mouse lactoferrin genes in the context of their natural promoters in this dissertation. Both human and mouse lactoferrin gene promoters encode imperfect EREs that are estrogen responsive in the uterus, but relatively little is understood about the molecular mechanisms that regulate lactoferrin expression in the mammary gland. To limit the contributions from the ligand, cellular environment, and ER subtype, we performed the ensuing experiments using the same ligand, diethylstilbestrol (DES), a potent, synthetic estrogen, the same receptor (ERα), and the same human mammary gland cell lines. As a result of our experimental design, we were able to show differential basal activity and estrogen responses of human and mouse lactoferrin gene EREs in the context of their natural promoters (Chapter 2). The dissimilar estrogen actions on these two lactoferrin gene promoters were not because of cell type- or species-specificity, but rather to the ERE sequence itself and the complexity of the natural promoter. Moreover, we determined that an estrogen response element extended half-site (ERRE) synergized with the ERE to achieve maximum estrogen response in the context of the human and mouse natural lactoferrin promoters (Chapter 3).

Table 1.1: Concentrations of Human, Bovine and Murine Whey Proteins in Various Milks

	Colostrum (mg/ml)		Mature Milk (mg/ml)			Involution (mg/ml)	
	Human	**Bovine**	**Human**	**Bovine**	**Murine**	**Human**	**Bovine**
LF	7	2	>2	0.25	0.25	40	20
αLA	1	3.6	2	1.2	0.8	ND	2
caseins	1.3	12	2	28	45	trace	trace
WAP	---	---	---	---	1	---	---
βLG	---	5	---	4	---	---	6
sIgA	20	70	2	0.03	ND	ND	50

Values are taken from the Handbook of Milk Composition (Jensen 1995) and Biochemistry of Lactation (Mepham 1993).

Abbreviations:

LF	lactoferrin
αLA	α-lactalbumin
caseins	total caseins (α_{S1}, α_{S2}, β, γ, κ)
WAP	whey acidic protein
β-LG	β-lactoglobulin
sIgA	secretory immunoglobulin A (predominant immunoglobulin in milk)
ND	not determined

Table 1.2: Estrogen Concentrations in Bovine Milks

	Colostrum **(ng/ml)**	Mature Milk **(ng/ml)**
17β estradiol	360	13
17α estradiol	470	160
Estrone	1032	28
Total Estrogen	1867	201

Values are taken from the Handbook of Milk Composition (Jensen 1995).

Table 1.3: Sequences of Natural EREs from Estrogen Responsive Genes and Corresponding ER Binding Affinities

Gene	**Sequence**	**ERα binding (Kd in nM)**	**ERβ binding (Kd in nM)**
Xenopus vitellogenin A2	5′-TCAGGTCAcagTGACCTGA	0.2-10	8
Xenopus vitellogenin B1	5′-GTA**A**GTCActgTGACCCCA	ND	ND
Human angiotensinogen	5′-ATAGG**G**CAtcgTGACCCGG	ND	ND
Human BRCA1	5′-TGGTCAggcTG**GT**CTTG	ND	ND
Human cathepsin D	5′-GGG**C**TC**G**ggcTGACCCCG	ND	ND
Human complement 3	5′-CCAGGT**GG**cccTGACCCTG	ND	ND
Human lactoferrin	5′-GCAGGTCAggc**C**GA**T**CTGT	ND	ND
Mouse lactoferrin	5′-ACAGGTCAaggT**A**ACCCAC	ND	ND
Human oxytocin	5′-CGGT**G**AcagTGACCGGC	ND	ND
Rat oxytocin	5′-GTGG**AA**CAgttTGACCCAA	ND	ND
Human Progesterone Receptor β	5′-AGGG**CAGG**agcTGACCAGC	ND	ND
Human pS2	5′-CAAGGTCAgcgTG**G**CCACC	ND	ND
Rat vasopressin	5′-CAGGG**C**CAgccTGACCGTG	ND	ND
Human VEGF	5′-**AA**TCAgacTGAC**T**GGC	ND	ND

Some genes encoding natural EREs in their promoter are given. Underlined nucleotides constitute the consensus ERE half-site sequences and nucleotides in bold are altered from the consensus ERE palindrome. ND = not determined. Taken from Klinge 2001 and references therein.

Table 1.4: Sequences of Natural EREs that Impart Changes in Nucleotides Critical for Receptor Binding

Gene	**Sequence**
Consensus Vitellogenin A2	** *** 5′-GGTCAnnnTGACC-3′
Human oxytocin	5′-GGT**G**AcctTGACC-3′
Vitellogenin B1	5′-**A**GTCActgTGACC-3′
Human pS2	5′-GGTCAcagTG**G**CC-3′

Some genes encoding natural EREs in their promoter are given. Asterisks denote the nucleotides that the ERs make critical contacts with when binding the ERE. The nucleotides in bold deviate from the consensus palindromic sequence

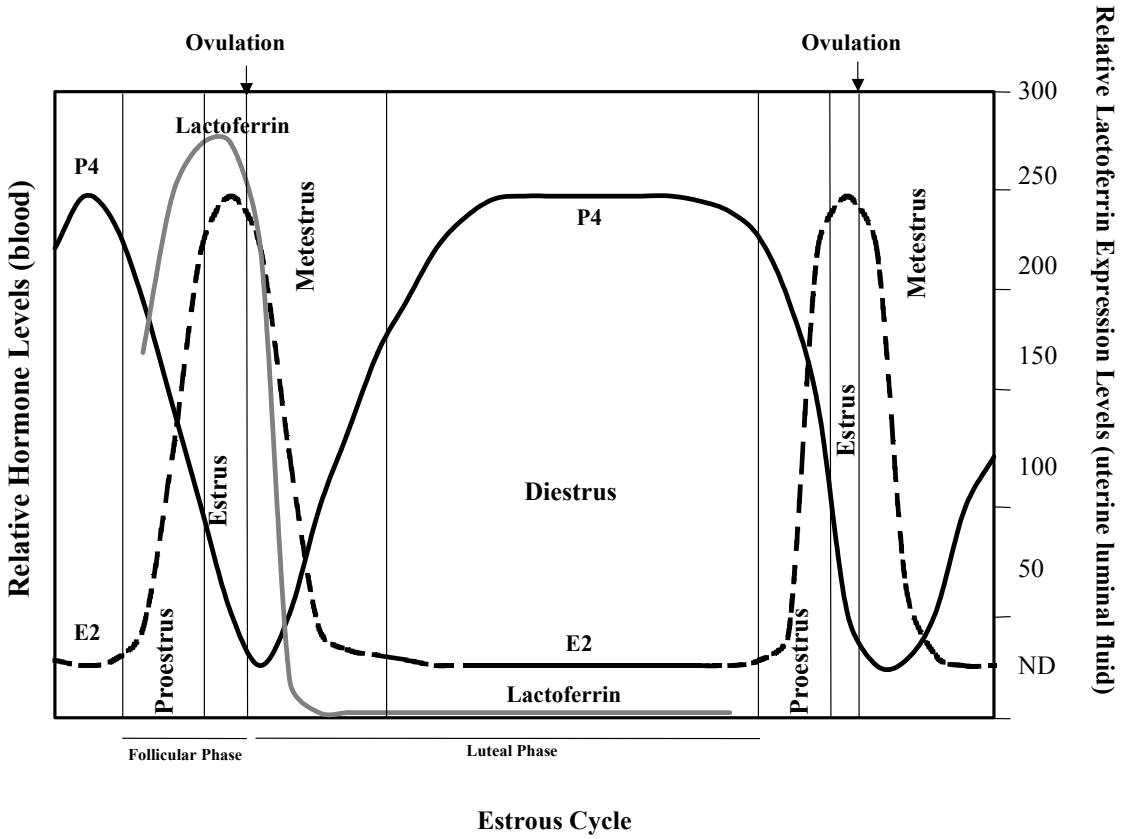
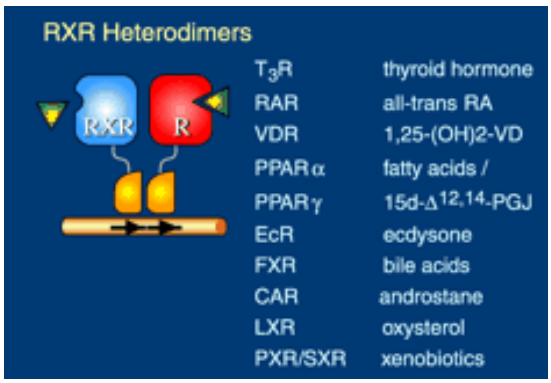


Figure 1.1: Reproductive hormones and lactoferrin levels during the estrous cycle
The reproductive hormone levels in circulating blood (P4, progesterone, solid black line and E2, 17β-estradiol, broken black line) are taken directly from Tucker 1979. The lactoferrin relative expression levels in uterine luminal fluid (solid grey line) during the various phases of the estrous cycle are plotted from Western Blot data published by Newbold *et al*. 1992, of normal, cycling, sexually mature female CD-1 mice.

A



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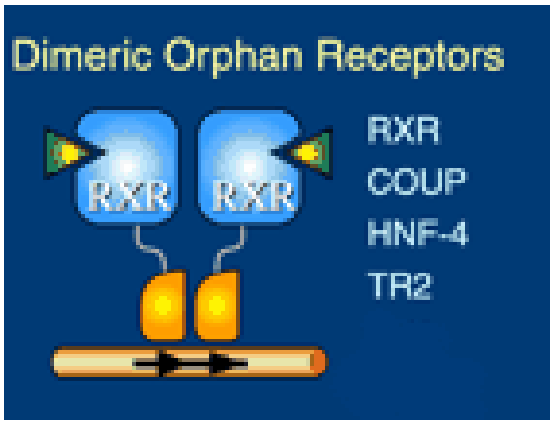


C

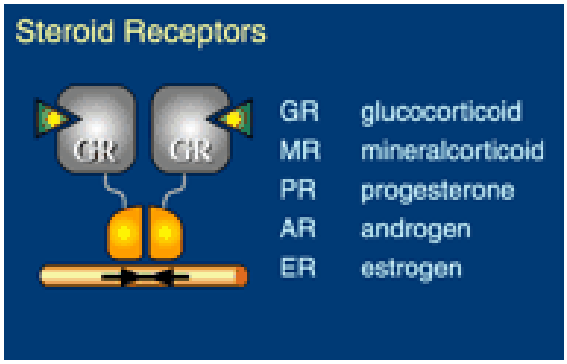


Figure 1.2: Binding of Nuclear Receptor Subfamilies to DNA response elements
Nuclear receptors can bind as heterodimers to palindromes, direct repeat or inverted repeat sequences (A) or as dimers to direct repeat sequences (B), or as homodimers to palindromic sequences (C). Taken from Olefsky 2001.

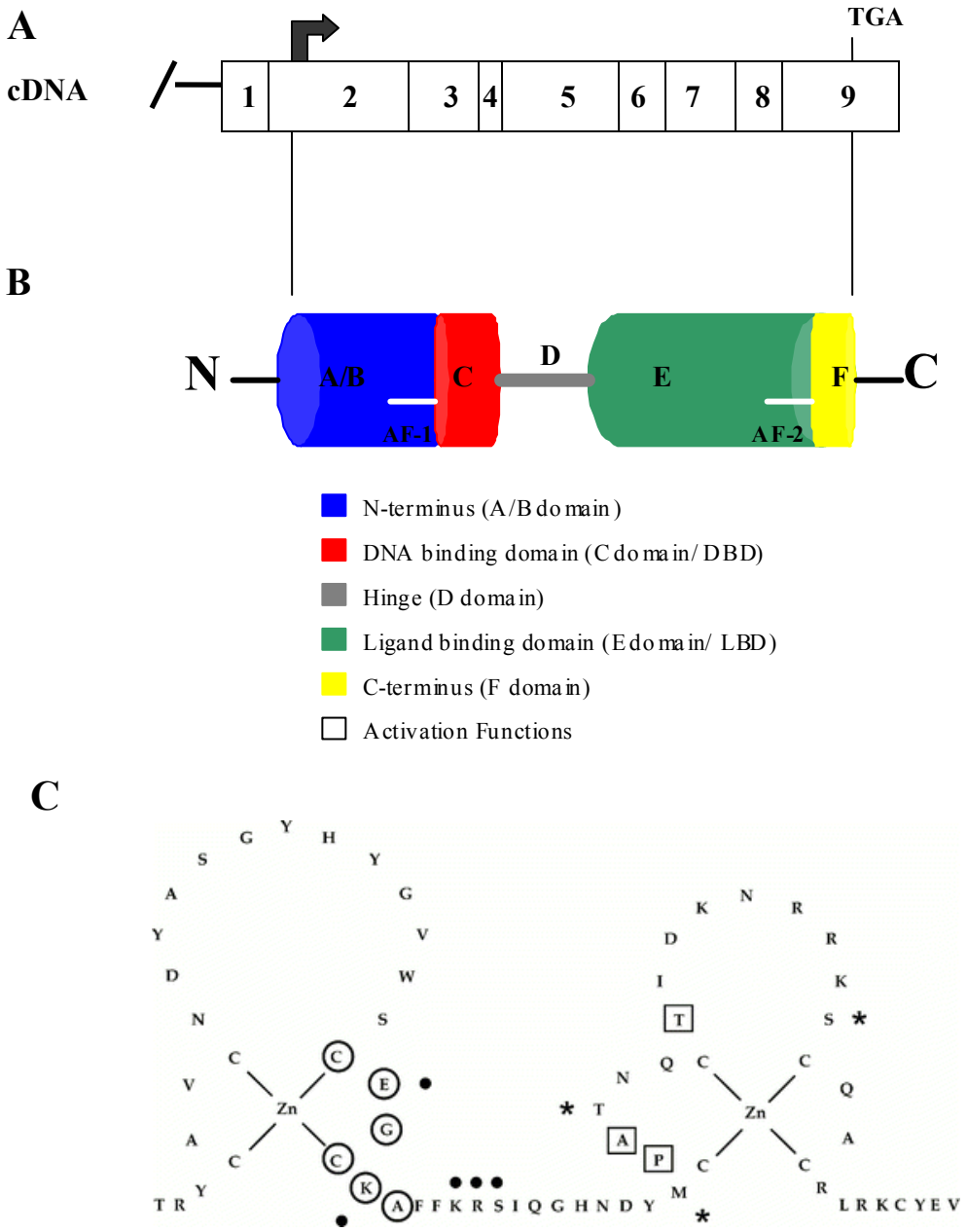


Figure 1.3: Estrogen receptor gene and protein organization

A. Organization of mouse ERα cDNA (taken from Couse and Korach 1999). B. Structural organization of estrogen receptor subtypes. The five major functional domains and two autonomous activation domains are indicated. Taken from McKenna and O'Malley 2002. C. Zinc finger organization of human ERβ. Circled residues signify the P-box. Residues indicated by a dot make direct base pair contacts with the estrogen responsive element (ERE). D-box Amino acids making direct contacts in the dimerization interface are boxed. Taken from Pettersson and Gustafsson 2001.

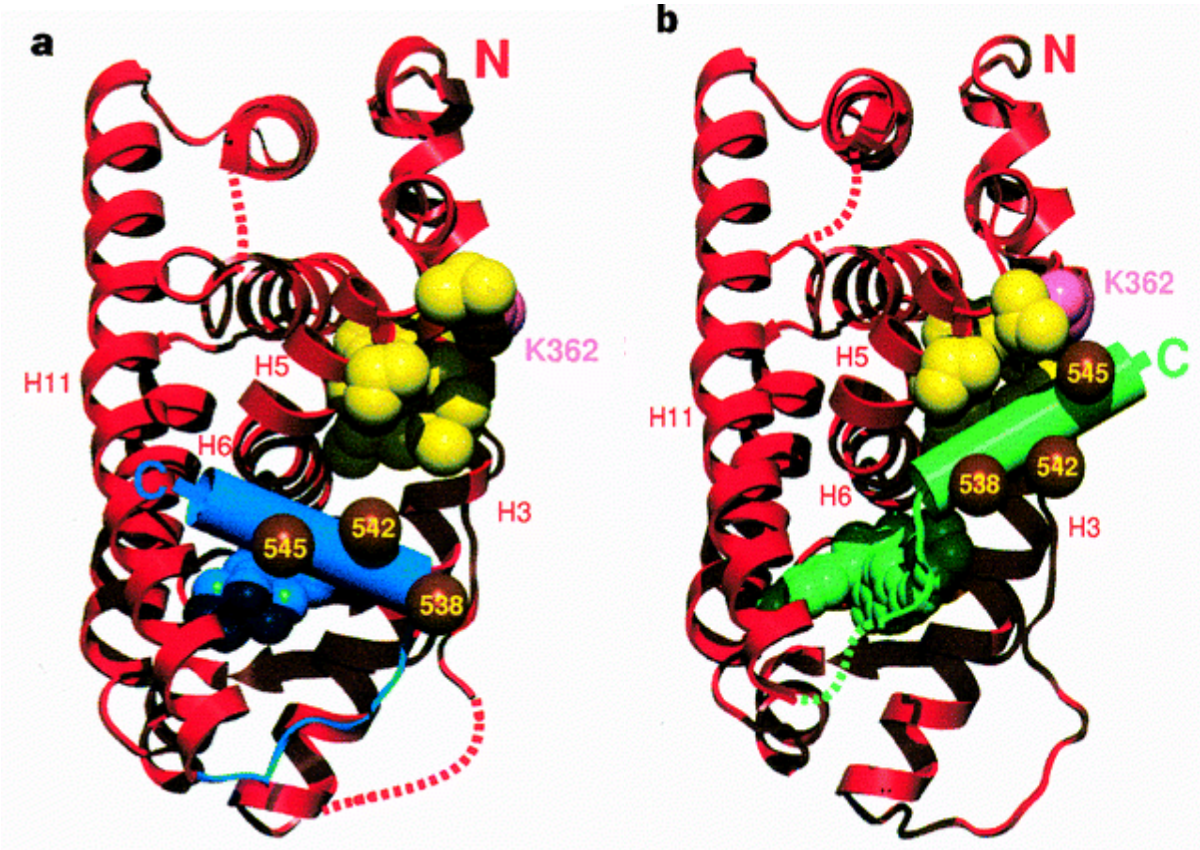
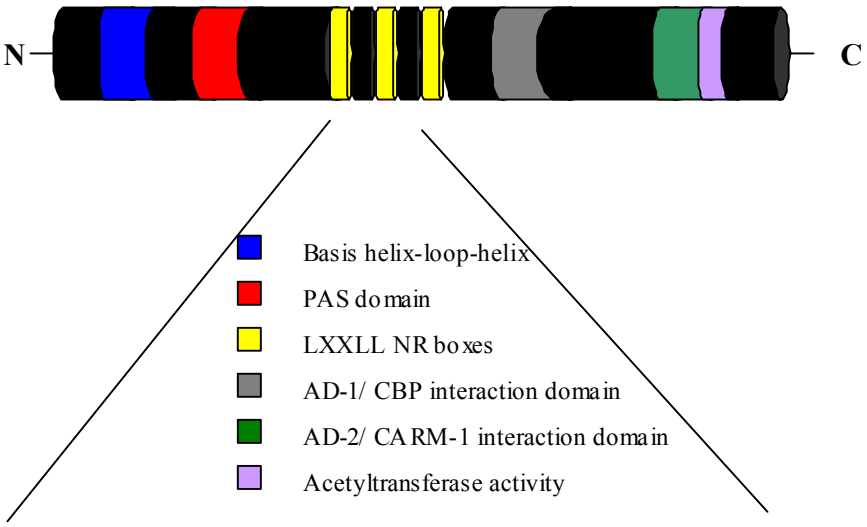


Figure 1.4: Positioning of helix 12 in ERα LBD complexed with an agonist or antagonist

A. The ERα LBD-E_2 complex. B. The ERα LBD-raloxifene complex. Helix12 is drawn as a blue colored (E_2 complex, A) or green cylinder (RAL complex, B). The remainder of the ERα LBD is shown in red. Dotted lines indicate unmodelled regions of the structures. Hydrophobic residues located in the groove between H3 and H5 (yellow) and Lys 362 (K362, pink) are depicted in space-filling form. The locations of Asp 538, Glu 542 and Asp 545 are highlighted (brown spheres) along with the helices that interact with H12 in the two complexes. Taken from Brzozowski *et al*. 1997.

A



B

	NR BOX 1	NR BOX 2	NR BOX 3
SRC-1	…SQTSHKLVQLLTTTAEE…	LTERHKILHRLLQEG.SP…	KKKESKDHQLLRYLLDKDE.KD
SRC-2	…SKGQTKLLQLLTTRSDD…	LKEKHKILHRLLQDSSSP…	KKKE...NALLRYLLDKDDTKD
SRC-3	…SKGHKKLLQLLTCSSDD…	LQEKHRILHKLLQNGNSP…	KKKESKDHQLLRYLLDKDE.KD

Figure 1.5: Structure of SRC nuclear receptor coactivator family.
A. Schematic presentation of the structural domains of SRC coactivators. The N-terminus contains the highly conserved bHLH and PAS A/B domains and the centrally located nuclear receptor boxes containing three LXXLL motifs. The specific domains for interaction with P/CAF, CBP/p300, and CARM1, as well as the histone acetyltransferase (HAT) domain, are indicated. Taken from McKenna and O'Malley 2002. B. Sequence alignment of the SRC family LXXLL motifs. The NR boxes of each SRC coactivator are boxed. Taken from Leo and Chen 2000.

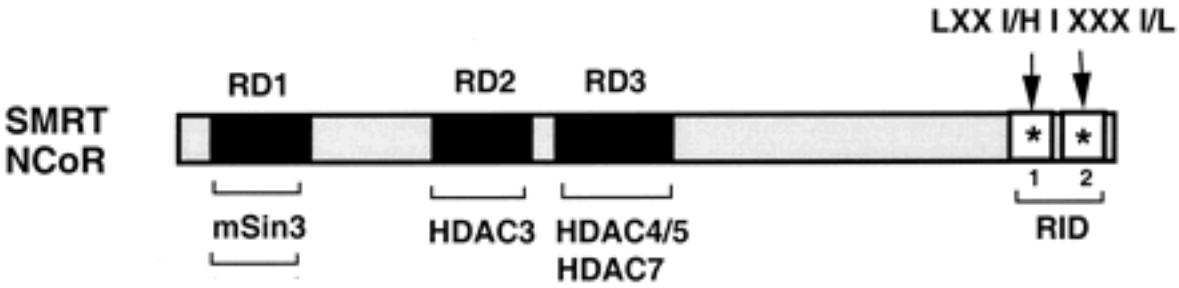


Figure 1.6: Structure of the nuclear receptor corepressors SMRT and NCoR
Schematic presentation of a corepressor showing the location of the repressor domains (RD1, RD2, and RD3) and the two receptor interacting domains (RIDs). The RIDs located at the C-terminus contain the extended helical motif LXX I/H I XXX I/L indicated by asterisks. The RD1 interacts with mSin3A, which in turn recruits class I deacetylases (HDAC1 and -2). HDAC3 has been shown to interact directly with RD2. Class II deacetylases bind at RD3 without Sin3 as a mediator. Taken from Aranda and Pascual 2001.

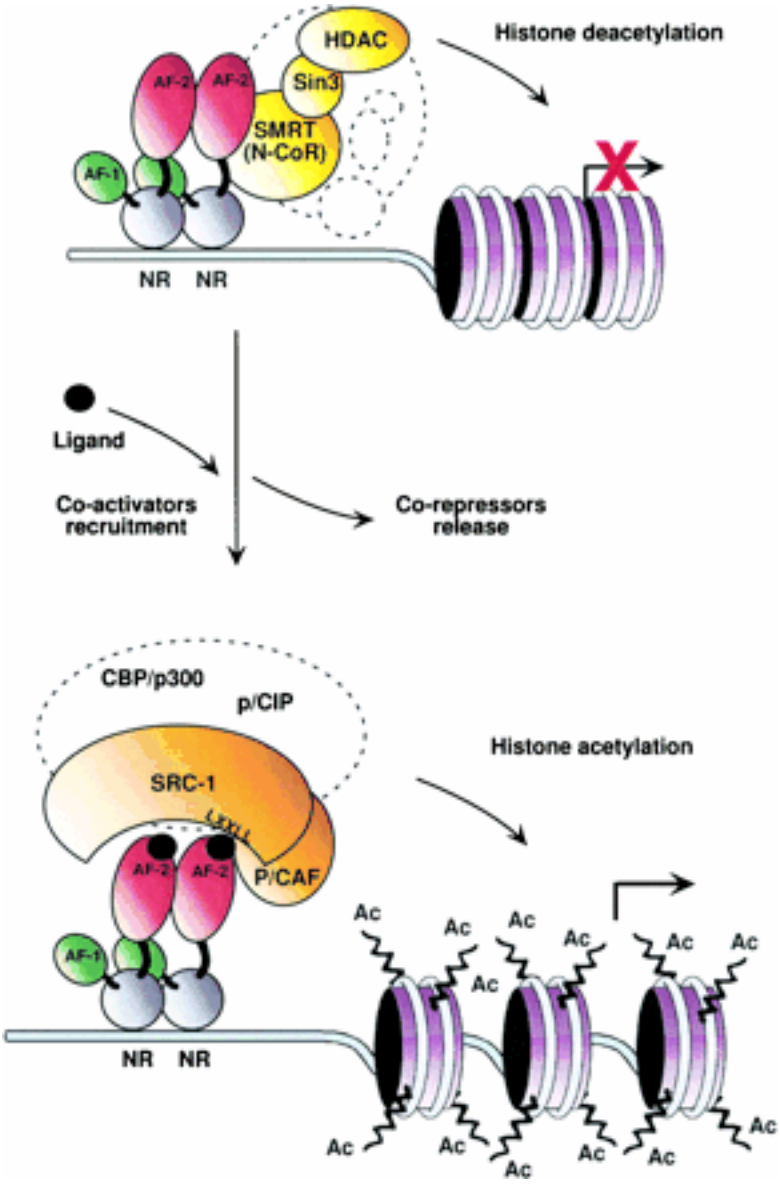


Figure 1.7: Ligand-dependent switch between a nuclear hormone receptor associated either with a corepression or a coactivation complex
The nuclear hormone receptor (NR) is associated with a corepressor (NCoR, SMRT), which in turn recruits a histone deacetylase (HDAC) through its interaction with Sin3. Deacetylation of histone tails leads to transcriptional repression. Addition of the ligand disrupts this repression complex in favor of the association of a coactivation complex (SRC-1, P/CAF, p300/CBP, pCIP, and others). These proteins possess a histone acetyltransferase activity that allows chromatin decompaction through histone modifications. The interaction between the nuclear hormone receptor AF-2 domain and the coactivation complex occurs through the LXXLL motif found in many coactivators. The coactivator and corepressor complexes are represented with dashed lines since their exact composition *in vivo* is not determined. Taken from Robyr *et al*. 2000.

Chapter 2

The Promoter Context of the Human and Mouse Lactoferrin Genes Influence Basal Activity in Mammary Gland Cells

I. Abstract

Relatively little is understood about the molecular mechanisms that regulate lactoferrin gene expression in the mammary gland, although it is well documented that estrogen regulates its expression in the uterus. To investigate whether estrogen could induce lactoferrin gene expression in mammary gland cells, we first detected the basal mRNA levels of lactoferrin and nuclear receptors known to mediate estrogen actions (estrogen receptors ERα and ERβ, and estrogen-related receptor-α (ERRα) in four human mammary gland cell lines by RT-PCR. We found very low levels of lactoferrin mRNA in the ERα positive MCF-7 and ERα negative MCF-10a cell lines. Furthermore, lactoferrin gene expression was estrogen-inducible in MCF-7 cells. Next we employed transient transfection assays with these same mammary gland cell lines to determine which regions of the natural human and mouse lactoferrin gene promoters influenced basal reporter activity. In general, the basal activities of the mouse lactoferrin-CAT reporters were more active than the human lactoferrin-CAT reporters, whereas the basal activity of individual reporters varied among each cell line. In general, higher basal reporter activity levels observed from cell lysates of transfected MCF-7 and MCF-10a cell lines, while lower reporter activities were detected from transfected MDA-MB-231 and HBL-100 cell lines.

Both human and mouse lactoferrin gene promoters encode overlapping COUP/ERE sequences (estrogen response modules, ERMs) at the same position relative to the transcription start site. Transient transfections of MCF-7 cells showed that liganded ERα activated transcription of the human lactoferrin ERM at least 4-fold higher than the mouse lactoferrin ERM in the context of their natural promoters. The same trend was observed in MCF-10a cells where the ERα-mediated estrogen response of the human lactoferrin reporters containing the ERM was greater than that of the mouse lactoferrin reporters. In an attempt to determine whether the observed differences in ERα transcription from the human and mouse lactoferrin ERMs in human mammary gland cells resulted from tissue- or species-specificity, the reporters were transfected in human endometrial and mouse mammary gland cells, and found that they behaved the same in the different cell types. Based on these results, we conclude that the ERM and surrounding cis-acting promoter sequences of the natural 400 bp region of the lactoferrin gene promoters influence ERα-mediated transactivation activity in mammary gland cells.

II. Introduction

In recent years, milk has been recognized not only for its nutritional value for infants, as it supplies almost all the nutrients required for growth and development of the neonate, but also as a "biological fluid." Milk promotes the development of passive immunity in the young, aids in gastrointestinal growth and mucosal defenses, and protects against the development of some cancers (reviewed in Koldovsky 1989, Schanbacher *et al.* 1997, Gill *et al.* 2000). The beneficial effects of breast-feeding have been well documented, but the specific bioactive milk components that contribute to these health benefits have not been completely characterized. Lactoferrin is one of the milk proteins that has been implicated in playing roles in a broad range of host defense mechanisms, including anti-microbial, anti-tumor, anti-inflammatory and physiological immune responses (reviewed in Brock 2002 and Ward *et al.* 2002). Lactoferrin has been shown to stimulate intestinal growth and iron absorption in infants (Davidson and Lönnerdal 1988, Nichols *et al.* 1987) and it is postulated that lactoferrin cleaves arginine-rich sequences in bacterial virulence proteins to combat intestinal pathogens (Hendrixson *et al*. 2003). This mechanism of virulence factor degradation may explain how lactoferrin significantly inhibited the adhesion of the enteric pathogen *Escherichia coli* to HeLa cells by 14% (de Araujo and Giugliano 2001). Additionally, lactoferrin resisted acid digestion in the neonatal intestine and has been purified from the urine of pre-term infants fed human breast milk (Knapp and Hutchens 1994). Based on these projected physiological functions in the digestive tract, several putative mammalian lactoferrin receptors have been characterized from a variety of cell lines including fetal and human intestinal cells (reviewed in Suzuki and Lönnerdal 2002). However, data proving

direct binding of lactoferrin to these receptors subsequent functional receptor studies have not been described to date.

There are four stages of mammary gland development, mammogenesis (differentiation and growth of epithelial cells during puberty and then pregnancy), lactogenesis (initiation of colostrums secretion just after parturition), full lactation (secretion of mature milk), and involution (glandular regression to a de-differentiated state). Given that the amount of lactoferrin found in bovine colostrum, the first milk, was detected at 10-fold higher levels than in mature milk (Table 1.1) and bovine colostrum also contains almost a 10-fold higher concentration of total estrogens compared to mature milk (Table 1.2), it is plausible that lactoferrin may have a specific biological activity during the first few days of infancy. The expression of the major milk-specific proteins (caseins, α-lactalbumin and β-lactoglobulin) is highest during lactation, while the lactoferrin content decreases during this stage compared to levels found in colostrums. Moreover, the mammary gland becomes unresponsive to estrogen during the lactation stage of development (Shyamala and Ferenczy 1982), whereas the involution stage of mammary gland development is characterized by decreased expression of milk-specific proteins (Welty *et al.* 1976) and increased sensitivity to estrogen. Accordingly, lactoferrin concentrations in dry secretions during involution were 100-fold higher than levels in milk (Welty *et al.* 1976) and estrogen accelerated involution in cows during late lactation, increased lactoferrin concentrations during the first week of involution, and is clinically prescribed to prevent lactation in mothers who do not wish to nurse the child (Athie *et al.* 1996, Llewellyn-Jones 1975). Because the pattern of lactoferrin expression is contrary to other milk proteins and parallels circulating estrogen levels, it seems

probable that estrogen, not lactogenic hormones, regulate lactoferrin gene expression during all stages of mammary gland development.

To explore the possibility that estrogen stimulates lactoferrin gene expression in the mammary gland, we used biologically relevant cell types established from a human milk epithelial cell (HBL-100, Gaffney 1982), benign fibrocystic disease (MCF-10a, Soule *et al.* 1990) and pleural effusions from malignant cancers (MCF-7, Soule *et al.* 1973 and MDA-MB-231, Cailleau *et al.* 1978) in transient transfection assays. In this report, we examined the 1.0 kb and 2.7 kb promoter regions of the human and mouse lactoferrin genes and found potential positive and negative regulatory regions that influenced the basal activity of the reporters in transfected mammary gland cells. Furthermore, we demonstrated differential ERα-mediated estrogen responses of the human and mouse lactoferrin ERMs in their natural promoter contexts.

III. Materials and Methods

Reagents

Diethylstilbestrol (DES) was purchased from Sigma (St. Louis, MO). Proteinase K (lot # D1734221) was purchased from Pierce Biotechnology (Rockford, IL). [^{14}C]-chloramphenicol was purchased from NEN Life Sciences (Perkin Elmer, Boston, MA).

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 2.1. The plasmids used in transient transfection assays were purified and amplified as follows. The DNA (50ng) was transformed into competent *E. coli* TOP cells (Invitrogen, Carlsbad, CA) by incubation on ice for 30 min, heat shock treatment at 42°C for 1 min, and then incubation on ice for an additional 2 min. SOC medium (250 μl) was added to the mixture and the bacteria were allowed to grow for 1 hr at 37°C with shaking. The transformation reaction (50 μl) was plated on luria broth (LB) plates containing ampicillin and incubated at 37°C overnight. The following day, several colonies from each transformation were picked and grown in LB and ampicillin broth (5 ml) overnight and then the plasmid DNA was isolated and purified (Qiagen quick spin miniprep kit, Valencia, CA). The presence of the correct sized plasmid was verified by digestion with BamH1 restriction endonuclease. The digested plasmids were separated on 1% agarose TBE gels (NuSieve GTG, BioWhitaker Molecular Applications, Rockland, ME, Fig. 2.1). If the plasmids were of the correct size, the original amplified cell cultures (750 μl) were inoculated into 750 ml LB and ampicillin broth and the cells were grown overnight at 37°C with shaking. Plasmid

DNA from these larger cell cultures was first purified using the Qiagen QIAfilter midi/maxiprep kit (Valencia, CA) and then re-purified by equilibrium centrifugation in cesium chloride- ethidium bromide continuous gradients (Molecular Cloning, third edition).

Cell Culture

The human MCF-7 (ATCC #HTB-22), MCF-10a (ATCC #CRL-10317), MDA-MB-231 (ATCC #HTB-26) and HEC-1B (ATCC #HTB-113) cell lines were obtained from the ATCC (Manassas, VA). The human HBL-100 cell line was a gift from J. Iglehart, Duke University. The mouse C57MG cell line was a gift from R. DiAugustine, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC). All cell lines were grown as monolayer cultures in 75 cm^2 tissue culture treated flasks in a humidified atmosphere at 37°C and 5% CO_2. MCF-10a cells were cultured in a 1:1 mixture of Ham's F12: Dulbecco's minimum essential medium (DME) media supplemented 10 ng/ml insulin, 500 ng/ml hydrocortisone, 20 ng/ml epidermal growth factor, 1% penicillin-streptomycin and 5% fetal bovine serum (FBS). MCF-7 and HBL-100 cells were cultured in Eagle's minimum essential medium (EMEM) containing 10ng/ml insulin, 1% penicillin-streptomycin and 10% FBS. HEC-1B and MDA-MB-231 cells were cultured in EMEM, 1% penicillin-streptomycin and 10% FBS. C57MG cells were cultured in high glucose DME supplemented with 1% penicillin-streptomycin and 10% FBS.

Transient Transfection

Cells were transferred into 6-well plates in phenol-red free medium containing charcoal-stripped FBS at 30-40% confluency. The following day, the cells were washed with phosphate buffered saline, fresh medium was then added, and the cells were transfected using the FuGENE 6 reagent (Roche Molecular Biology, Indianapolis, IN) according to the manufacturer's instructions. For basal reporter activity studies, a DNA mixture consisting of 500 ng reporter plasmids, 100 ng pCH110 (β-gal expression plasmid to measure transfection efficiency) and carrier DNA up to a total of 750 ng/well was prepared (Table 2.1) and then added to 2.25 μl FuGENE 6 diluted in 100 μl base media (3:1 ratio FuGENE 6 to DNA). After a 1 hr incubation of DNA and FuGENE 6, the complex was added drop-wise to the cells in 2 ml charcoal-stripped serum media and transfection proceeded for 40 hrs at 37°C in a humidified atmosphere with 5% CO_2. For hormone response studies, a DNA mixture consisting of 500 ng reporter plasmid, 100 ng pCH110, 100 ng ERα expression plasmid and carrier DNA up to a total of 750 ng/well was prepared. Sixteen hours after transfection, 10 nM DES was added for an additional 24 hr.

The cells were harvested in reporter lysis buffer (150 μl, Promega, Madison, WI) and cell lysates were collected. The β-galactosidase assay was performed in 100 μl reactions (50 μl cell lysate, 50 μl 2X assay buffer (200 mM sodium phosphate buffer pH 7.3, 2 mM $MgCl_2$, 100 mM β-mercaptoethanol, 1.33 mg/ml ONPG)) that were incubated for 1 hr at 37°C and then the absorbance was read at 420 nm (Ultra Mark Microplate Imaging System, BioRad). The CAT enzyme activity assay was performed in 150 μl reactions mixtures (50 μl cell extract heated at 55°C to inactivate endogenous deacetylases, 78 μl 0.25M Tris-HCl pH 7.5, 20 μl n-butyryl coenzyme A (4 mM) and 2 μl ^{14}C chloramphenicol) that were incubated

for 1 hr at 37°C. After the incubation period, xylene (320 μl) was added to the CAT reactions, the mixture was vortexed for 30 sec and then centrifuged at 14,000 rpm for 7 min. The organic phase was removed and then added to eppendorf tubes containing 0.25M Tris-HCl pH 8.0 (200 μl). The mixture was vortexed for 30 sec and then centrifuged at 14,000 rpm for 7 min. The organic phase (200 μl) containing butyryl-^{14}C chloramphenicol, indicative of CAT activity, was added to a scintillation cocktail (2 ml) and counted by liquid scintillation counting. CAT activity normalized to β-galactosidase activity is reported as the relative CAT activity for the indicated experiments.

RNA Extraction and RT-PCR analysis of endogenous gene expression

Cells were cultured in complete media to determine endogenous levels transcripts indicated in each experiment, or in charcoal-stripped medium for four days and then treated with or without 10 nM DES for 48 hr to detect induced levels of endogenous lactoferrin mRNA. Total cellular RNA was isolated from ~1 x 10^6 cultured cells using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The yield and purity of RNA samples were assessed by the ratio of absorbance at 260 nm and 280 nm. For RT-PCR reactions, purified RNA was reverse transcribed into cDNA and then amplified in the same tube using the Titan One Tube RT-PCR Kit (Roche Molecular Biology, Indianapolis, IN). Total RNA (1 μg), forward and reverse primers (0.4 μM each), dNTP (0.2 mM each), dithiothreitol (5 mM), buffer containing $MgCl_2$ (1.5 mM), enzyme mix (reverse transcriptase and DNA polymerase) and water were added for a final volume of 50 μl. The primers were synthesized by Sigma Genosys and designed to span introns in the original DNA sequence that should not be present in the cDNA after the reverse transcription

reaction. The resulting RT-PCR products (10 μl or 20 μl for lactoferrin) were analyzed by electrophoresis with 3% low melting temperature agarose TAE gels (NuSieve GTG, BioWhitaker Molecular Applications, Rockland, ME). The primers used for amplification, expected product size, and cycling parameters are described in Table 2.2.

IV. Results

Endogenous lactoferrin gene expression is estrogen inducible in MCF-7 cells

In order to determine whether estrogen induced lactoferrin gene expression in mammary gland cells, we examined endogenous expression of lactoferrin, ERα, ERβ, and ERRα mRNAs in human mammary epithelial (MCF-7, HBL-100, MCF-10a and MDA-MB-231) and endometrial (HEC-1B) cell lines using primers sets spanning intronic sequences in DNA. ERα and ERβ mediate transcriptional responses to estrogen (Green *et al*. 1986, Kuiper *et al.* 1996, Mosselman *et al.* 1996, Tremblay *et al*. 1997, Walter *et al*. 1985), while ERRα shares target genes, coregulatory proteins, ligands and sites of action with the ERs (Johnston *et al.* 1997, Lu *et al.* 2001, Vanacker *et al*. 1999a and 1999b, Yang *et al*. 1996, Zhang and Teng 2000, reviewed in Giguere 2002). The cell lines were cultured in complete media containing fetal bovine serum. As shown in Fig. 2.2A, ERRα was expressed in all cell lines, ERβ transcripts were detected in MCF-7 and HBL-100 cells, and ERα transcripts were detected in MCF-7 cells only. Endogenous lactoferrin mRNA was expressed in MCF-7 and MCF-10a cells, but only after the number of amplification cycles was increased from 25 to 40 and the amount of RT-PCR product analyzed by gel electrophoresis was increased from 10 μl to 20 μl (Fig. 2.2A). Next, we demonstrated that expression of endogenous lactoferrin is estrogen inducible in the ERα and ERβ positive MCF-7 cell line (Fig. 2.2B).

The cell and promoter context influence the basal CAT activities of the various human and mouse lactoferrin reporters

Previously, our laboratory generated several human and mouse lactoferrin gene reporters containing variable lengths of the natural promoter regions. The relative position of some of the identified elements contained in the human lactoferrin gene promoter (Fig. 2.3A) and those in the mouse lactoferrin gene promoter (Fig. 2.3B) are illustrated. We wanted to first establish that these lactoferrin-CAT reporters were active in mammary gland cells, so we transiently transfected the sequential 5' deletion fragments of the natural human (hLF-CAT) and mouse (mLF-CAT) lactoferrin reporters in the various cell lines. The human endometrial carcinoma cell line HEC-1B served as a positive control since the basal activity and estrogen responses of the human lactoferrin reporters have been already established (Zhang and Teng 2000). In general, the basal activities of the mLF-CAT reporters were higher than the hLF-CAT reporters (compare Figs. 2.4A and 2.4B). For the hLF-CAT reporters, we did not detect any major differences in the basal activity of an individual construct within the same cell line (Fig. 2.4A, compare the CAT activities of the four hLF reporters in the HEC-1B (white), MCF-7 (dotted), HBL-100 (gray), MDA-MB-231 (checker) cells). The only difference in reporter activity was noted from the cell lysates of MCF-10a cells transfected with the shortest human lactoferrin reporter (0.1 hLF-CAT, Fig. 2.4A). For the mLF-CAT reporters, each reporter had a distinct basal promoter activity in each cell line (Fig. 2.4B, compare the CAT activities of the eight mLF constructs in the HEC-1B (white), MCF-7 (dotted), HBL-100 (gray), MDA-MB-231 (checker), and MCF-10a (black) cells). Based on the basal reporter activities, we identified potential mouse lacoferrin regulatory elements that were active in the mammary gland (Fig. 2.4B). In transfected MCF-10a cells

(black bars), we saw a decrease in basal CAT activity when the mouse lactoferrin promoter region upstream of -1731 was deleted, suggesting that positive regulatory elements were deleted. The basal activity in transfected MCF-10a cells gradually increased as the promoter region between -1731 and -396 was deleted, suggesting that possible negative regulatory elements were removed. Additionally, sequential deletion of implicated the presence of negative regulatory elements located between the regions -291 and -103 upstream of the transcription start site. The same overall trend of mLF-CAT basal activities reported in MCF-10a cells was observed in all other cell lines tested.

The promoter context mediates the differential estrogen response of human and mouse lactoferrin gene promoters in mammary gland cells

The ER exists in two genetically distinct subtypes, ER alpha (ERα) and ER beta (ERβ), that have distinct expression patterns and functions on target genes (Green *et al*. 1986, Kuiper *et al*. 1996, Mosselman *et al*. 1996, Tremblay *et al*. 1997, Walter *et al*. 1985). In the present study, we focused on ERα because this subtype mediates the major biological functions estrogen during mammary gland development (Bocchinfuso and Korach 1997, Krege *et al*. 1998) and ERα potently transactivates lactoferrin in cell culture systems. Based on RT-PCR analyses and basal lactoferrin reporter activity data, we chose to use the tumorigenic MCF-7 ($ER\alpha^+$) and the normal MCF-10a ($ER\alpha^-$) cell lines to determine the ERα-mediated transcriptional activity of the human and mouse lactoferrin reporters in response to estrogen. First, we determined that 100 ng of human ERα expression vector and 10 nM DES conferred optimal ERα-mediated estrogen response in MCF-7 (left panel) and MCF-10a (right panel) cells transfected with the 0.4 hLF-CAT reporter (Fig. 2.5). Next, we

examined the estrogen response of the lactoferrin ERMs in the context of their natural promoters. We found that DES treatment of cells transfected only with the various reporters did not produce a detectable estrogen response in MCF-7 or MCF-10a cells (Figs. 2.6B and 2.6C, dotted bars). This result was expected because the MCF-10a cells do not express either ER subtype transcript (Fig. 2.2A) and transient transfection assays of ER positive MCF-7 cells have routinely relied on overexpression of ERα to stimulate reporter gene transcription in response to estrogen. Therefore, the human ERα expression vector was co-transfected in these cell lines to observe an estrogen response (Figs. 2.6B and 2.6C, black bars).

Data from cells transiently transfected with the human and mouse lactoferrin reporters showed that estrogen-induced transactivation is dependent upon the ERM in the promoter of the lactoferrin gene. There were marked differences between the estrogen activity of the hLF-CAT and mLF-CAT reporters in the mammary gland cells overexpressing ERα. There was a strong ERα-mediated increase in estrogen-induced CAT activity (>21-fold) in MCF-7 cell lysates transfected with the 1.0 hLF-CAT and 0.4 hLF-CAT reporters containing the overlapping COUP/ERE sequence and deletion of this sequence rendered the reporter (0.3 hLF-CAT) insensitive to estrogen (Fig. 2.6B). However, we observed variable CAT activity in response to estrogen with the mLF-CAT reporters containing the COUP/ERE (2.6, 1.7, 0.9, 0.6, and 0.4 mLF-CAT). Only the 2.6 mLF-CAT and 0.4 mLF-CAT reporters were modestly activated by liganded ERα in MCF-7 cells, having 3-fold and 5-fold increases in estrogen-induced CAT activity in MCF-7 cells (Fig. 2.6B). A similar trend was observed in MCF-10a cells transfected with either the human or mouse lactoferrin reporters (Fig. 2.6C). Overexpression of ERα conferred a strong increase in estrogen-induced reporter activity compared to untreated MCF-10a cells transfected with the reporter

alone (1.0 hLF-CAT and 0.4 hLF-CAT), whereas only the 2.7 mLF-CAT reporter containing the ERM was responsive to estrogen. In contrast to the 400 bp human lactoferrin promoter, the 0.4 mLF-CAT reporter in MCF-10a cells overexpressing ERα was unresponsive to estrogen. Since the basal activity of the 0.4 mLF-CAT reporter is 6-fold higher than 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 (Fig. 2.6C, compare CAT activities of 0.4 hLF and 0.4 mLF reporters alone in MCF-10a cells, white bars). We found that lowering the amount of the 0.4 mLF-CAT reporter transfected into the cells from 500 ng to 200 ng did not produce a strong estrogen response in MCF-10a cells overexpressing ERα (compare Figs. 2.6C and 2.6D, no fold increase versus 2-fold increase). On the contrary, the ERα-mediated estrogen response of the 0.4 hLF-CAT reporter increased in MCF-10a cells transfected with a lower amount of the reporter (compare Figs. 2.6C and 2.6D, 42-fold increase versus 20-fold increase). Human endometrial carcinoma (HEC-1B, Fig. 2.7, left panel) and mouse mammary epithelial (C57MG, Fig. 2.7, right panel) cells overexpressing ERα and the 400 bp mouse lactoferrin reporter were also unresponsive to estrogen, hence in its natural promoter context in the tested cell lines, the mLF ERM was unresponsive to estrogen. Taken together, these data suggest that additional positive regulatory elements that may work in tandem with the ERM to increase transactivation may be absent or negative regulatory elements that may block estrogen-induced transcription may be present within the natural mouse lactoferrin promoter.

V. Discussion

DES induces endogenous lactoferrin gene expression in mammary epithelial cells

The overall goal of this research was to determine whether estrogen stimulated human and mouse lactoferrin gene expression in mammary epithelial cells. We first characterized several mammary epithelial cell lines for endogenous expression of an estrogen-inducible gene in the uterus, lactoferrin (Pentecost and Teng 1987, Teng *et al*. 1989, 1992, 2002a, and 2002b), and nuclear receptors involved in estrogen action, ERα, ERβ, and ERRα (reviewed in Couse and Korach 1999, reviewed in Giguere 2002). We were able to demonstrate that lactoferrin is an estrogen-inducible gene in the $ER\alpha^+$/ $ER\beta^+$ MCF-7 cells (Fig 2.2B). Our RT-PCR data showing endogenous expression of lactoferrin mRNA in MCF-10a cells cultured in complete media in the presence of whole fetal bovine serum (Fig. 2.2A) was unexpected because this cell line does not express endogenous ERα or ERβ transcripts. Although the MCF-10a cells were cultured in media supplemented with EGF (20 ng/ml), hydrocortisone (500 ng/ml) and insulin (10 ng/ml) and EGF has been shown to stimulate mouse lactoferrin promoter activity in transiently transfected cell lines (Shi and Teng 1994 and 1996), MCF-10a is a human cell line and to date, an EGF responsive region has not been characterized in the human lactoferrin gene promoter. Since our laboratory has cloned and sequenced and characterized the proximal 1 kb region of the human lactoferrin gene promoter (Fig. 2.3A), we do not know whether EGF, GR, or insulin responsive elements are located further upstream. In addition to hormone and growth factor response elements in the gene promoter, the locus control region (LCR), also regulates transcription of milk protein genes (human α-lactalbumin and bovine αs_1-, αs_2-, β-, and κ-casein) by directing position-independent gene expression in transgenic mice (Ellis *et al*. 1996, reviewed in Kioussis and

Festenstein 1997). The LCR is the region of the gene that and is usually located several kb upstream or downstream of the transcription start site and is thought to act to open the chromatin structure for access to transcription factors by preventing heterochromatin formation (Ellis *et al*. 1996, Kioussis and Festenstein 1997). The identification of additional regulatory regions within the lactoferrin gene may help explain the endogenous expression of lactoferrin in the ER negative MCF-10a cell line.

Identification of potential positive and negative regulatory elements within the human and mouse lactoferrin gene promoters

To define potential regulatory elements within the lactoferrin gene promoter, we performed transient transfection assays to determine the basal transcription level from varying lengths of the 5' flanking sequence of the gene in several mammary gland cell lines. We observed differential basal activity of the human and mouse lactoferrin gene promoters. The variable levels of CAT activity detected from an individual cell line transfected with the human or mouse lactoferrin promoters suggested the presence of positive and negative regulatory sequences distributed throughout the promoter region (Figs. 2.4A and 2.4B). We also detected high basal activity from the 100 bp and 400 bp mouse lactoferrin reporters in MCF-10a cells (Fig. 2.4B). The mitogen response unit, composed of adjacent cAMP response element (CRE) and epidermal growth factor response element (EGFRE), mediates transcriptional activation of the 100 bp mouse lactoferrin promoter in response to EGF and forskolin (Shi and Teng 1996). Both the 100 bp and 400 bp promoters regions of the mouse lactoferrin gene contain an EGF response element, explaining the elevated basal activity of these reporters (0.1 mLF-CAT and 0.4 mLF-CAT) in MCF-10a cells cultured in media

supplemented with EGF. In addition to the EGFRE, there are other regulatory elements involved in the regulation of tissue-specific lactoferrin gene expression. The 100 bp proximal region of the mouse lactoferrin gene promoter contains a CCAAT enhancer binding protein (C/EBP) C/EBP regulatory element that positively regulates stage-specific expression of the human lactoferrin gene in human hematopoietic cells (Khanna-Gupta *et al*. 1997 and 2003, Verbeek *et al*. 1999). Although we do not know whether the various C/EBPs are expressed ubiquitously in the tested mammary gland cell lines, it has been reported that C/EBP-β regulates expression of the milk protein gene bovine αs_1-casein in primary mammary gland cells (Jolivet *et al*. 2001, Raught *et al*. 1995, Robinson *et al*. 1998), supporting a potential role for the various C/EBPs in stage-specific regulation of lactoferrin gene expression during mammary gland development. In addition to the EGF and C/EBP elements, the 400 bp mouse lactoferrin promoter contains the COUP/ERE that functions in estrogen-induced gene transcription in MCF-7 cells (Fig. 2.6B), but is unresponsive to estrogen in MCF-10a cells (Fig 2.6C) that lack endogenous ERα or ERβ transcripts (Fig. 2.2A). The mouse lactoferrin COUP/ERE has been described as a functional retinoic acid response element (RARE) in transfected mammary gland cell lines, but not in hematopoietic cell lines (Lee *et al*. 1995), suggesting that the high basal activity of the 400 bp mouse lactoferrin-CAT reporter construct may result from retinoic acid action mediated by its nuclear receptor. Further support comes from Northern blot analysis showing expression of retinoic acid receptor gene transcripts (alpha, beta, and gamma) and their heterodimeric binding partner retinoid X receptor gene MCF-7, MCF-10a, HBL100, and MDA-MB-231 cells (Roman *et al*. 1992, Rosenauer *et al*. 1998, Seewaldt *et al*. 1997, Swisshelm et al.

1994). We speculate that these hormone responsive units located throughout the lactoferrin gene promoters may cooperate with the ERM to regulate basal transcriptional activity.

In addition to positive regulatory elements, one negative regulatory element that functions during hematopoietic cell differentiation has been characterized by transient transfection and EMSA experiments. Lactoferrin gene expression is induced during the second stage of neutrophil development and detection of the lactoferrin protein is an essential method to identify mature neutrophils (Johnston *et al*. 1992). The CCAAT displacement protein (CDP) is ubiquitously expressed and was found to bind three repetitive regulatory sequences found between -916 bp and -726 bp region of the human lactoferrin gene promoter in immature myeloid cells by EMSA (Khanna-Gupta *et al*. 1997). Overexpression of CDP abrogated basal promoter activity while transfection of immature myeloid cells with a lactoferrin reporter gene construct in which the three repetitive CDP binding elements were deleted restored basal promoter activity. Conversely, upon neutrophil maturation, CDP no longer bound the three repetitive CDP binding elements and overexpression of CDP did not result in suppression of basal promoter activity. Although the mechanism of CDP-induced repression of lactoferrin gene expression in immature neutrophils is unknown, these experiments have demonstrated temporal regulation of lactoferrin gene expression.

Promoter-specific transcriptional activity of ERα on the human and mouse lactoferrin genes

In the present study, we demonstrated differential ERα-mediated estrogen responses of the human and mouse lactoferrin gene promoters in transfected human mammary gland cells. The most surprising result in this study was that the human lactoferrin gene ERM was potently stimulated by estrogen in the context of its natural promoter, while the mouse

lactoferrin ERM were barely responsive to estrogen in transfected mammary gland cells (compare Figs. 2.6B and 2.6C). Data from transient transfection studies of human endometrial carcinoma (HEC-1B) and mouse mammary epithelial (C57MG) cells with the ERα expression vector and the 400 bp mLF-CAT reporters demonstrated that the differential estrogen effects on the human and mouse lactoferrin EREs were not due to tissue- or species-specificity (Fig. 2.7). However, i*n vivo* studies have already documented the estrogen responsiveness of the mouse lactoferrin gene in both the male and female reproductive tract. Normal male mice did not express lactoferrin mRNA in their seminal vesicles, but after their testes were removed, low levels of lactoferrin mRNA were detected and levels increased further when the castrated animals were exposed to DES (Pentecost *et al.* 1988). Furthermore, prenatal exposure to DES induced lactoferrin protein expression in the seminal secretions of both normal and castrated mice (Pentecost *et al.* 1988, Newbold *et al.* 1989). *In vivo* studies conducted with mature female mice correlated the expression of the lactoferrin mRNA and protein in uterine luminal fluid with circulating levels of estrogen during the estrous cycle (Newbold *et al.* 1992, Walmer *et al.* 1992). Lactoferrin expression was up-regulated during proestrus and estrus, stages of the estrous characterized by peak estrogen levels. Additionally, *in vitro* based transient transfection studies showed that estrogen induced a 9-fold increase in reporter activity in human mammary epithelial cell lines ZR-751 and Hs578T overexpressing ERα and the mouse COUP/ERE linked to a heterologous promoter (Lee *et al.* 1995). Thus, the mouse lactoferrin ERE is functional, but other factors may coordinately regulate ERα-mediated promoter activity in our transient transfection system.

Collectively, the above data suggested that the disparities in estrogen responses of the natural human and mouse lactoferrin gene promoters might result from three possible scenarios. First, putative negative regulatory sequences identified in the mouse lactoferrin gene promoter between regions -234 to -396 and -587 to -1731 (Fig. 2.4B) may act to repress ERα-mediated transcription. Second, positive estrogen responsive elements that may work in tandem with the ERE to increase transactivation may be absent in the cloned region of the mouse lactoferrin gene promoter. Third, the CAT reporter gene used in our transient transfection assays may repress the estrogen response of the mouse lactoferrin gene.

Negative regulatory elements are often associated with tissue- and stage-specific control of gene expression as observed in CDP-mediated regulation of human lactoferrin gene expression in hematopoietic cell lines (Khanna-Gupta *et al*. 1997). A putative CDP binding element located in the region -321 to -313 of the mouse lactoferrin gene promoter was identified using Genomatix Mat Inspector transcription factor binding site program. Although mutational studies have not been performed to assess the functionality of this element, the DNA sequence (-320 AGTGATTC -313) is similar to the sequences of the three repetitive CDP binding elements (-856 ATGTATTT -849, -842 ATGTATTC -835, -827 AGTATTCT -820) that have been characterized as repressing elements that are bound by CDP during the inactivation of the human lactoferrin gene expression in immature myeloid cells (Khanna-Gupta *et al*. 1997). An alternative explanation for the differential estrogen responses of the human and mouse lactoferrin ERMs in the context of their natural promoters is the presence of additional positive regulatory sequences that are unique to the human lactoferrin gene. Two possible elements are the GATA binding factor-1 element (GATA) and the estrogen response element extended half-site (ERRE), both of which are located just

upstream of the COUPP/ERE in the human lactoferrin gene promoter but not the mouse lactoferrin gene (compare Figs. 2.3A and 2.3B). GATA binding proteins are usually associated with gene expression in hematopoietic cells, therefore we will not attempt to address the function of these transcription factors in the estrogen response of the lactoferrin gene in mammary gland cells. The ERRE has been shown to play a role in the estrogen response of the human lactoferrin gene in human endometrial RL-95 cells (Yang *et al*. 1996) and DNase I footprint analysis and EMSA revealed that ERα binds the ERRE of the human lactoferrin gene (Zhang and Teng 2000), thus the ERRE is a candidate element that may work in tandem with the ERM to stimulate ERα-mediated transcriptional activity of the lactoferrin gene and its role in ERα-mediated transactivation of the lactoferrin genes in mammary gland cells is the focus of Chapter 3.

Characterization of promoter functions of various eukaryotic genes usually utilizes heterologous reporter genes including chloramphenicol transferase (CAT), β-galactosidase (β-gal), and luciferase (LUC). In our transfection assays, we have observed that the same promoter region linked to different reporter genes resulted in different promoter activities. Transient transfection of the 2.7 mLF-CAT reporter in MCF-7 and MCF-10a cells overexpressing ERα resulted in a 3-fold and 2-fold increase in reporter activity in response to DES (Figs. 2.6B and 2.6C). In contrast, transfection of this 2.7 kb region of the mouse lactoferrin gene promoter linked to the luciferase reporter gene and ERα expression vector in the human hepatoma cell line HepG2 resulted in nearly a 25-fold increase in relative transcriptional activity in response to 17β-estradiol (Hall and Korach 2002), which is similar to that observed with 1.0 hLF-CAT and 0.4 hLF-CAT reporters in our assays with mammary gland cells. Although the discrepancies in CAT and LUC reporter activities may be due to

cell-type specificity (mammary gland versus hepatic cell times), a recent report suggests an alternative explanation. The authors have identified silencer activity associated with the CAT reporter plasmid (Zhang *et al*. 2003), suggesting that this repressing activity may produce the observed low levels of estrogen responsiveness of the mLF-CAT reporters. The transfection experiments in this report utilized either a long (800 bp) or short (80 bp) region of the 5' regulatory element of the human blood coagulation factor IX (hFIX) gene linked to CAT or β-gal heterologous reporter genes or its autologous hFIX cDNA and CAT, β-gal (Zhang *et al*. 2003). The hFIX-CAT reporters showed variable reporter activity between constructs containing the short (80 bp) and long (800 bp) hFIX gene promoter sequences. The 80 bp hFIX promoter region was functional in transient transfection experiments, but the basal CAT activity of 800 bp reporter was 20% of the level observed with the short hFIX reporter. On the contrary, the long and short hFIX gene promoters linked to the β-gal and hFIX cDNA reporters showed comparable reporter activities in various transfected cell lines, suggesting that the silencer activity of the hFIX gene promoter may represent an artifact of the CAT reporter gene. These transfection data with the hFIX-CAT reporters are analogous to our MCF-7 transfection data with the short (0.1 mLF-CAT) and long (0.6, 0.9, 1.7, and 2.7 mLF-CAT) reporters, where the constructs containing the longer promoter sequences gave the lower levels of relative transcriptional activity (Fig. 2.4B). The basal transcriptional activities of the four human lactoferrin-CAT reporter genes did not significantly vary within a given transfected cell line (Fig. 2.4A, compare 0.1, 0.3, 0.4 and 1.0 hLF-CAT reporters) or among the cell lines (Fig. 2.4A, compare HEC-1B, MCF-7, HBL-100, MDA-MB-231 cells), further supporting the possibility that the putative negative regulatory regions within the mouse lactoferrin gene reporters are artifacts of the CAT assay system.

Table 2.1: Description of Plasmids Used in Transient Transfection Experiments

Plasmid	**Description**	**Reference**
pCAT-Basic	cloning vector without promoter	Promega, Madison, WI
pSV40-CAT	cloning vector with SV40 promoter	Promega, Madison, WI
pCH110	β-gal expression vector	Amersham Biosciences, Piscataway, NJ
2.7 mLF-CAT	mouse lactoferrin promoter (-2661/ -21)	Liu and Teng 1991
1.7 mLF-CAT	mouse lactoferrin promoter (-1731/ -21)	Liu and Teng 1991
0.9 mLF-CAT	mouse lactoferrin promoter (-922/ -21)	Liu and Teng 1991
0.6 mLF-CAT	mouse lactoferrin promoter (-587/ -21)	Liu and Teng 1991
0.4 mLF-CAT	mouse lactoferrin promoter (-396/ +1)	Liu and Teng 1991
0.3 mLF-CAT	mouse lactoferrin promoter (-291/ -21)	Liu and Teng 1991
0.2 mLF-CAT	mouse lactoferrin promoter (-234/ -21)	Liu and Teng 1991
0.1 mLF-CAT	mouse lactoferrin promoter (-103/ +1)	Liu and Teng 1991
1.1 hLF-CAT	mouse lactoferrin promoter (-1069/ +69)	Yang and Teng 1994
0.4 hLF-CAT	human lactoferrin promoter (-414/ +69)	Yang and Teng 1994
0.3 hLF-CAT	human lactoferrin promoter (-311/ +69)	Yang and Teng 1994
0.1 hLF-CAT	human lactoferrin promoter (-151/ +69)	Yang and Teng 1994

Table 2.2: Description of Reverse Transcription Polymerase Chain Reaction Primers and Cycling Parameters

Gene	Primer Set Sequence	Cycling Parameters
Human Lactoferrin	Forward- 5'GACCGCAGACATGAAACTTGTC3' Reverse- 5'GCCTCGGGTTGGGATACG3' Primers span exons 1 and 2 of the gene and a 101 bp region of the cDNA is amplified	RT- 50°C for 30 min denaturation- 94°C for 10 min 40 cycles of the following denaturation- 94°C for 15 sec annealing- 60°C for 30 sec extension- 68°C for 15 sec
Human ERα	Forward- 5'TGCCCTACTACCTGGAGAACGA3' Reverse- 5'GCCATACTTCCCTTGTCATTGG3' Primers span exons 1 and 2 of the gene and a 142 bp region of the cDNA is amplified	RT- 50°C for 30 min denaturation- 94°C for 10 min 25 cycles of the following denaturation- 94°C for 15 sec annealing- 62°C for 30 sec extension- 68°C for 15 sec
Human ERβ	Forward- 5'GGTGTGAAGCAAGATCGCTAGA3' Reverse- 5'GTGAGCATCCCTCTTTGAACCT3' Primers span exons 1 and 2 of the gene and a 122 bp region of the cDNA is amplified	RT- 50°C for 30 min denaturation- 94°C for 10 min 25 cycles of the following denaturation- 94°C for 15 sec annealing- 62°C for 30 sec extension- 68°C for 15 sec
Human ERRα	Forward- 5'AGATGTCAGTACTGCAGAGCGT3' Reverse- 5'GCTTCATACTCCAGCAGG3' Primers span exons 6 and 7 of the gene and a 322 bp region of the cDNA is amplified	RT- 50°C for 30 min denaturation- 94°C for 10 min 25 cycles of the following denaturation- 94°C for 15 sec annealing- 60°C for 30 sec extension- 68°C for 15 sec
Human βactin	Forward- 5'GACAGGATGCAGAAGGAGATCAC3' Reverse- 5'GCTGATCCACATCTGCTGGAA3' Primers span exons 5 and 6 of the gene and a 144 bp region of the cDNA is amplified	RT- 50°C for 30 min denaturation- 94°C for 10 min 25 cycles of the following denaturation- 94°C for 15 sec annealing- 60°C for 30 sec extension- 68°C for 15 sec

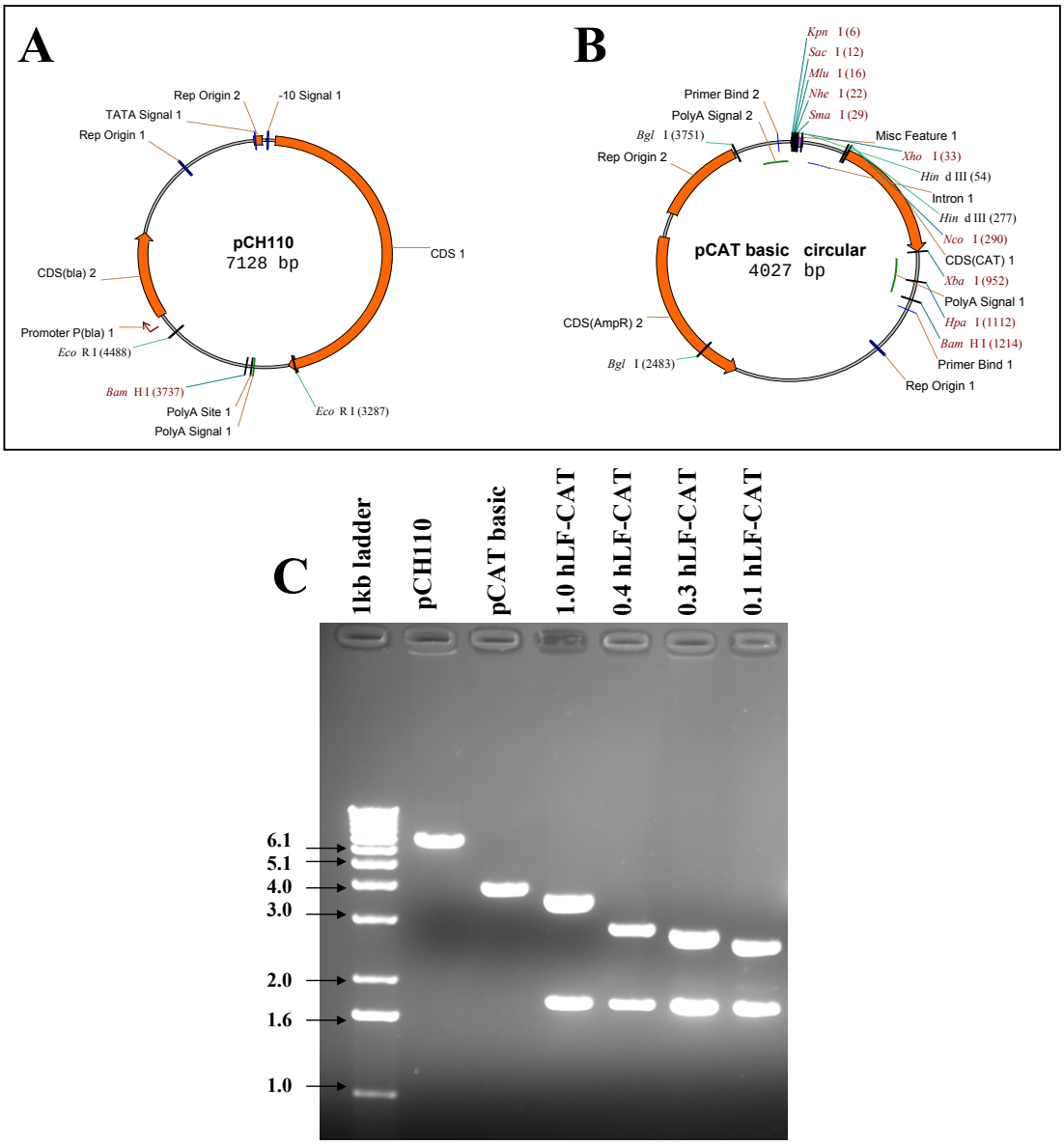


Figure 2.1: Restriction enzyme digestion of transformed, amplified and cesium-chloride purified reporter constructs

(A) Schematic of the pCH110 plasmid used in transfection experiments for expression of the β-galactosidase gene under the control of the SV40 early promoter (www.amersham.com). (B) Schematic of the pCAT basic promoter (www.promega.com) used for insertion of the various lengths of the human and mouse lactoferrin gene promoters in the restriction cloning sites upstream of the CAT gene. C. The indicated purified human LF plasmids were digested with BamH1 producing the following correct sized fragments: pCH110, 7 kb; pCAT basic, 4 kb; 1.0 hLF-CAT, 3.9 kb and 1.4 kb; 0.4 hLF-CAT, 3.4 kb and 1.4 kb; 0.3 hLF-CAT, 3.3 kb and 1.4 kb; 0.1 hLF-CAT, 3.2 kb and 1.4 kb.

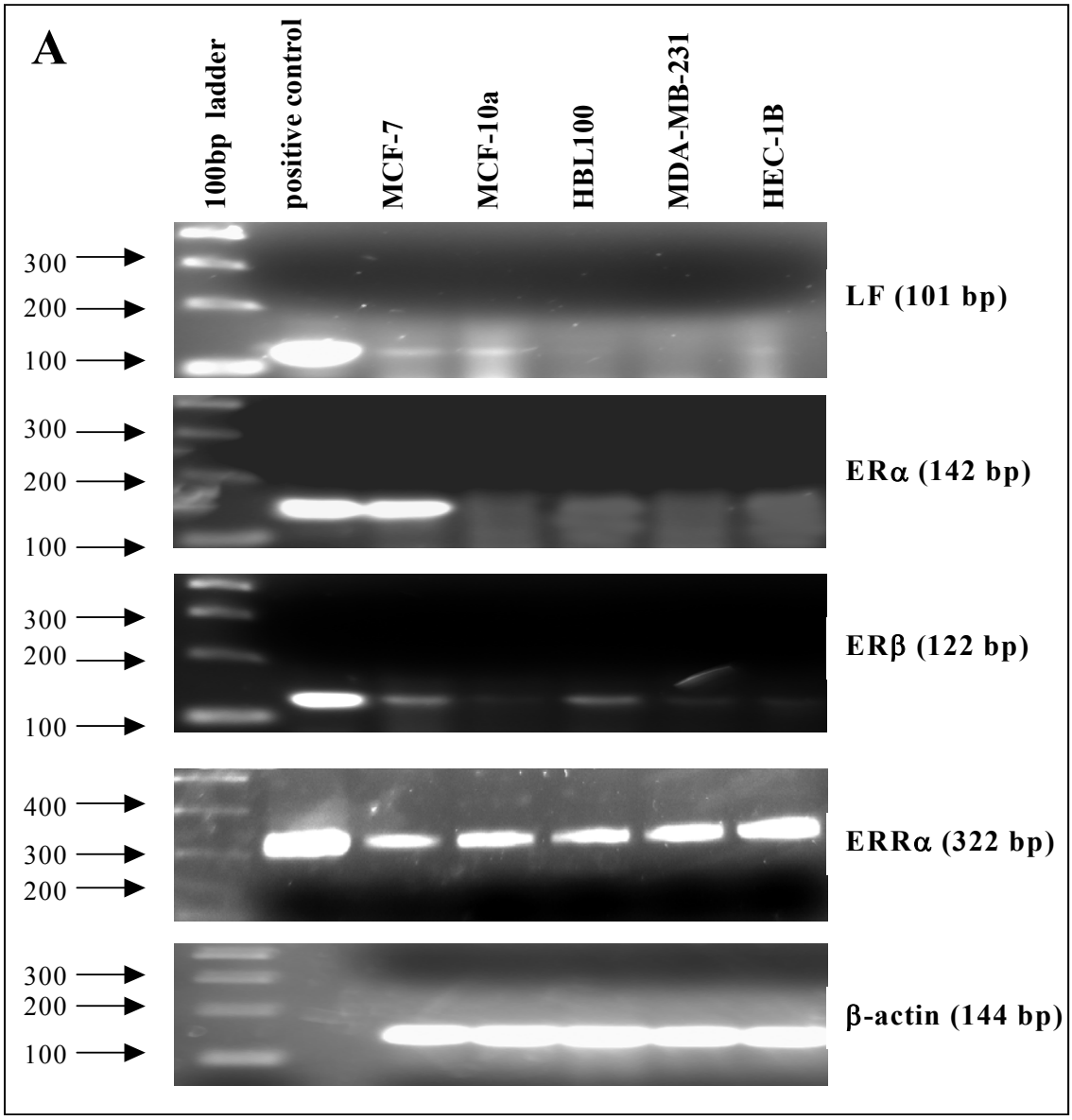
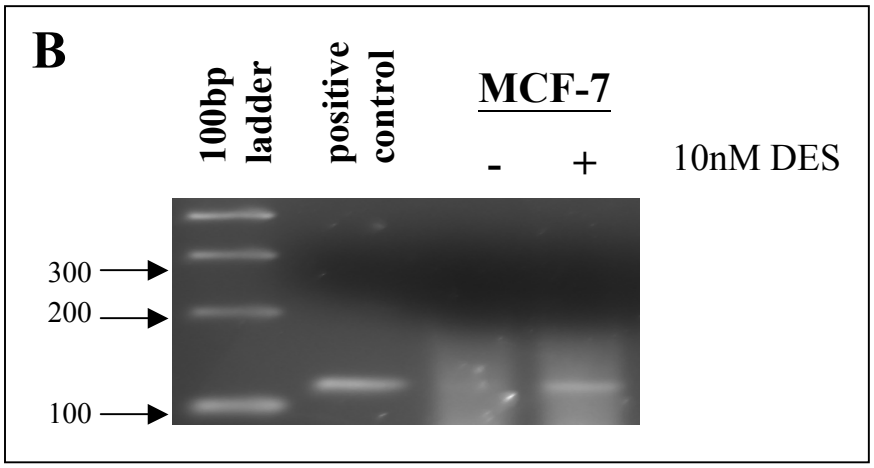


Figure 2.2: RT-PCR detection of lactoferrin and nuclear receptor gene expression in human mammary gland cells

A. Indicated cell lines were cultured in complete media. B. MCF-7 cells were grown in charcoal-stripped media for 4 days and then treated with or without 10 nM DES for 48 hr. Total RNA was isolated and the indicated transcripts were reverse transcribed and amplified as described in the materials and methods section using the primers described in Table 2.2. The indicated cDNA expression vectors served as positive controls to verify the size of the PCR products. The expression of β-actin mRNA was used as a control to confirm equal amounts of total RNA that was reverse transcribed and equal loading of PCR products. Data are representative of at least two independent experiments.

Figure 2.2: (continued)



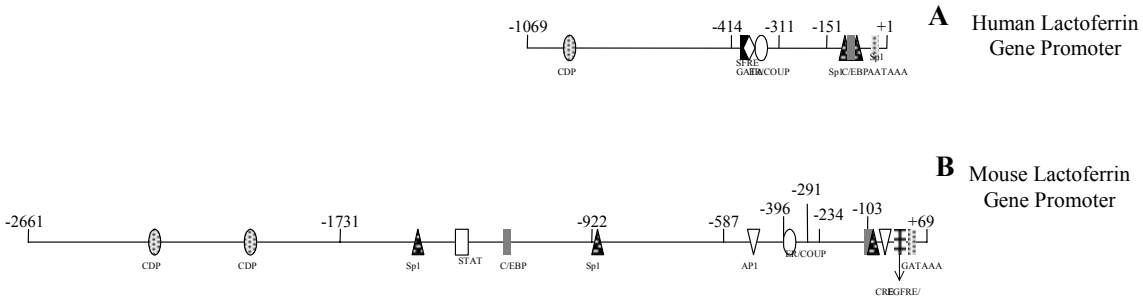


Figure 2.3: Comparison of the putative transcription factor binding sites in lactoferrin gene promoters

A. 1 kb region of the human lactoferrin gene promoter indicating the nucleotide location of the four reporters. B. 2.7 kb region of the mouse lactoferrin gene promoter indicating the nucleotide location of the eight mouse reporters.

AP1- activator protein 1
CDP- CCAAT displacement protein
C/EBP- CCAAT enhancer binding protein
COUP- chicken ovalbumin upstream promoter element
CRE- cAMP response element
EGFRE- epidermal growth factor response element
ERE- estrogen response element
ERRE- estrogen response element extended half-site
SP1- stimulatory protein 1
STAT- signal transduction activator of transcription

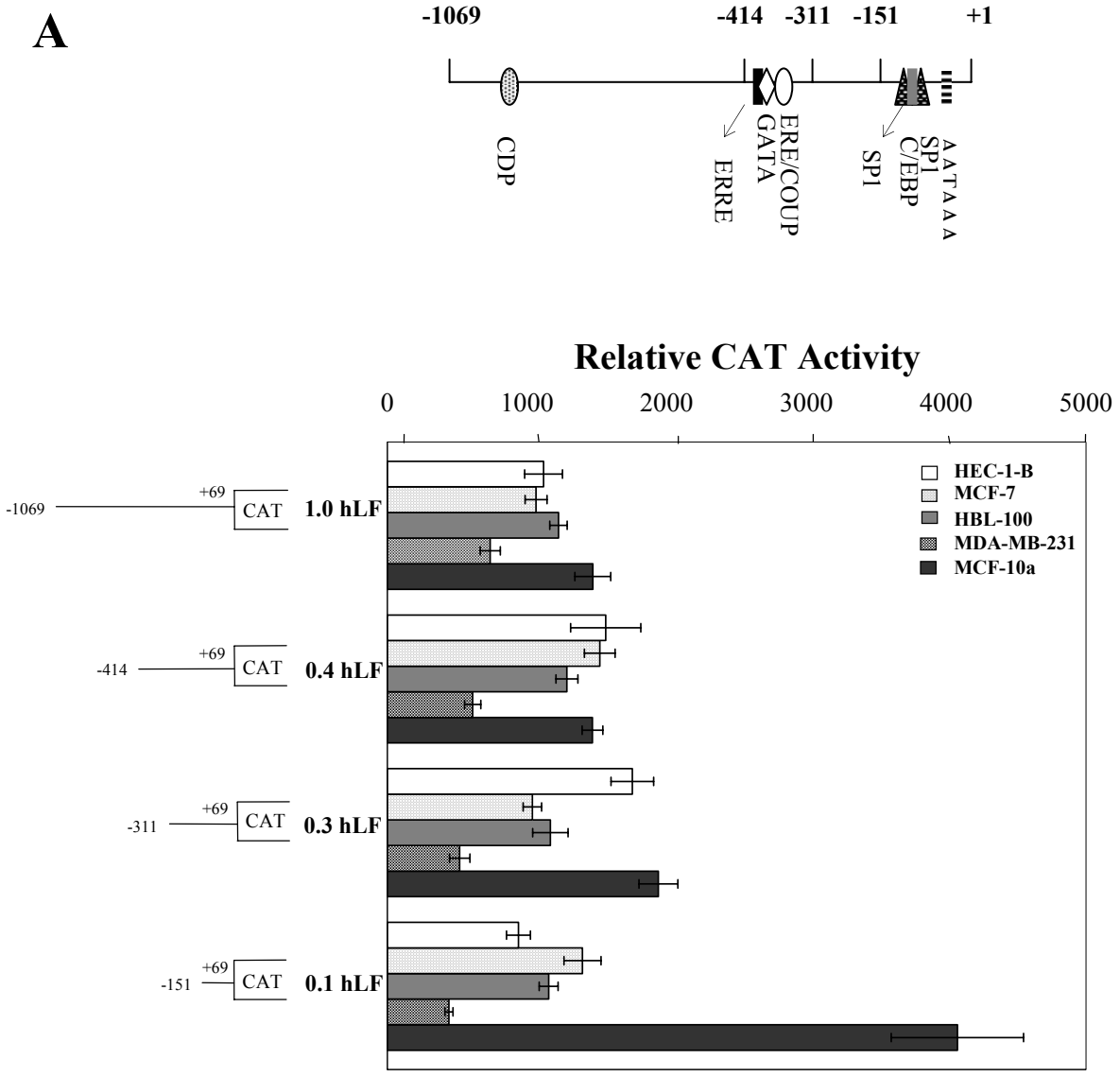
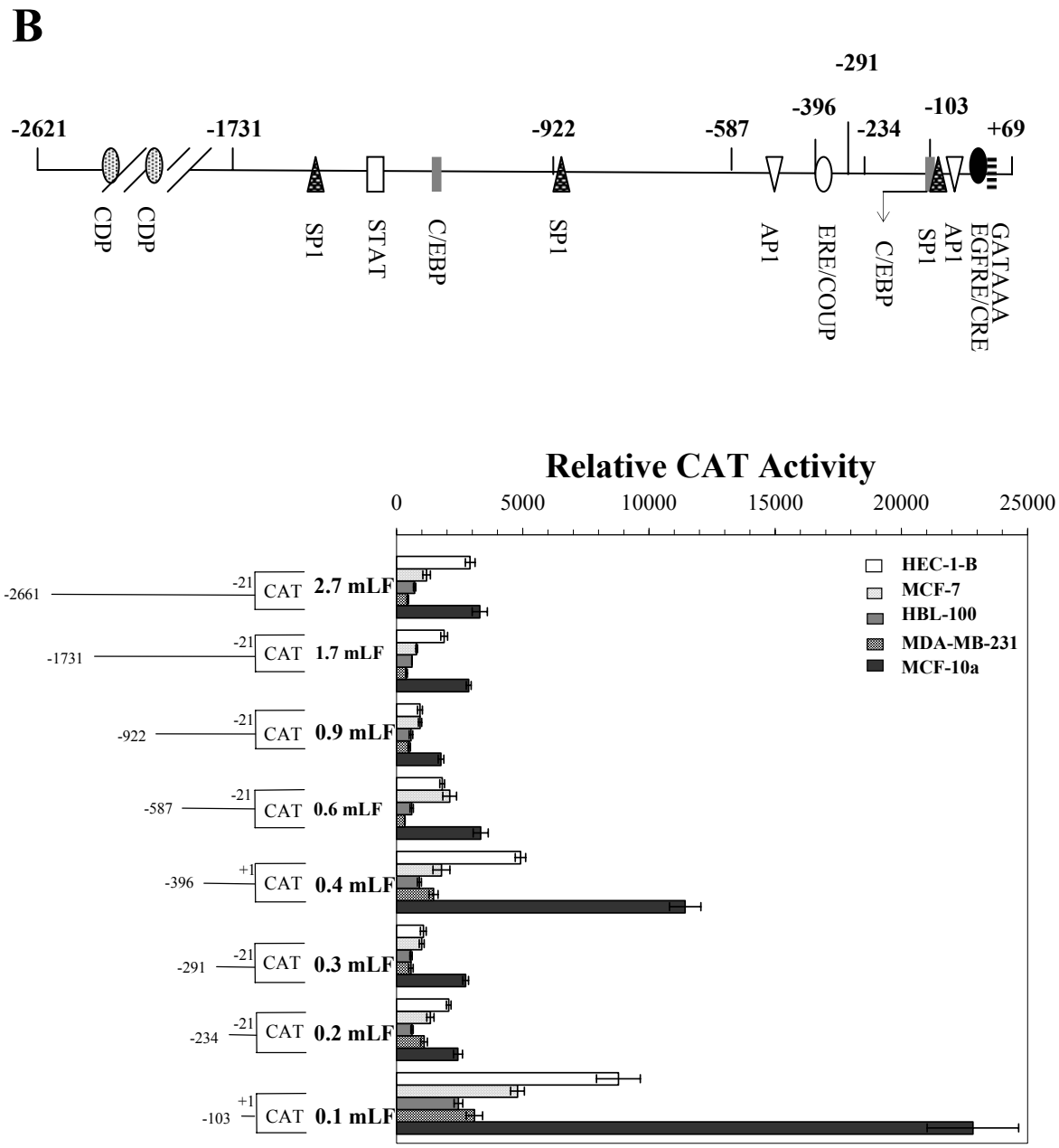


Figure 2.4: Basal activities of the various human and mouse lactoferrin reporters differ in the mammary gland cells

A. Top, Schematic presentation of the 1 kb region of the native human lactoferrin gene promoter indicating the relative positions of the CDP, ERRE, GATA, COUP/ERE, SP1, C/EBP and TATA-like elements. Bottom, Relative CAT activity normalized to β-gal activity is the value expressed as the mean ± SEM of four independent assays in duplicate. B. Top, Schematic presentation of the 2.7 kb region of the native mouse lactoferrin gene promoter indicating the relative positions of the CDP, SP1, AP1, COUP/ERE, C/EBP, EGFRE, CRE, and TATA-like elements. Bottom, Relative CAT activity normalized to β-gal activity is the value expressed as the mean ± SEM of four independent assays in duplicate.

Figure 2.4: (continued)



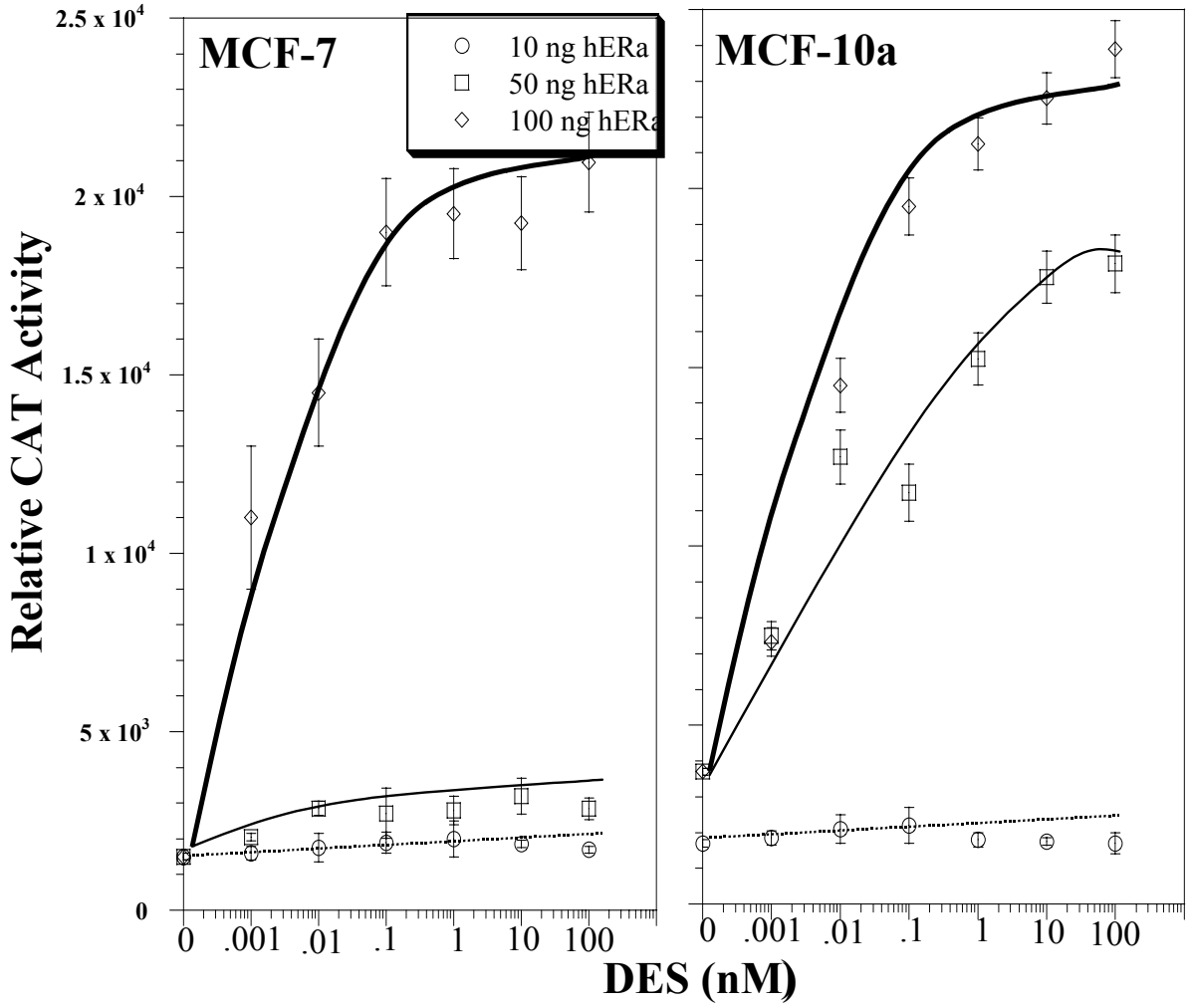


Figure 2.5: Dose response effects of ERα and DES on 400 bp human lactoferrin reporter activity in transfected mammary gland cells

Relative CAT activity normalized to β-gal activity is the value expressed as the mean ± SEM of three independent assays in duplicate. Open circles and broken lines, 10 ng ERα. Open squares and thin solid line, 50 ng ERα. Open diamonds and thick solid line, 100 ng ERα.

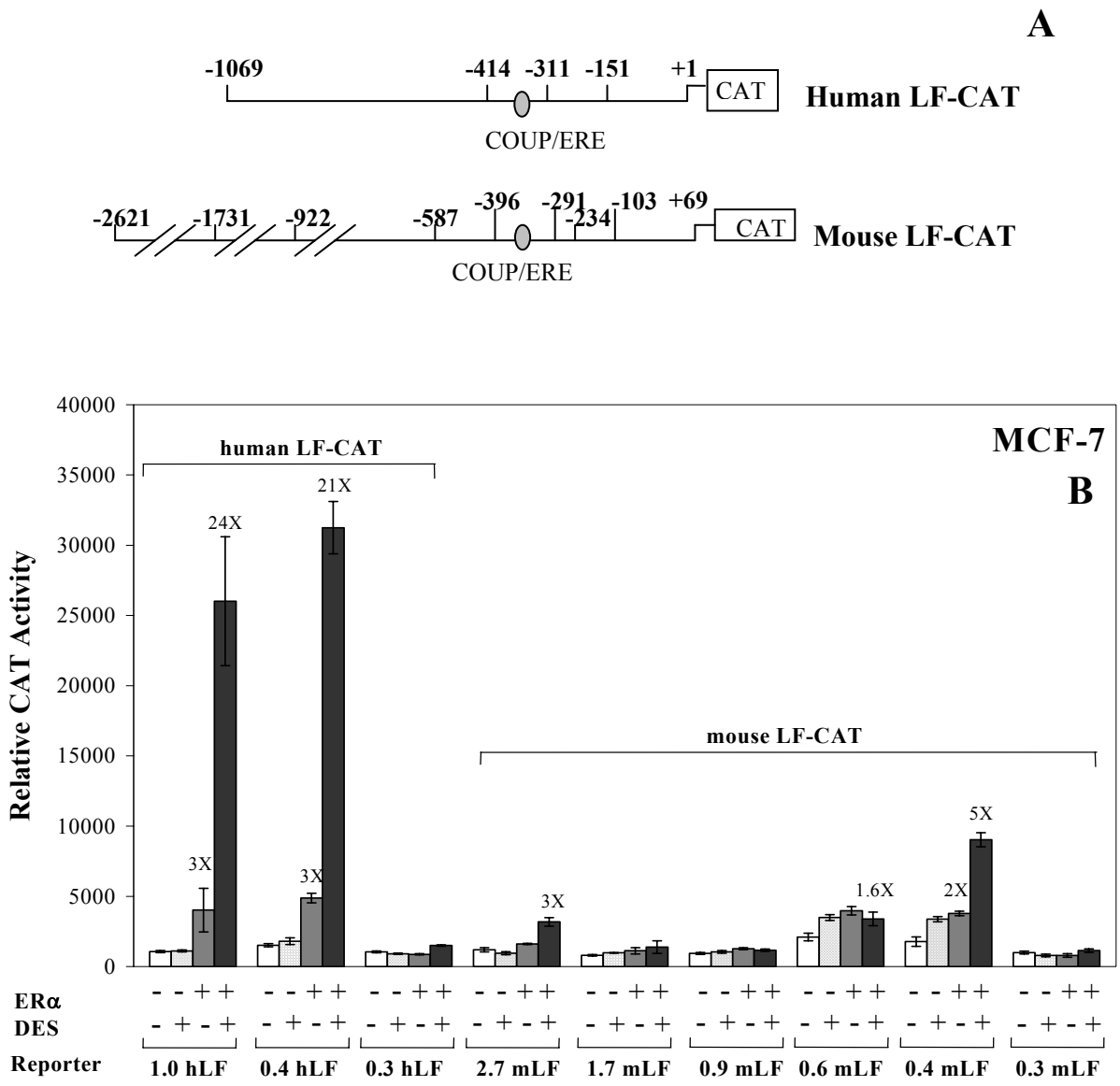
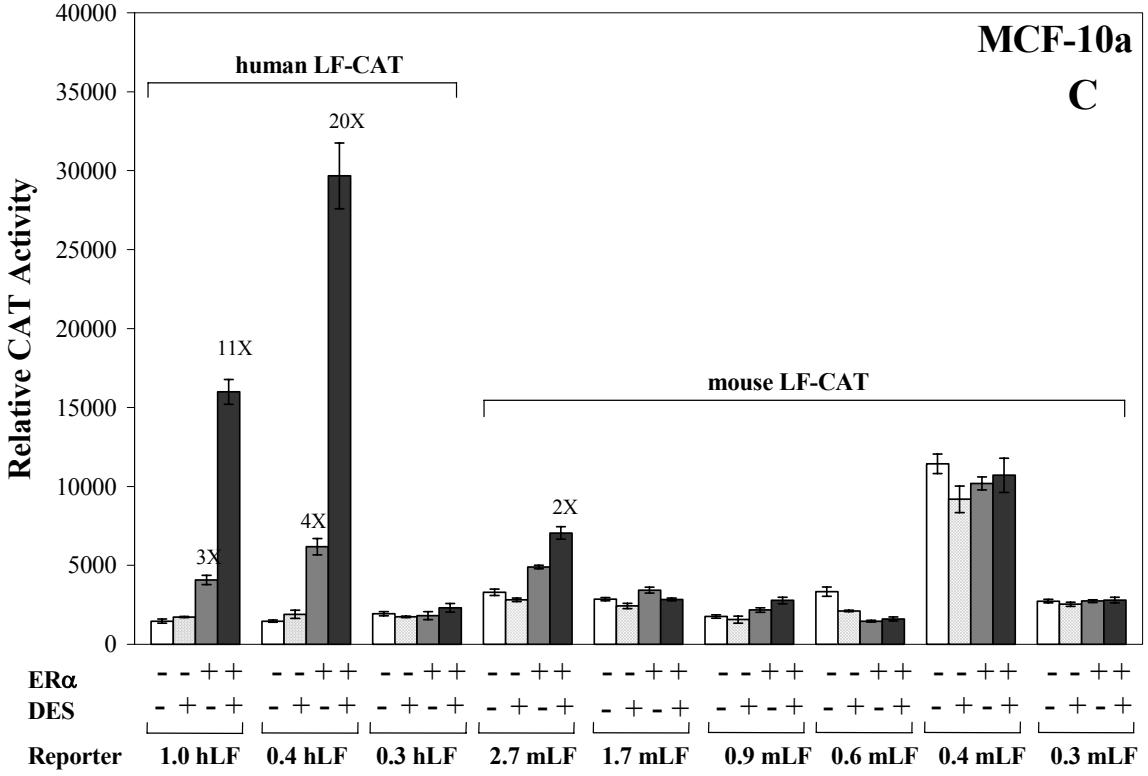
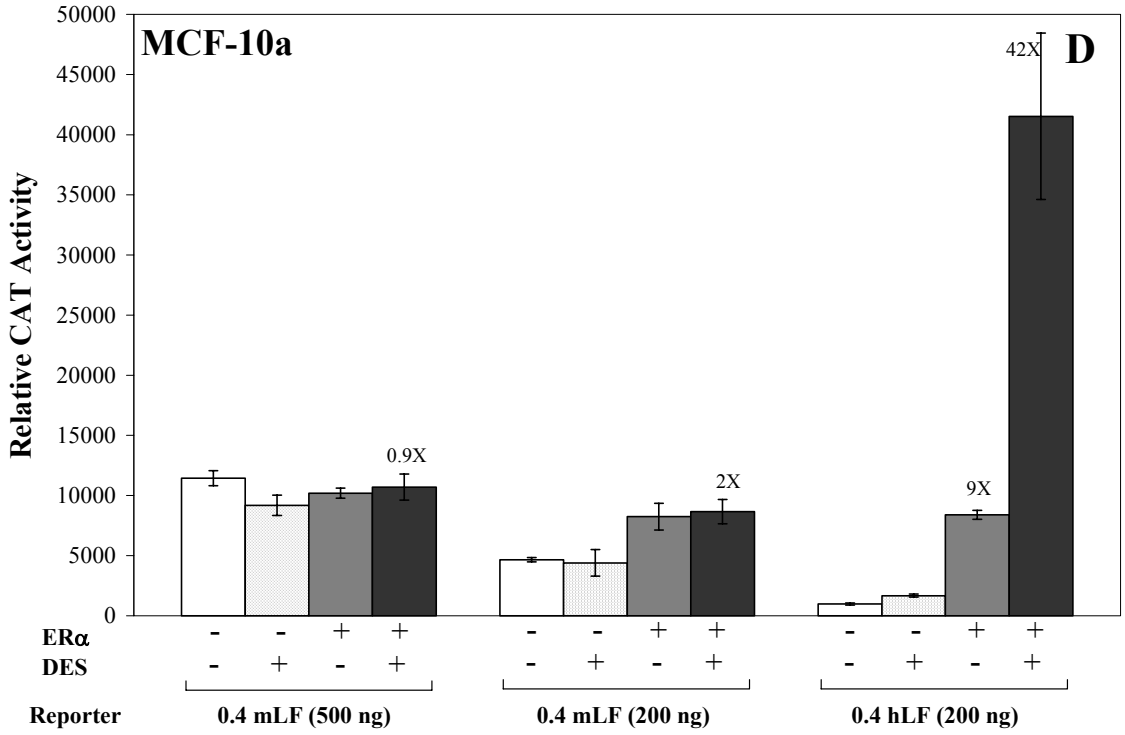


Figure 2.6: Differential estrogen response of the human and mouse lactoferrin ERMs in their natural promoter in transfected mammary gland cells

A. Schematic presentation of the 1 kb and 2.7 kb regions of the native human and mouse lactoferrin gene promoters indicating the relative positions of COUP/ERE element. B. (MCF-7), C and D. (MCF-10a). Relative CAT activity normalized to β-gal activity is the value expressed as the mean ± SEM of four independent assays in duplicate. Fold activation in reference to control (transfection of reporter alone) is indicated above the error bars.

Figure 2.6: (continued)





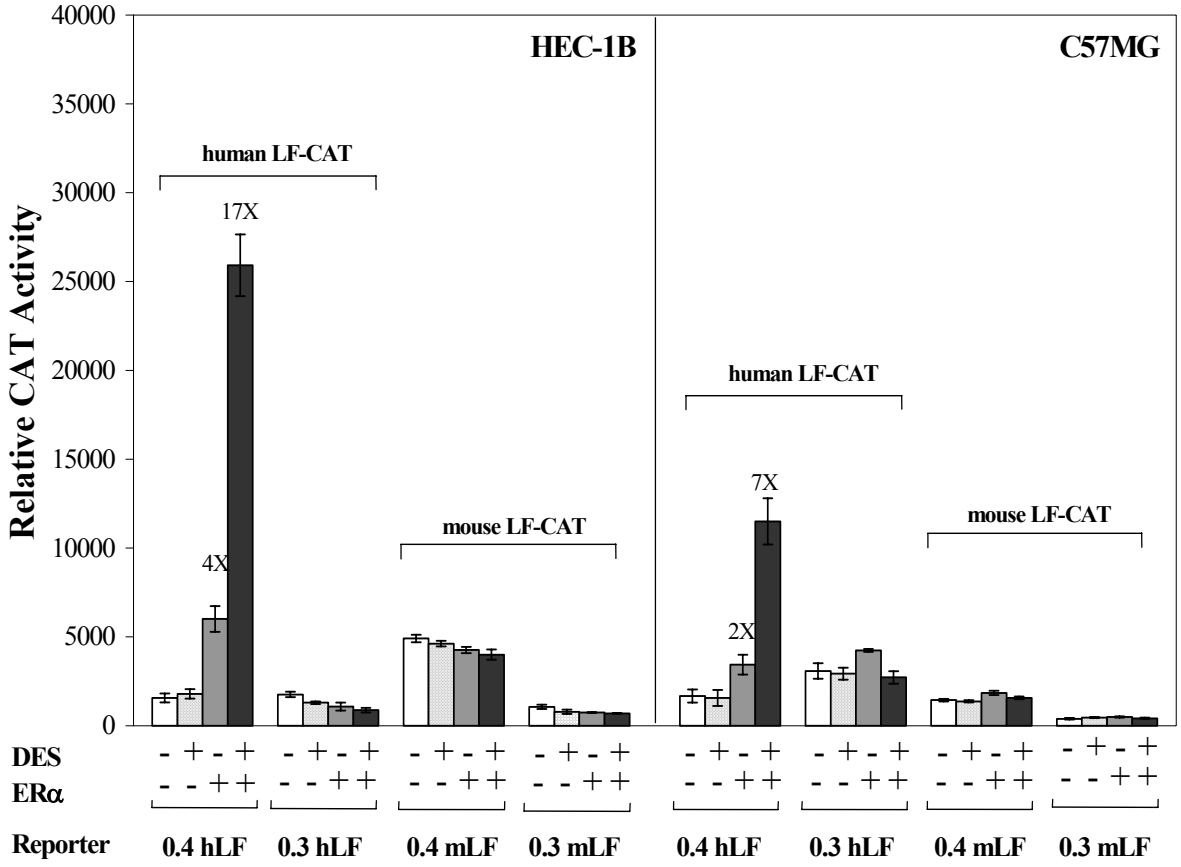


Figure 2.7: Tissue- or species-specificity do not account for the lowered estrogen response of the mouse lactoferrin ERM compared to the human lactoferrin ERM in their natural promoter contexts

The indicated human and mouse lactoferrin reporters were transfected in human endometrial (HEC-1B) or mouse mammary gland (C57MG) cells. Relative CAT activity normalized to β-gal activity is the value expressed as the mean ± SEM of four independent assays in duplicate. Fold activation in reference to control (transfection of reporter alone) is indicated above the error bars.

Chapter 3

Estrogen Response Element and the Promoter Context of the Human and Mouse Lactoferrin Gene Influence Estrogen Receptor α-Mediated Transactivation Activity in Mammary Gland Cells

I. 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 EREs in the context of their natural promoters. We demonstrated that one copy of the isolated human and mouse lactoferrin overlapping COUP/ERE sequence functioned as enhancers to a heterologous promoter in response to estrogen stimulation mammary gland cells overexpressing ERα and the estrogen-induced ERα-mediated transactivation activity was higher from the mouse COUP/ERE than the human COUP/ERE. However, in the context of their natural promoters, liganded ERα activated transcription of the human lactoferrin COUP/ERE 4-fold higher than the mouse lactoferrin COUP/ERE in transiently transfected MCF-7 cells. Since the ERRE of the human lactoferrin gene naturally occurs 18 bp upstream from the COUP/ERE and is absent in the mouse lactoferrin gene promoter, we tested the ERα-mediated estrogen response of the human lactoferrin COUP/ERE containing a mutated ERRE sequence and also created a chimeric mouse lactoferrin CAT reporter, which now encodes the ERRE in the identical arrangement as found in the human lactoferrin gene. The data revealed that the

ERRE played a role in the estrogen response of the human and mouse lactoferrin gene promoters. Mutation of the ERRE sequence in the human lactoferrin promoter reduced the ERα-mediated estrogen response by half, whereas the addition of the ERRE in the mouse lactoferrin plus rendered this reporter extremely responsive to estrogen stimulation. Using electrophoretic mobility shift assays (EMSAs) and limited protease digestions, we showed that ERα binds to the various lactoferrin estrogen response modules (ERMs) with different stability and the conformation of ERα bound to the human or mouse lactoferrin ERM with and without the ERRE differed. Furthermore, the presence of the ERRE influences the selectivity of coactivators in liganded ERα-mediated transcriptional activity. When the receptor is bound to human and mouse plus genes, which contain the ERRE, SRC-2 was preferred, while SRC-1 and SRC-3 coactivators were selectively enhancing the mouse lactoferrin gene activity. Moreover, PGC-1α and PERC coactivators robustly increase the transcriptional function of ERα in the presence of the ERRE. In conclusion, these data showed that the ERRE cooperates with the ERM of the lactoferrin gene to regulate ERα-induced transcription by influencing different receptor conformations and subsequently coactivator recruitment.

II. 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 (Krege *et al*. 1998, Lubahn *et al*. 1993, reviewed in Couse and Korach 1999). The biological actions of estrogens are mediated by two genetically different ligand-inducible receptors (Green *et al.* 1986, Kuiper *et al*. 1996, Mosselman *et al*. 1996, Tremblay *et al*. 1997, Walter *et al*. 1985) that have distinct expression patterns and functions on target genes. The estrogen receptors alpha (ERα) and beta (ERβ), members of the nuclear receptor superfamily, share only 47% overall peptide sequence identity, but their DNA binding domains share 94% peptide sequence identity (Kuiper *et al*. 1996, Mosselman *et al*. 1996, Tremblay *et al*. 1997). Binding of the natural estrogen 17β-estradiol to the receptor induces conformational changes, resulting in the departure of the receptor departure 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 and Norris 2002).

Estrogen action is a result of the activities of the receptor, the hormone response element and the cell context, which collectively influence transcription. The regulatory role of the ERE sequence in estrogen action has received a growing amount of attention over the past several years. 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'GGTCAnnnTGACC3' (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 reduced ERα transcriptional activation compared to the vitellogenin ERE (reviewed in Klinge 2001). 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. EMSA studies of ERα binding to three or four tandem repeats of the estrogen response element suggested that receptor binding is stabilized by dimers at adjacent sites and this cooperative binding promotes transcriptional synergy (Tyulmenkov *et al*. 2000).

Among the genes encoding imperfect EREs in their promoter regions are the human and mouse lactoferrin genes (Liu and Teng 1991, Teng *et al.* 1992). Promoter analysis of the human and mouse lactoferrin gene has identified several regulatory sequences that promote tissue-specific transcription in response to different stimuli including an overlapping COUP/ERE, AP1, CRE and C/EBP binding sites. Considerable work has been done in recent years to elucidate the mechanism of estrogen action on lactoferrin gene expression in the mouse and human uteri. Clinical studies showed that estrogen induced endogenous lactoferrin gene expression in the endometrium women normal cycling women (Teng *et al.* 1992, Teng *et al.* 2002b) and in the uterus of immature mice and mature mice during specific stages of the estrous cycle (Newbold *et* al. 1992, Pentecost and Teng 1987, Teng *et al*. 1989,

Teng *et al.* 2002a, Walmer *et al*. 1992). Molecular studies of the mouse lactoferrin gene showed that hormones and mitogens regulated lactoferrin gene expression in the uterus (Liu *et al*. 1993, Shi and Teng 1994 and 1996). Studies of the overlapping COUP/ERE 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-TF competing with ERα for DNA binding (Liu *et al*. 1993). In addition to the imperfect ERE element that enhanced ERα-mediated transactivation of the SV40 promoter in response to estrogen (Teng *et al*. 1992), the human lactoferrin gene promoter also contains an extended estrogen response element half-site (ERRE) that mediated ligand-independent transactivation of the human lactoferrin gene by estrogen-related receptor α (ERRα) (Yang *et al*. 1996, Zhang and Teng 2000). Considering that most imperfect EREs exhibit weaker ERα binding affinities (Curtis and Korach 1991, Darwish *et al*. 1991, Hall and Korach 2002, Wood *et al*. 2001, 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. Since the ERα and ERβ P box sequence (CEGCKA) is very similar to the estrogen-related receptor (ERRα) P box sequence (CEACKA), which recognizes the extended core DNA element ERRE, ERα may recognize and bind this extended half-site. Indeed, DNase I footprint protection analysis and EMSA revealed that ERα also binds the ERRE of the human lactoferrin gene, however less efficiently than ERRα (Zhang and Teng 2000).

In addition to its regulated expression in the uterus, lactoferrin is also highly expressed in milk secreted from mammary epithelial cells (reviewed in Teng 2002).

Relatively few data have been published on the hormonal regulation of lactoferrin gene expression in the mammary gland (Athie *et al.* 1996, Green and Pastewka 1978, Kumura *et al.* 2001). In general, prolactin, insulin and glucocorticoids are the principal hormones that coordinately regulate milk-specific protein gene expression during lactation. Thus, prolactin was originally thought to induce lactoferrin gene expression since lactoferrin protein was secreted into the medium in response to increasing concentrations of prolactin in cultured mammary explants from mid-pregnant mice (Green and Pastewka 1978). Additionally, the mouse lactoferrin gene encodes a potential Stat5 binding element (Genomatix/MatInspector), although its functionality has not been demonstrated to date. Recently, data from cultured porcine mammary epithelial cells suggested that lactoferrin gene expression occurs through prolactin-independent mechanisms in the pig (Kumura *et al.* 2001), contradicting the 20-year old report by Green and Pastewka (1978). Moreover, promoter analysis of human (Teng *et al.* 1992), bovine (Seyfert *et al.* 1994), porcine (Wang *et al.* 1998), and bubaline (water buffalo, Das *et al.* 1999) lactoferrin genes did not identify the specific prolactin-like DNA elements that may mediate lactogenic hormone signaling. Analysis of milk protein concentrations from several species showed that lactoferrin expression in human milk is higher than that in mouse milk (Masson and 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 10-fold higher level of total circulating estrogens just prior to parturition, when colostrum is secreted compared to established lactation that produces milk (Lönnerdal et al. 1976, Nagasawa et al. 1972). Taken together, these data suggest that the human and mouse lactoferrin gene expression may be regulated by estrogen in mammary gland cells.

In this report, we focus on the role of the estrogen responsive module in ERα-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 response 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 robust ERα-mediated transcriptional activity. Furthermore, we showed that the addition of the ERRE in the natural lactoferrin promoter sequence influences receptor conformation and the recruitment pattern of coactivators to the liganded receptor.

III. Materials and Methods

Reagents

Diethylstilbestrol (DES), 17β-estradiol, α-chymotrypsin (lot # 109H74853), and trypsin were purchased from Sigma (St. Louis, MO). Proteinase K (lot # D1734221) was purchased from Pierce Biotechnology (Rockford, IL). [^{14}C]-chloramphenicol was purchased from NEN Life Sciences (Perkin Elmer, Boston, MA) and [α^{32}P]-dCTP was purchased from Amersham Biosciences (Piscataway, NJ). Baculovirus-expressed recombinant human estrogen receptor α (lot #023K0784) was purchased from Sigma (St. Louis, MO).

Plasmids and Oligonucleotides

The 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 3.1. The DNA oligonucleotides used in EMSA were synthesized by Sigma Genosys (The Woodlands, TX) and are described in Table 3.2.

Site-directed Mutagenesis

To create the chimera reporter 0.4 mLF plus-CAT, the 12 bp ERRE half-site sequence located 18 bp upstream of the imperfect COUP/ERE in the human lactoferrin promoter was inserted 18 bp upstream of the imperfect COUP/ERE in the 0.4 mLF-CAT reporter using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The following selected primer set was synthesized by Sigma Genosys (The Woodlands, TX): (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 insert. The PCR-based mutagenesis reaction (50 μl) contained 0.4 mLF-CAT (20 ng), mutagenic primers (125 ng each), dNTPs (0.2 mM each), 1X reaction buffer, Pfu Turbo polymerase (2.5 U) and water. The cycling parameters for insertion of multiple nucleotides were denaturation at 95°C for 30 sec, 18 cycles of denaturation at 95°C for 30 sec, mutagenic primer annealing at 55°C for 1 min and extension at 68°C for 5 min. The PCR product was placed on ice for 2 min and then treated with Dpn I for 1 hr to digest the parental methylated DNA and select for the synthesized DNA containing the mutation. The mutagenic DNA (50 ng) was then transformed into XL1-Gold supercompetent cells (Stratagene, La Jolla, CA) and plated on LB-ampicillin plates. After incubation at 37°C overnight, the plates contained over 300 colonies and 4 colonies were picked and grown up in 5 ml LB-ampicillin. The plasmid DNA from the overnight culture was purified and used as the template (100 μg) was used in a Big Dye PCR sequencing reaction (Applied Biosystems, Foster City, CA). The PCR product was purified using post-reaction purification columns (Sigma, St. Louis, MO), dried under a vacuum and then sent to the DNA Sequencing Core (NIEHS, National institutes of Health, Research Triangle Park, NC) for sequencing. Three of the 4 clones that were sequenced contained the desired insert (Fig. 3.1B).

The 0.4 mLF plus-CAT mutant was constructed by mutating the guanines in the 12 bp ERRE sequence TCAAGGTCATCT to adenines in the 0.4mLF plus reporter using the following mutated primer set:

forward 5'GGGGATTTGCTTCAAAATCATCTTGCTCCATGC3'

and reverse 5'GCATGGAGCAAGATGATTTTGAAGCAAATCCCC3'. The mutagenesis reaction was carried out as reported above with the following cycling parameters for creating point mutations: denaturation at 95°C for 30 sec, 12 cycles of denaturation at 95°C for 30 sec, mutagenic primer annealing at 55°C for 1 min and extension at 68°C for 5 min. The mutagenic DNA was transformed into supercompetent *E. coli* cells and plated on LB-ampicillin plates. After an overnight incubation, over 100 colonies were counted. Eight colonies were amplified, purified and then sequenced and 4 clones contained the desired base pair mutations (Fig. 3.1C).

Cell Culture and Transient Transfection

The human MCF-7 (ATCC #HTB-22) and MCF-10a (ATCC #CRL-10317) cell lines were obtained from the ATCC (Manassas, VA). All cell lines were grown as monolayer cultures in 75 cm^2 tissue culture treated flasks in a humidified atmosphere at 37°C and 5% CO_2. MCF-10a cells were cultured in a 1:1 mixture of Ham's F12: Dulbecco's modified Eagle's medium (DME) supplemented 10 ng/ml insulin, 500 ng/ml hydrocortisone, 20 ng/ml epidermal growth factor, 1% penicillin-streptomycin and 5% fetal bovine serum (FBS). MCF-7 and HBL-100 cells were cultured in Eagle's minimum essential medium (EMEM) containing 10ng/ml insulin, 1% penicillin-streptomycin and 10% FBS. MCF-7 cells (ATCC #HTB-22, Manassas, VA) were cultured in EMEM supplemented with 10 ng/ml insulin and 10% FBS. MCF-10a cells (ATCC #CRL-10317) were cultured in a 1:1 mixture of Ham's F12: DME supplemented with 10 ng/ml insulin, 500 ng/ml hydrocortisone, 20ng/ml epidermal growth factor and 5% FBS. All media were supplemented with 100 U/ml penicillin and 0.1 mg/ml streptomycin and cells were maintained at 37ºC in 5% CO_2. 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). A DNA mixture consisting of 500 ng reporter plasmids, 100 ng pCH110, 100 ng ERα expression plasmid and carrier DNA up to a total of 750 ng/well was prepared and added to 2.25 μl FuGENE 6 diluted in 100 μl base media (3:1 ratio FuGENE6 to DNA). For coactivator studies, 200 ng coactivator expression plasmids, 300 ng reporter plasmids, 100 ng pCH110, 50 ng ERα expression plasmid and carrier DNA up to a total of 750 ng/well was prepared. After a 1 hr 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 was added for an additional 24 hr. CAT reporter activities were measured and normalized with the β-galactosidase (pCH110) activities as previously described in the Materials and Methods Section in Chapter 2.

Statistical Analyses

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

In Vitro Translation

The human wild-type ERα cDNA in the pSG5 vector was transcribed and translated *in vitro* using a coupled rabbit reticulocyte system (TNT, Promega, Madison, WI) according to the manufacturer's instructions.

Preparation of Nuclear Protein Extracts

MCF-7 cells were transfected with the human ERα expression vector (2 μg) or carrier DNA (2 μg) using FuGENE 6 and grown to 90% confluency in 60 mm tissue culture plates. Nuclear protein extracts were prepared by collecting the cells (8 plates) and centrifugation at 3000 x g for 10 min at 4ºC. The cells were washed once with cold PBS (10 ml) and centrifuged again at 3000 x g for 10 min. The volume of the cell pellets were estimated (~800 μl) and then the cells were resuspended in 4 ml hypotonic lysis buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 2 mM dithiothreitol, and 1% (v/v) protease cocktail). After a 10 min incubation on ice, the cell suspension was centrifuged for 10 min at 2000 x g and the supernatant was discarded. Next, the same hypotonic lysis buffer (1.6 ml) was added to the cells, which were then lysed using a dounce homogenizer. The cell suspension was checked under a microscope to verify that at least 90% of the cells were lysed and then centrifuged at 2000 x g for 10 min. The supernatant (cytosolic protein fraction) was removed and the pellet was centrifuged again at 15,000 x g for 20 min to remove residual cytosolic proteins. The nuclei were resuspended in nuclear 500 μl extraction buffer (20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 0.55 M NaCl, 0.2 mM EDTA, 20% (v/v) glycerol, 2 mM dithiothreitol, and 1% (v/v) protease cocktail) and lysed with at least 10 strokes of a dounce homogenizer. The cell suspension was checked under a microscope to verify that at least 90% of the cells were lysed and then incubated on ice for 30 min and vortexed in 5 min intervals. The nuclear suspension was centrifuged at 15,000 x g for 30 min and the resulting supernatant containing the nuclear proteins was collected, the protein concentration (4.3 μg/μl) was determined using the D_c non-detergent-based protein assay (BioRad) and aliquots were stored at -70°C.

Electrophoretic Mobility Shift Assays

Complementary oligonucleotide DNA sequences containing the estrogen-responsive regions in the lactoferrin and vitellogenin A2 genes were synthesized by Sigma Genosys (The Woodlands, TX) as described in plasmids and oligonucleotides section. The single-stranded oligonucleotides (21 μg each) were annealed by boiling for 5 min in annealing buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 200 mM NaCl) in 50 μl reactions and then slowly cooling to room temperature in the same water that was removed from the heat source over a period of 3 hours. The resulting double-stranded probes (1.25 μg) were labeled in 25 μl reactions consisting of α^{32}P dCTP (10 μl, ~50μCi), 0.25 mM each dATP, dGTP, dTTP, Klenow DNA polymerase (2 μl) and 1X Klenow reaction buffer for 30 min at room temperature. A chase solution (2 μl) consisting of 1 mM each dATP, dCTP, dGTP and dTTP was then added for 5 min and the reaction was stopped by the addition of 500 mM EDTA (1 μl). The labeled probes were purified through G-25 spin columns (Amersham Biosciences, Piscataway, NJ) to remove unincorporated nucleotides and then 2 μl samples were counted on a scintillation counter. The specific activity of the probes was ~ 0.8 to 2 x 10^8 cpm/μg DNA. The probes were then purified through a preparative 5% non-denaturing acrylamide gel (0.5X TBE, 5% acrylamide gel mix, 0.07% APS, 0.07% TEMED) in 0.5X TBE buffer. The gel was run at 170 V for 1 hr 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 placed into a 1.5 ml tube. The gels were crushed with a flat-tip wooden stick and the DNA was eluted from the gel overnight at 4ºC in 200 μl elution buffer (10 mM Tris-HCl pH 8.0, 10 mM NaCl). The next morning, the gel-elution buffer mixtures were centrifuged at 14,000 rpm for 10 min and the supernatant was removed and then passed through a G-25 column for purification. A

2 μl sample was counted to determine the ^{32}P counts and the probes contained at least 1 x 10^5 cpm/μl.

The binding reactions (10 μl) included the ^{32}P-labeled probes (0.2 to 0.5 ng DNA at 2 x 10^4 cpm) and binding buffer (4 μg poly dI-dC (Amersham Biosciences, Piscataway, NJ), 8 mM HEPES pH 7.9, 8% glycerol, 2% Ficoll-400, 50 mM KCl, 10.8 mM dithiothreitol, 1 μg bovine serum albumin, 80 μM EDTA, 2 mM $MgCl_2$), 10^{-6} M 17β-estradiol, ERα and either protease or antibody. For limited proteolysis, liganded-*in vitro* translated ERα (2 μl) and baculovirus-expressed ERα (1.5 pmoles) were incubated with the binding buffer and DNA for 30 min at room temperature. Then proteinase K (0, 1.25, 2.5, 5, 10, 20, or 40 ng) α-chymotrypsin (0, 25, 30, 35, or 40 ng), or trypsin (0, 1.25, 2.5, 5, 10, 20 or 40 ng) in a 1 μl volume was added to the binding reactions for an additional for 10 min at room temperature. For antibody supershift experiments, nuclear protein extract from MCF-7 cells overexpressing ERα, liganded *in vitro* translated ERα, or liganded baculovirus-expressed ERα was pre-incubated with one of the antibodies described in Table 3.3 for 30 min on ice and then the other components of the binding reaction were subsequently added and further incubated for 10 min at room temperature. Reactions were resolved on a 5% non-denaturing polyacrylamide gel at 170 V for 2 hr. Gels were dried under vacuum and visualized by autoradiograph on Kodak Bio-Max MR film with an intensifying screen at -70ºC overnight.

IV. Results

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

To characterize the estrogen response of the lactoferrin genes in mammary gland cells, the human (hLF COUP/ERE) and mouse (mLF COUP/ERE) reporters (Table 3.1 and Fig. 3.2A, top panel) were transiently transfected into normal (MCF-10a/ ERα negative) and tumorigenic (MCF-7/ ERα positive) human mammary epithelial cells that express detectable levels of the endogenous lactoferrin gene by RT-PCR (Fig. 2.2A). One copy of either the human or mouse lactoferrin ERM functions as a transcriptional enhancer in transiently transfected MCF-7 (Fig. 3.2B) and MCF-10a (Fig. 3.2C) cell lines overexpressing human ERα. There is a 36-fold increase with the mLF COUP/ERE versus a 16-fold increase with the hLF COUP/ERE relative to untreated MCF-7 cells (Fig. 3.2B) and a 10-fold increase with the mLF COUP/ERE versus a 6-fold increase with the hLF COUP/ERE relative to untreated MCF-10a cells (Fig. 3.2C). The basal activity of the simplified hLF ERE reporter was higher than that of the mLF ERE, hence, we observed a higher fold increase in response to estrogen with the mLF ERE, although the estrogen-induced relative CAT activity values are almost identical. The estrogen response of the mLF COUP/ERE in MCF-7 cells overexpressing mouse ERα is exactly the same as cells overexpressing human ERα (Fig 3.2B, compare black dotted bar with black solid bar). In MCF-10a cells overexpressing mouse ERα, the estrogen response of the mLF COUP/ERE in declined from a 10-fold increase observed with human ERα-mediated to a 6-fold increase (Fig 3.2C, compare black dotted bar with black solid bar).

Next, we examined the 400 bp region of the lactoferrin gene promoter, which includes the COUP/ERE and the natural minimal lactoferrin promoter. The human lactoferrin gene promoter contains an ERRE upstream of the COUP/ERE while the mouse gene contains the COUP/ERE at the same position as the human lactoferrin promoter, but lacks the ERRE (Fig. 3.3A). 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 ERα showed that estrogen-induced transactivation is dependent upon the ERE in the promoter of the lactoferrin gene. Overexpression of ERα 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. 3.3B, compare 0.4 hLF and 0.3 hLF CAT activities). In contrast, the 0.4 mLF-CAT reporter, which contains only the COUP/ERE, had a 5-fold increase in estrogen-induced CAT activity in MCF-7 cells, which is 4-fold lower than the response of the 0.4 hLF-CAT construct (Fig. 3.3B left panel, compare 0.4 hLF and 0.4 mLF CAT activities). MCF-10a cells overexpressing ERα and the mLF-CAT reporter with or without the COUP/ERE were unresponsive to estrogen (Fig. 3.3B right panel, compare 0.4 mLF and 0.3 mLF CAT activities), although the baseline activity of the 0.4 mLF-CAT reporter was higher in MCF-10a cells. As we reported in Chapter 2, the lack of estrogen response of the mLF COUP/ERE in the context of its natural promoter is not a consequence of masking by elevated basal activity, tissue-specificity, or species-specificity in the cell lines tested. Rather, additional positive promoter elements within the natural 400 bp promoter context that 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.

Alignment of the 400 bp regions of the human and mouse lactoferrin gene promoters revealed the presence of an estrogen response element extended half-site (ERRE) and GATA binding element in the human lactoferrin promoter only, while the presence of a GC-rich region (CRE/AP1) in the minimal 100 bp promoter region containing a cAMP response element and an AP1 site is unique to the mouse lactoferrin promoter (Fig 3.4). Since GATA binding proteins are usually associated with gene expression in hematopoietic cells we have already published data showing that the GC-rich region in the mouse is responsive to mitogens (Shi and Teng 1994 and 1996) and that ERα binds the ERRE in response to DES treatment (Yang *et* al. 1996, Zhang and Teng 2000), we decided to directly examine the contribution of the ERRE in estrogen action on the lactoferrin genes in mammary gland cells. We mutated the guanine dinucleotide critical for nuclear receptor binding to either the ERRE (m1), ERE (m6), or in combination (m1/m6) in the human lactoferrin gene promoter (Fig 3.5A, top panel, Yang *et al*. 1996). Mutation of the ERRE in the 0.4 hLF-CAT construct (m1) reduced the estrogen response in MCF-7 cells by 50% and in MCF-10a cells by 25% (Fig. 3.5A). Mutating the ERE alone (m6) or in combination with the ERRE (m1/m6) further reduced the estrogen response in both cell lines to ~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 COUP/ERE into the natural 0.4 mLF promoter (Fig. 3.5B, top panel). The correct spatial insertion and sequence of this construct designated as 0.4 mLF plus-CAT was

verified by DNA sequencing (Fig 3.1B). MCF-7 cells transfected with the 0.4 mLF plus-CAT construct had an extremely robust estrogen response, which was significantly greater than either of the responses of the 0.4 mLF-CAT and 0.4 hLF-CAT reporters (compare Figs. 3.5B and 3.3B, black bars, left panels). As further support of this enhanced sensitivity to estrogen, the limited amount of endogenous ERα in MCF-7 cells activated the 0.4 mLF plus-CAT reporter 4-fold compared to a 2-fold induction reported with the 0.4 mLF-CAT reporter (Fig. 3.5B, compare 0.4 mLF plus and 0.4 mLF dotted bars, left panel) and overexpression of ERα conferred a 60-fold increase in estrogen-induced reporter activity compared to untreated cells transfected with the reporter alone (Fig. 3.5B, left panel). In fact, the estrogen response of this reporter was also elevated in MCF-10a cells co-transfected with ERα compared to the native mouse lactoferrin reporter lacking the ERRE (Fig. 3.5B, compare 0.4 mLF plus and 0.4 mLF, black bars, right panel). Also, the estrogen-induced relative CAT activity of the 0.4 mLF plus-CAT reporters was comparable to the 0.4 hLF-CAT reporter in MCF-10a cells (~30,000, compare Figs. 3.3B and 3.5B, black bars, right panels).

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. 3.5B top panel, an asterisk represents mutated nucleotides). Interestingly, destroying the added ERRE in the 0.4 mLF plus-CAT showed a loss of strong estrogen response and the estrogen stimulated activity was reduced to nearly an identical level as the wild-type 0.4 mLF in either MCF-7 or MCF-10a (Fig. 3.5B, compare 0.4 plus mutant and 0.4 mLF, black bars). If any potential inhibitory sequences were

disrupted, then the ERα-mediated estrogen response of the 0.4 mLF plus mutant reporter would have remained. Thus, the apparent differences in estrogen response of human and mouse lactoferrin EREs in its natural promoter context were resulted from the presence of the ERRE sequence in the human lactoferrin promoter.

We previously demonstrated that both ERα and ERRα bound the ERRE and that these two nuclear receptors expressed in nuclear extracts of a human endometrial cell line interacted *in vitro* (Yang *et al*. 1996), therefore we had to rule out the possibility that ERRα, not ERα, robustly transactivates the mouse lactoferrin gene containing the added ERRE in response to estrogen. Although there is no evidence to date supporting the formation of ERα-ERRα heterodimers *in vivo* or *in vitro*, we overexpressed ERRα in the $ERα^{+}$/ $ERRα^{+}$ MCF-7 cell line. Transient transfections of MCF-7 cells show that ERRα conferred a low level of ligand-independent transactivation of the human lactoferrin and mouse lactoferrin plus reporter genes containing the ERM (Fig. 3.6, solid and spotted black bars). Furthermore, mutation of the ERRE in the mouse lactoferrin plus reporter (0.4 mLF plus mutant) showed a similar strength of ERRα-mediated transcriptional activity, suggesting that the constitutive activity of ERRα does not predominantly influence the estrogen response of the lactoferrin gene.

The binding affinity of ERα to the different lactoferrin ERMs differs

To investigate the mechanism of ERRE contribution in estrogen action, we examined MCF-7 nuclear protein binding to the double stranded oligonucleotides of human (hLF), mouse (mLF) and mouse plus (mLF plus) lactoferrin ERMs and the vitA2 consensus ERE (Table 3.2). By EMSA studies (Fig. 3.7), 17β-estradiol was added to the nuclear extract

preparation, which was incubated with either no antibody (lanes 1, 6, 11, and 16), pre-immune serum (PRE, lanes 2, 7, 12, and 17), ERα antibody (H222, lanes 3, 8, 13, and 18), COUP-TF antibody (COUP, lanes 4, 9, 14, and 19), or ERRα antibody (PEP3, lanes 5, 10, 15, or 20) for 30 min on ice prior to the addition of the various ^{32}P-labeled oligonucleotides. We detected several specific protein-DNA complexes with all four oligos, but our antibodies could only supershift complexes containing COUP-TF bound to the lactoferrin ERMs (COUP, lanes 4, 9, and 14) and ERα bound to vitA2 (H222,lane 18). Since we could not demonstrate specific binding of ERα from MCF-7 cells overexpressing ERα to the various lactoferrin ERMs using the ERα antibody (H222), we examined the binding of ERα from MCF-7 nuclear protein extracts (MCF-7), *in vitro* translated receptor (In vitro trl), and baculovirus-expressed receptor (Recombinant) binding to the double stranded oligonucleotides with either no antibody (lanes, 2, 6, 10, 15, 19, 23, 28, 32, 36, 41, 45, and 49), ERα antibody H222, (lanes, 3, 7, 11, 16, 20, 24, 29, 33, 37, 42, 46, and 50), ERα antibody Ab-10 (lanes, 4, 8, 12, 17, 21, 25, 30, 34, 38, 43, 47, and 51), or ERα antibody H-184 (lanes 5, 9, 13, 18, 22, 26, 31, 35, 39, 44, 48, and 52). MCF-7 ERα formed several specific protein-DNA complexes with all four oligos, but none of the ERα-specific antibodies (H222, lanes 3, 16, 29, and 42; Ab-10, lanes 4, 17, 30, and 43; H-184, lanes 5, 18, 31, and 44) could supershift ERα-specific complexes. Recombinant ERα formed one ERa-DNA complex with the hLF (lanes 10-13) and mLF plus (lanes 23-26) ERMs, whereas no protein-DNA complexes were observed with the mLF (lanes 36-39) and vitA2 (lanes 49-52) oligos. I*n vitro* translated ERα formed two specific complexes with all four oligonucleotides (lanes 6, 19, 32, 45) that could be supershifted by all three ERα-specific antibodies (hLF, lanes 7-9; mLF plus, lanes 20-22, mLF, lanes 33-35; vitA2, lanes 46-48).

Based on the previous data set, we chose to use *in vitro* translated ERα for subsequent EMSA studies and verified the results (Fig. 3.9). Liganded *in vitro* translated ERα was pre-incubated with either no antibody (lanes 2, 7, 12, and 17), ERα antibody (H222, lanes 3, 8, 13, and 18), a mouse lactoferrin antibody (LF, lanes 4, 9, 14, and 19) or pre-immune serum (PRE, lanes 5, 10, 15, and 20) prior to the addition of the various ^{32}P-labeled oligonucleotides. Again, ERα formed two specific complexes with all four oligonucleotides, suggesting that the receptors bind to the DNA elements (monomer, lower band; homodimer, upper band) and can be supershifted by the ERα-specific antibody (H222) but not with non-relevant LF or PRE. The results indicated that the *in vitro* translated receptor binds specifically and effectively to the four EREs because the receptor binds specifically and effectively to the four ERMs because the receptor bands are not detected with the in vitro translation mixture (TNT, lanes 1, 6, 11, and 16), which produced non-specific bands (NS). In time course studies, ERα-DNA complexes were initially detected after a 5 min incubation period and remained stable for more than 90 min for all four oligonucleotides (Fig. 3.10). Competition studies revealed weaker binding of the receptor to the hLF and mLF probes than with the mLF plus and vitA2 probes (3.11). The labeled mLF-ERα complex is completely competed off by a 10 fold molar excess of cold probe (Fig. 3.11A, lane 3), whereas a higher molar excess of cold oligonucleotide is needed to completely compete with the hot mLF plus (50X, lane 12) and vitA2 (25X, lane 17) probes for receptor binding. The more stable interaction of the mLF plus and vitA2 ERMs with ERα are also shown in Figure 3.11B. Addition of 100X cold oligonucleotide quickly displaced ERa to hLF (lanes 1-7), while the receptor was never completely displaced from the labeled mLF, mLF plus and vitA2 ERMs. A 5 min incubation with excess cold self-

competitor was required to decrease ERα binding to labeled mLF by ~ 50% (compare lanes 15 and 17), whereas 10 min was required for mLF plus (compare lanes 8 and 11) and over 20 min was required for vitA2 (compare lanes 22 and 26). To determine the percentage of free probe shifted by ERα relative to the total input, the relative intensity of each band (Fig. 3.9, lanes 2, 7, 12, and 17) was quantitated by pixel histogram analysis using Adobe Photoshop® and the sum of the ERα bands (ERα-shifted) was divided by the sum of total bands (ERα-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 38% for mLF plus, 37% for mLF and vitA2, and 24% of hLF (Table 3.4). These EMSA data (Figs. 3.9 and 3.10) showing a lower percentage of receptor-hLF complexes (24%) compared to the receptor-mLF complexes (37%) (Table 3.4) are inconsistent with the functional studies in that the ERα-mediated transactivation from the natural 400 bp human was much more robust than the mouse lactoferrin gene promoter (Fig. 3.3B), suggesting that other cis-acting elements may be involved.

ERE sequence modulates the conformation of ERα

Next, we investigated whether ERα conformation differed when bound to the various estrogen responsive modules by using the protease sensitivity assays. Baculovirus-expressed ERα was added to the *in vitro* translated protein mix in order to increase the concentration of ERα and different amounts of protease were added to the binding reactions for 10 min at room temperature. Representative limited protease digestion EMSAs of ERα bound to the ^{32}P-labeled hLF, mLF, mLF plus, or vitA2 oligonucleotides are shown (Fig. 3.12). Addition of baculovirus-expressed ERα to the protein mix resulted in an increase in

the amount of the receptor forming homodimers, which bind the DNA more tightly as shown in the pattern of undigested specific bands when ERα is incubated with the mLF and vitA2 ERMs (compare Fig. 3.9, lanes 12 and 17 to Fig. 3.12A, lanes 15 and 22). Proteinase K cleaves at peptide bonds adjacent to the carboxyl groups of aliphatic and aromatic amino acids and digestion of ERα bound to the various DNA elements produced proteolytic complexes having distinct migration patterns (Fig. 3.12A). Under identical binding conditions where the only difference is the sequence of the oligonucleotides, we observed differences in ERα sensitivity to proteinase K (hLF, lanes 4-7; mLF plus, lanes 11-14; mLF, lanes 18-21; vitA2, lanes 25-28). Incubation of the DNA-bound receptor with as little as 1.25 ng proteinase K was sufficient to produce some proteolytic cleavage fragments (lanes 2, 9, 16, and 23). Digestion of ERα bound to the mLF and mLF plus ERMs produced proteolytic complexes of similar mobility at lower protease amounts (lanes 4, 5, 11, 12, 18, 19, 25, and 26), but distinct faster migrating complexes were formed with higher amount of protease (lanes 6, 7, 13, 14, 20, 21, 27, and 28). Overall, the receptor bound to the vitA2 oligonucleotide appeared most resistant to proteinase K digestion. A second protease was also utilized to examine the accessibility of ERα to proteolysis when bound to the four oligonucleotides (Fig. 3.12B). α-Chymotrypsin hydrolyzes the peptide bonds adjacent to the carboxyl groups of aromatic residues and leucine. Similar differences in protease sensitivity of ERα bound to the four oligonucleotides were obtained by α-chymotrypsin digestion (hLF, lanes 3-5; mLF plus, lanes 8-10; mLF, lanes 13-15; and vitA2, lanes 18-20). Overall, ERα bound to the vitA2 oligonucleotide was more resistant to digestion, whereas the receptor bound to the hLF and mLF plus oligonucleotides was highly susceptible to protease cleavage. A third protease, trypsin, which hydrolyzes the peptide bonds adjacent to the carboxyl groups

of arginine and lysine, was also used limited protease digestion EMSAs. Trypsin cleavage also resulted in unique cleavage patterns of ERα bound to the various ERMs (Fig. 3.12C). The receptor bound to the three lactoferrin ERMS was highly susceptible to trypsin digestion, evidenced by the loss of ERα-DNA complexes at higher amounts of protease (lanes 6, 7, 13, 14, 20, and 21). Similar to the limited digestion experiments using proteinase K and α-chymotrypsin, the receptor bound to vitA2 was more resistant to trypsin (Fig. 3.12C). However, unique patterns of ERα-DNA digestion were generated when the same oligonucleotide was incubated with the different proteases and when the receptor bound to the different ERMs was digested with the same protease, indicating that the liganded receptor assumed different conformations when bound to the different ERMs.

ERE sequences influence the recruitment of coactivators to ERα

It is well documented that ligand binding to ERα induces conformational changes affecting the position of α-helices within the LBD allowing recruitment of multiple cofactor complexes to the target gene promoter (Brzozowski *et al.* 1997, Shiau *et al.* 1998, Tsai and O'Malley 1994). 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-1α) and PGC-1 related estrogen receptor coactivator (PERC) (reviewed in Robyr *et al*. 2000).

Recruitment of coactivator is a major step in ERα-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. To assure that our assay system could detect

coactivator enhancement of ERα-mediated transactivation activity, the cells were transfected with a lower amount of reporter and ERα expression vector (where indicated) and with or without saturating amounts of a single p160 family or PGC family coactivator in the presence or absence of 10 nM DES. The level of ERα-mediated transcription of the natural lactoferrin genes were lower than those values reported in our previous transfection experiments (compare Fig. 3.5B, left panel, fold activation above black bars to Table 3.5, values in Fold Relative CAT Activity column and ERα+DES row). Each coactivator enhanced reporter activity independent of ligand treatment compared to transfection of the reporter alone with substantially higher transcriptional activities stimulated by the PCG family coactivators (Table 3.5, compare Fold Relative CAT Activity column and (-) or DES rows). 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 SCR-3 enhanced ERα-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 ERα-mediated transcription of this reporter ($p<0.001$) (Fig. 3.13A, left panel). The recently discovered inducible coactivator PGC-1α and its related family member PERC potently stimulated the hormone-dependent activity of several nuclear receptors including ERα (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. 3.13A, right panel). Thus, it appeared that the pattern of coactivator recruitment by liganded ERα 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 Figs. 3.13A and 3.13B), SRC-2 ($p<0.001$) was more efficient than SRC-1 and SRC-3 ($p<0.05$). The PGC-1α and PERC coactivators stimulated CAT activity of 0.4 hLF and PGC-1α or PERC comparable to that observed from cells transfected with 0.4 mLF plus (compare right panels of Figs. 3.13A and 3.13B). Collectively, these experiments showed that the presence of the ERRE in the natural human and mouse lactoferrin gene promoters conferred preferential enhancement of liganded ERα by SRC-2, PGC-1α and PERC coactivators to the estrogen receptor, whereas SRC-3, SRC-1 and PGC-1α selectively enhanced liganded ERα-mediated transcription of the natural mouse lactoferrin gene promoter that does not contain the ERRE more efficiently.

V. Discussion

Several studies focusing on the effects of the minimal ERE sequence on ER-mediated transactivation have demonstrated three major points: 1) variations in the consensus ERE sequence occur naturally and may elicit unpredictable transcriptional activity; 2) ER binding to ERE does not always result in a corresponding level of transcriptional activity; and 3) 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 ERα-mediated transactivation by altering receptor conformation and receptor interactions with cofactors.

Estrogen response elements located on heterologous versus natural promoters confer different ERα-mediated transcriptional activity

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. Several studies have reported 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 (Figs. 3.2B and 3.2C). As expected, the mLF ERM, which has an 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 to the hLF ERE that has two base-pair

changes (T to C and C to T in the 3' arm). Surprisingly, the mouse lactoferrin ERE in the context of its natural promoter did not efficiently enhance reporter activity in response to estrogen in the transiently transfected mammary gland cells (Fig. 3.3B). Since EREs are usually located in gene promoters containing multiple cis-acting elements, we reasoned that the complexity of the natural 400 bp region of the lactoferrin gene promoters influence ERα-mediated transactivation activity in mammary gland cells.

Role of ERRE in ERα-mediated transcriptional activity of the estrogen responsive lactoferrin genes in the context of their natural promoters

We next examined the role of the ERRE in the estrogen response of the lactoferrin gene promoters. It is well established that ERα action at EREs is mediated by two separate, inherent transactivation functions, ligand-independent AF-1 and ligand-dependent AF-2 activities. Mutation of the ERRE in the 0.4 hLF-CAT reporter (m1) reduced both the AF-1 and AF-2 transcriptional activities of the receptor in mammary gland cells (Fig. 3.5A, compare gray and black bars of 0.4 hLF to m1). Moreover, the addition of the ERRE in the 0.4 mLF plus reporter substantially increased the AF-1 and AF-2 activity of ERα in MCF-7 cells (Fig. 3.5B, left panel, compare gray and black bars of 0.4 mLF plus to 0.4 mLF).

The human lactoferrin gene promoter naturally contains the ERRE (Yang and Teng 1994), which was initially characterized as a steroidogenic factor-1 binding element (Lala *et al.* 1992, Rice *et al.* 1991) and later as an estrogen-related receptor binding element (ERRE, Johnston *et al*. 1997, Sladek *et al*. 1997, Yang and Teng 1994, Yang *et al.* 1996) and an ERα binding element (Vanacker *et al*. 1999a) Zhang and Teng 2000). The ERR family was found as constitutive active nuclear receptors closely related to estrogen receptors (Giguere *et al*.

1988, reviewed in Laudet 1997), but more recent reports demonstrated that serum components (Vanacker *et al*. 1999b, Zhang and Teng 2000), DES (Lu *et al*. 2001) and 4-hydroxytamoxifen (Coward *et al*. 2001, Tremblay *et al*. 2001) bind these receptors to modulate transactivation of target genes. MCF-7 cells express endogenous ERα and ERRα mRNA (Green *et* al. 1986, Lu *et al*. 2001). EMSA data performed with MCF-7 nuclear extracts or *in vitro* translated receptor did not show distinctive receptor-lactoferrin DNA binding patterns in the presence or absence of the ERRE (Fig. 3.8, MCF-7 and Fig. 3.9 lanes 2, 7, 12, and 17), and overexpression of ERRα in MCF-7 cells mainly influenced estrogen-independent reporter activity (Fig. 3.6, compare white bars to black bars). Collectively, these data demonstrated that endogenous ERRα does not affect the outcome of our transient transfection experiments. Interestingly, our laboratory has recently shown that ERRα gene expression is induced by estrogen in the mouse uterus and heart (Liu *et al*. 2003). Promoter analysis of the ERRα 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 ERα with the MHRE of the endogenous ERRα gene in estrogen-treated MCF-7 cells. Since the spatial organization of the three hormone responsive elements in the ERRα gene is comparable to the three estrogen response half-sites of the lactoferrin genes (ERRE extended half-site plus two ERE half-sites), it is likely that ERα functions similarly on the lactoferrin gene promoters containing the ERRE to robustly stimulate estrogen responses. 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 ERα-dependent transactivation have demonstrated synergism between the ERE and AGGTCA half-site motifs in these, genes. The half-site motif in the pS2 gene is located 124 bp downstream of an imperfect ERE and mutation of the ERRE reduced the ERα-mediated estrogen response by half (Lu *et al.* 2001). In the rainbow trout ER gene, the half-site motif is located 16 bp downstream of an imperfect ERE and deletion of this motif reduces reporter activity by ~70% in yeast cells overexpressing rat ER (Petit *et al*. 1999). These reports support our data showing synergy between an imperfect lactoferrin ERE and ERRE to achieve maximum estrogen response in the context of the human and mouse natural lactoferrin promoters.

Interactions between ERMs and minimal promoter elements

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 and Maniatis 1997, reviewed in Carey 1998 and Grosschedl 1995). Recently, Guberman *et al*. (2003) showed that an AP1 site (-354) and two CREs (-156 and -75) located in the 900 bp proximal promoter region of the 5-aminolevulinate synthase (ALAS) gene cooperatively activated strong reporter activity in the presence of TPA and the coactivator p300. We have previously identified and characterized an AP1/CRE protein binding site (-56 to -36) in the minimal mouse lactoferrin gene promoter that conferred the basal activity of the gene (Shi and Teng 1994). The arrangement of the AP1 and CRE binding elements in the ALAS gene is quite similar to the relative positions of the imperfect ERE and CRE in the mouse lactoferrin gene promoter (Fig. 3.4), therefore a similar mechanism could function during the estrogen response of the 400 bp mouse

lactoferrin gene. Also, the ERα-mediated activity of the 0.4 mLF plus-CAT reporter is higher than that of the 0.4 hLF-CAT reporter in MCF-7 cells (60-fold versus 21-fold, compare Fig. 3.5B to Fig. 3.3B, left panel). Based on these observations, we speculate that the minimal promoter regions and the hormone responsive units may cooperatively regulate the estrogen response of the lactoferrin genes.

ERM sequence alters liganded ERα conformation

Several recent studies have examined the conformation of the liganded receptor bound to perfect and imperfect EREs by limited protease digestion EMSAs. Using the vitA2, pS2, vitB1 and oxytocin EREs, Loven *et al*. showed that ERα and ERβ structural changes were mediated by the DNA elements (Loven *et al*. 2001a and 2001b, Wood *et al*. 2001). These structural changes were not influenced by the ligand since digestion of ERα bound to a given ERE alone or complexed with agonists or antagonists did not produce different digestion patterns, although the various ligand conveyed variable transcriptional activities (Yi *et al*. 2002). Our limited protease digestion experiments also demonstrated that ERα assumed distinct conformations when bound to the different lactoferrin ERMs (Fig. 3.12). The receptor bound to EREs with an upstream ERRE was more sensitive to digestion by α-chymotrypsin and receptors bound to only the consensus ERE or the mouse lactoferrin imperfect ERE are most resistant to proteolysis (Fig. 3.12B). Of particular importance was the conformation of the receptor bound to mLF and mLF plus DNA fragments. The addition of the ERRE induced a different receptor conformation, which may explain the differences in coactivator recruitment patterns seen with these two reporters (Fig. 3.13A). Wood *et al*. (1998) utilized an alternative approach to the understanding receptor conformation when

bound to various EREs by incorporating ERα-specific antibodies directed at different receptor epitopes in their EMSA experiments. Using several antibodies directed against the N-terminus, DBD, or LBD in supershift experiments, they showed differences in the exposed ERα epitopes when bound to the pS2 or vitA2 EREs.

ERM sequence regulates coactivator recruitment to liganded ERα

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, Vienonen *et al*. 2003) and the phenotype of p160 family knock-out mice showed that these coactivators exhibit redundant biological functions (Gehin *et al*. 2002, Xu *et al*. 1998, Xu *et al*. 2000). Now, a growing number of studies have indicated the importance of the sequence of the DNA element in regulating ERα-mediated transcription (Hall *et al*. 2002, Loven *et al*. 2001a, Wood *et al*. 2001, 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 ERα-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 ERM was selectively enhanced by SRC-3 and SRC-2. (Figs. 3.13A and 3.13B). 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 ERα-mediated estrogen action when these coactivators were overexpressed (Figs. 3.13A and 3.13B). 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 ERα bound to the mLF, pS2, vitA2 and complement 3 EREs with a single LXXLL peptide motif from the SRC family coactivators. Even though these experiments were not performed with the natural promoter regions or the full-length coactivator, they specifically demonstrated 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.

Overexpression of the PGC family coactivators alone or in the presence of DES substantially increased the CAT activity of the reporters containing the ERRE extended half-site (Table 3.5, 0.4 mLF plus and 0.4 hLF). By definition, coactivators should not enhance transactivation in the absence of a nuclear receptor (reviewed in Robyr *et al*. 2000). Recently, our laboratory demonstrated that overexpression of PGC-1α alone in MCF-7 cells strongly stimulated the activity of the ERRα-CAT reporter containing the MHRE (C.T. Teng, unpublished data), suggesting that an endogenous NR binding partner must be present in these cells to mediate gene transactivation. Because the arrangement of the MHRE is similar to that of the lactoferrin promoters containing the ERRE and MCF-7 cells express endogenous ERα, we suspect that the coactivator enhanced transcription of the lactoferrin reporters via the endogenous receptor.

Table 3.1: Description of Plasmids Used in Transient Transfection Experiments

Plasmid	**Description**	**Reference**
pCAT-Basic	cloning vector without promoter	Promega, Madison, WI
pSV40-CAT	cloning vector with SV40 promoter	Promega, Madison, WI
pCH110	β-galactosidase expression vector	Amersham Biosciences, Piscataway, NJ
0.4 mLF-CAT	mouse lactoferrin promoter (-396/ +1)	Liu and 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 and Teng 1994
0.4 hLF-CAT m1	human lactoferrin ERRE mutant	Yang *et al.* 1996
0.4 hLF-CAT m6	human lactoferrin ERE mutant	Yang *et al.* 1996
0.4 hLF-CAT m1/m6	human lactoferrin ERRE and ERE mutant	Yang *et al.* 1996
hLF ERE/COUP	human lactoferrin ERM linked to SV40	Teng *et al.* 1992
mLF ERE/COUP	mouse lactoferrin ERM linked to SV40	Liu and Teng 1992
pSG5-hERα	human ERα expression plasmid	Migliaccio *et al*. 1991
pCR3.1-hSRC-1	human SRC-1 expression plasmid	Onate *et al*. 1995
pSG5-mSRC-2	mouse SRC-2 expression plasmid	Chen *et al*. 1999a
pSG5-hSRC-3	human SRC-3 expression plasmid	Chen *et al*. 1997
pcDNA3-hPGC-1α	human PGC-1α expression plasmid	Knutti *et al*. 2000
pcDNA3-hPERC	human PERC expression plasmid	Kressler *et al*. 2002

Table 3.2: Sequence of Double-Stranded ^{32}P-labeled Oligonucleotides Used in EMSA

Vitellogenin A2 (vitA2) 33 bp 5'CCCGAAGCTTCTAGGTCACAGTGAC**CTCGAGCG**3' 3'**GGGCTTC**GAAGATCCAGTGTCACTGGAGCTCGC5'
Human Lactoferrin (hLF) 78 bp (-396/-338) 5'CCCAAGCTTGGCACCTTCAAGGTCATCTGCTGAAGAAGATAGCAGTCTCACAGGTCAAGGCGATCTT**CACTCGAGGGG**3' 3'**GGGTTCGAACC**GTGGAAGTTCCAGTAGACGACTTCTTCTATCGTCAGAGTGTCCAGTTCCGCTAGAAGTGAGCTCCCC5'
Mouse Lactoferrin Plus (mLF plus) 82 bp (374/-322) 5'CCGAAGCTTATTTGCTTCAAGGTCATCTTGCTCCATGCAGCTTAAGTGTCACAGGTCAAGGTAACCCA**CAAATCTCGAGGGG**3' 3'**GGCTTCGAATAAAC**GAAGTTCCAGTAGAACGAGGTACGTCGAATTCACAGTGTCCAGTTCCATTGGGTGTTTAGAGCTCCCC5'
Mouse Lactoferrin (mLF) 46 bp (-351/-322) 5'CCGAAGCTTAGTGTCACAGGTCAAGGTAACCCACAAATCT**CGAGCG**3' 3'**GGCTTC**GAATCACAGTGTCCAGTTCCATTGGGTGTTTAGAGCTCGC5'

Complementary oligonucleotides (normal text) were annealed and nucleotides were filled-in (bold) during the labeling reaction. The ERE (underlined) and ERRE (double underlined) elements are indicated.

Table 3.3: Description of Antibodies Used in Supershift EMSA

Antibody	**Description**	**Reference**
H222	human ERα monoclonal antibody directed against ligand binding domain	Abbott Laboratories, Abbott Park, IL
Ab-10	human ERα monoclonal antibody directed against ligand binding domain	NeoMarkers, Fremont, CA
H-184	human ERα polyclonal antibody directed against amino terminus	Santa Cruz Biotechnology, Santa Cruz, CA
LF	mouse LF anti-serum (8344)	Teng *et al*. 2002b
COUP	human COUP-TF antibody	Tsai, SY, Baylor College of Medicine, Houston, TX
PEP3	human ERRα rabbit anti-serum	Teng, CT, NIEHS, NIH, Research Triangle Park, NC
PRE	rabbit pre-immune serum	Teng, CT, NIEHS, NIH, Research Triangle Park, NC

Table 3.4: Partial Nucleotide Sequences of ^{32}P-labeled Oligonucleotides used in EMSA

Double-stranded oligos	**ERM Sequence**	**% Shifted**
VitA2 (33 bp)	----------AGGTCACAGTGACC--	37%
Human LF (78 bp)	--TCAAGGTCATCT---AGGTCAAGG**C**GA**T**C--	24%
Mouse LF (46 bp)	----------AGGTCAAGGT**A**ACC--	37%
Mouse LF plus (82 bp)	--TCAAGGTCATCT---AGGTCAAGGT**A**ACC--	38%

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 ERα-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. Data are the averages of three independent assays.

Table 3.5: Effect of p160 and PGC families of Coactivators Interacting with ERα Bound to mouse LF, mouse LF plus, and human LF Gene Promoters on Reporter Activity

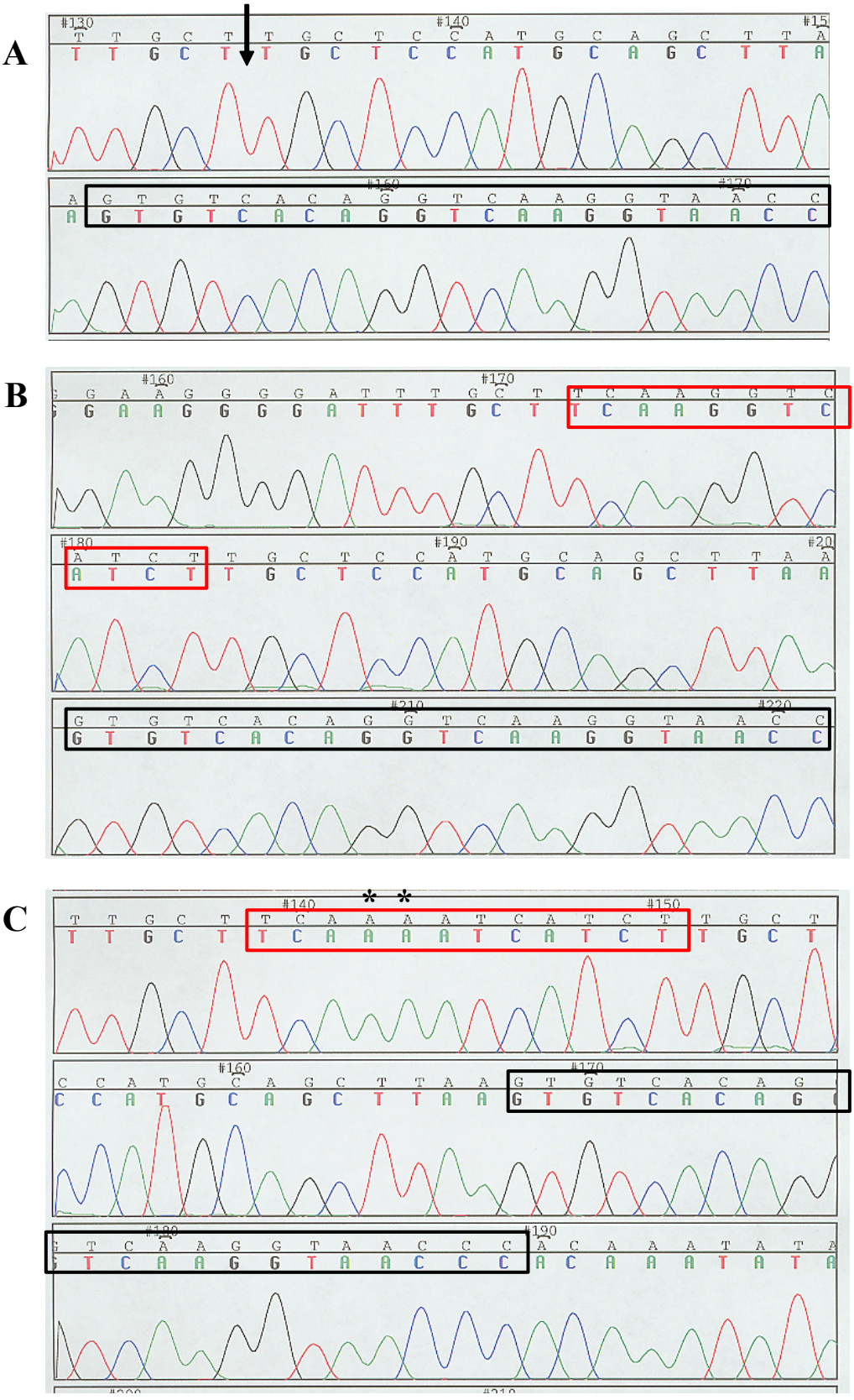
MCF-7		0.4 mLF plus-CAT			0.4 mLF-CAT			0.4 hLF-CAT		
	Co-Activator	Relative CAT Activity	Fold Relative CAT Activity	Fold Co-Acti-vation	Relative CAT Activity	Fold Relative CAT Activity	Fold Co-Acti-vation	Relative CAT Activity	Fold Relative CAT Activity	Fold Co-Acti-vation
-	-	1501	1	-	2315	1	-	446	1	-
DES	-	2063	1.4	-	2389	1	-	418	.9	-
ERα	-	3903	2.6	-	2885	1.2	-	670	1.5	-
ERα+ DES	-	11,917	7.9	1	4628	2	1	3644	8.2	1
-	SRC-1	4851	3.2	-	5485	2.4	-	1481	3.3	-
DES	SRC-1	6109	4.1	-	4788	2.1	-	1649	3.7	-
ERα+ DES	SRC-1	26,302	17.5	2.2	8154	3.5	1.8	6194	13.9	1.7
-	SRC-2	1826	1.2	-	2916	1.3	-	1066	2.4	-
DES	SRC-2	2447	1.6	-	3168	1.4	-	1083	2.4	-
ERα+ DES	SRC-2	38948	25.9	3.3	5266	2.3	1.1	8442	18.9	2.3
-	SRC-3	4488	3	-	8033	3.5	-	1090	2.4	-
DES	SRC-3	5078	3.4	-	5975	2.6	-	1362	3.1	-
ERα+ DES	SRC-3	25,849	17.2	2.2	11,969	5.2	2.6	5279	11.8	1.4
-	PGC-1α	33,500	22.3	-	5785	2.5	-	4366	9.8	-
DES	PGC-1α	35,726	23.8	-	6914	3	-	4322	9.7	-
ERα+ DES	PGC-1α	119,747	79.8	10	13,654	5.9	3	33,469	75	9
-	PERC	17,175	11.4	-	4773	2.1	-	3204	7.2	-
DES	PERC	16,042	10.7	-	4372	1.9	-	2322	5.2	-
ERα+ DES	PERC	104,732	69.8	8.8	8681	3.7	1.9	21,523	48.3	5.9

Relative CAT activity normalized to β-gal activity is the value expressed as the mean ± SEM of three independent assays in duplicate is reported in the column labeled Relative CAT Activity. Fold CAT activity in reference to control (relative CAT activity of reporter alone) is in the column labeled Fold Relative CAT Activity. Fold CAT activity of the relative CAT activity of co-transfections with reporter, liganded hERα and coactivator in reference to the relative CAT activity of co-transfections with the reporter and liganded hERα is reported in the column labeled Fold Co-Activation. Values are expressed as the mean ± SEM of three independent assays in duplicate and are also reported in Figure 3.13.

Figure 3.1: Sequence verification of the 0.4 mLF-CAT chimera (0.4 mLF plus) and mutant chimera (0.4 mLF plus mutant) reporters

A. Nucleotide sequence of the natural 0.4 mLF-CAT reporter outlining the COUP/ERE (black box). Denoted by an arrow is the position for insertion of the ERRE 18 bp upstream of the COUP/ERE. B. Nucleotide sequence of 0.4 mLF-CAT plus reporter depicting the insertion of the ERRE (red box) in the original 0.4 mLF-CAT reporter. C. Nucleotide sequence of 0.4 mLF-CAT plus mutant reporter in which the guanine dinucleotide in the inserted ERRE (red box) was mutated to adenines (asterisks).

Figure 3.1 (continued)



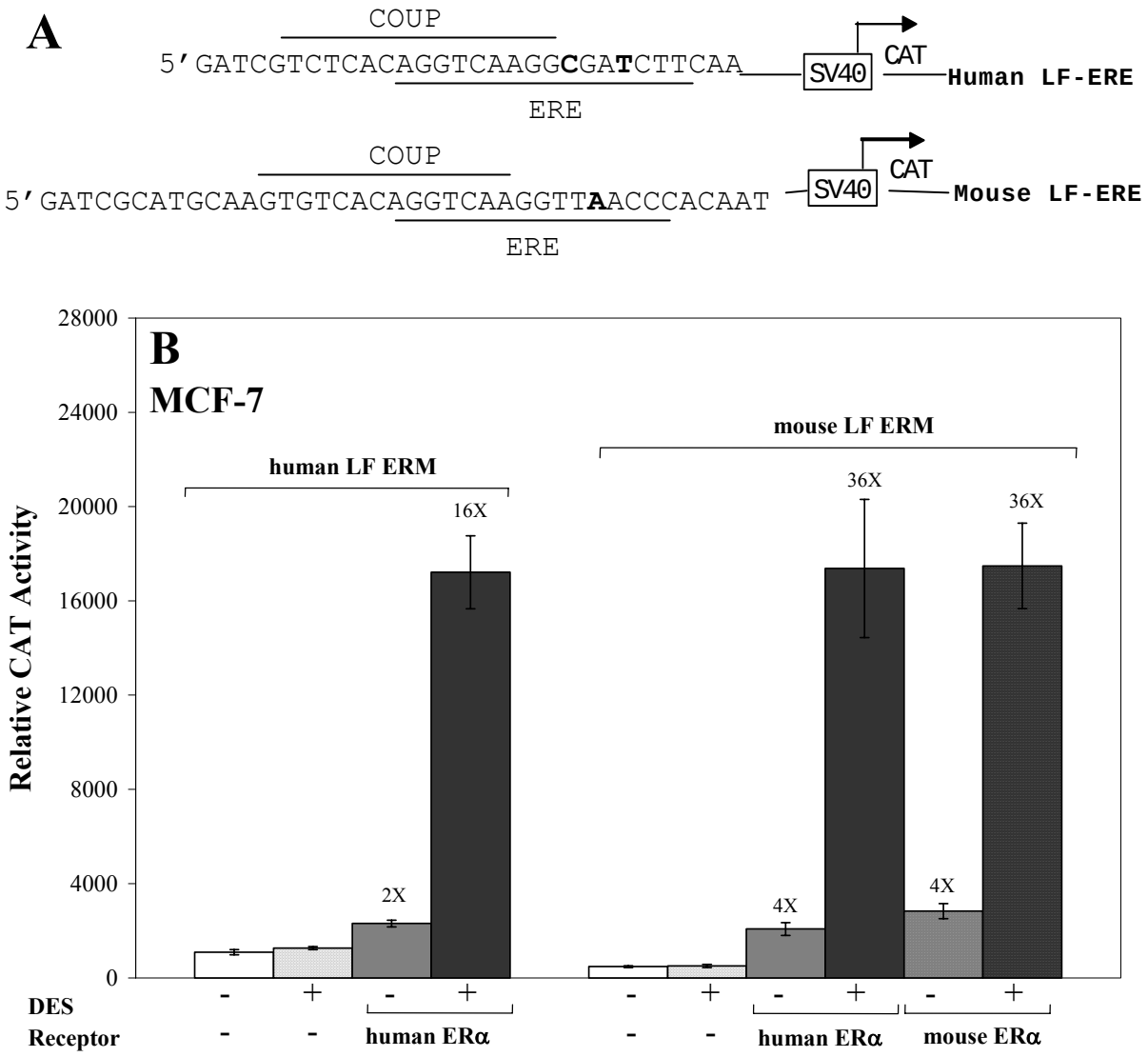
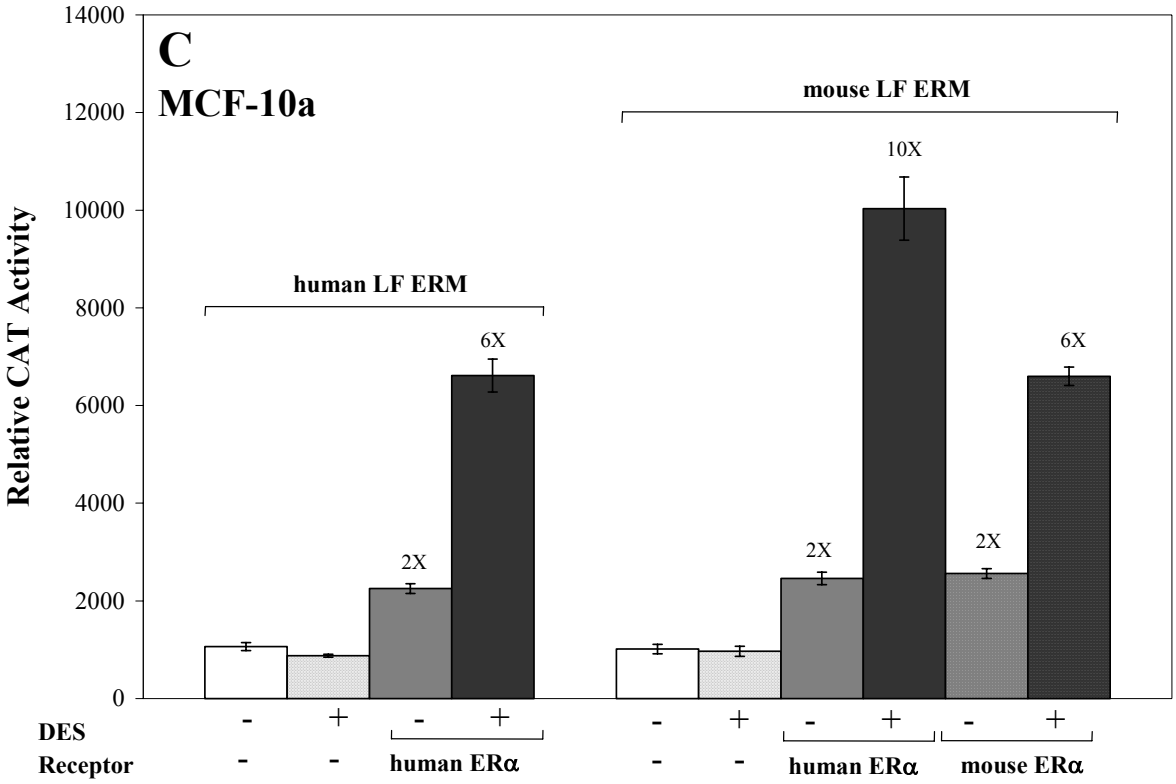


Figure 3.2: Human and mouse lactoferrin imperfect COUP/EREs function as enhancers to a heterologous promoter in human mammary gland cells

A. Schematic presentation of the human and mouse overlapping ERE/COUP module. Letters in bold denote the nucleotides that are mismatched from the consensus ERE. B. and C. Relative CAT activity normalized to β-gal activity is the value expressed as the mean ± SEM of three independent assays in duplicate. Fold activation in reference to control (transfection of reporter alone) is indicated above the error bars.

Figure 3.2 (continued)



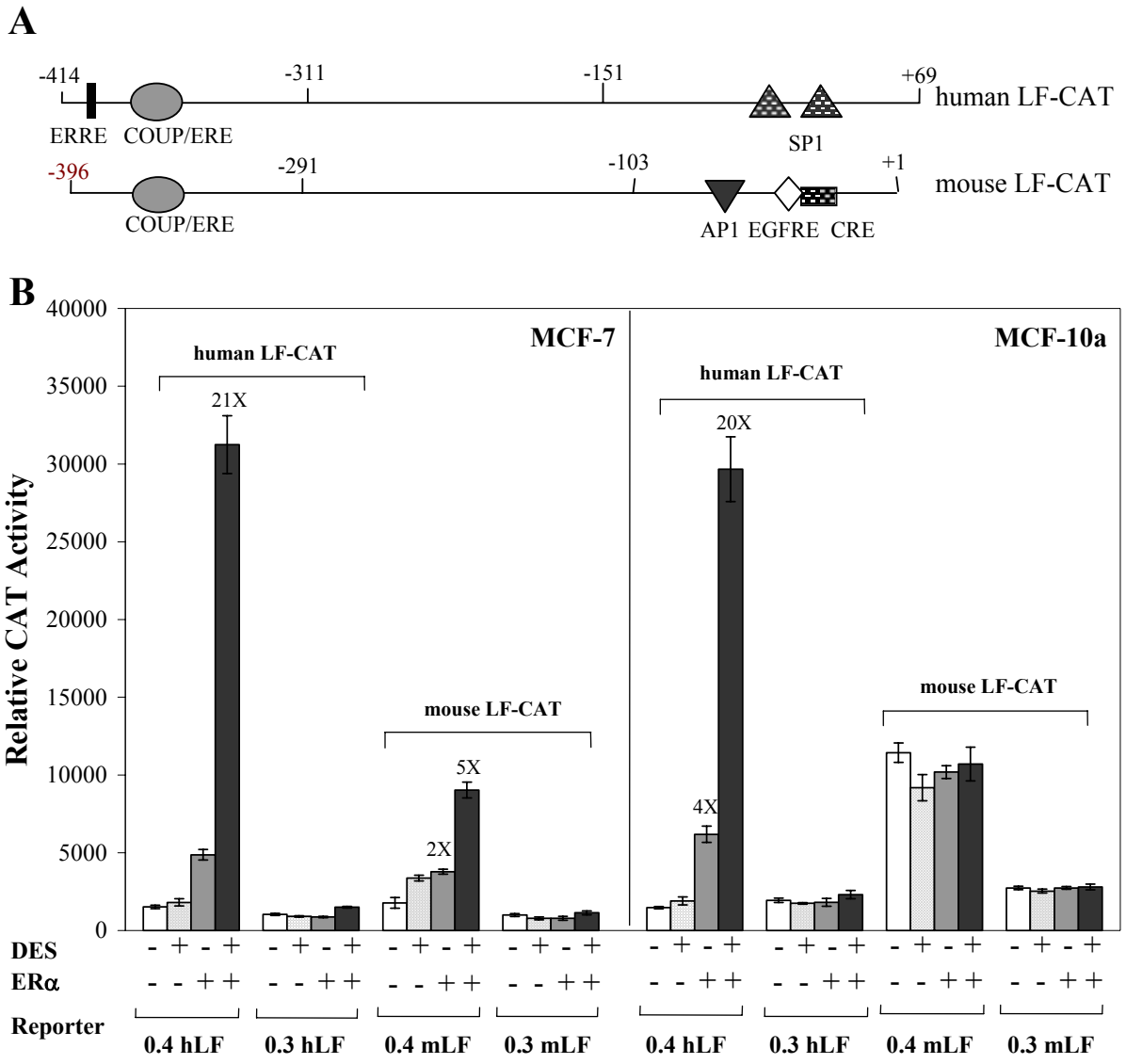


Figure 3.3: Human and mouse lactoferrin imperfect COUP/EREs function as enhancers in the context of their natural promoters in human mammary gland cells
A. Schematic presentation of the 400 bp region of native human and mouse lactoferrin gene promoters indicating the relative positions of the ERRE, COUP/ERE, AP1, EGFRE, SP1 and CRE elements. B. Relative CAT activity normalized to β-gal activity is the value expressed as the mean ± SEM of four independent assays in duplicate. Fold activation in reference to control (transfection of reporter alone) is indicated above the error bars.

Figure 3.4: Comparison of the 400 bp Human and Mouse Lactoferrin Gene Promotes

Characterized DNA elements are underlined (ERRE, GATA, COUP/ERE, CAAT, SP1, CRE, SP1, and TATA-like box). Numbers to the left of the sequences indicate the nucleotide position relative to the transcription start site (arrows at the +1 position. Taken from Teng *et al*. 1992.

Figure 3.5: ERRE synergizes with the ERM to enhance ERα mediated estrogen responses of the human and mouse 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 COUP/ERE 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 ± SEM of four independent assays in duplicate. 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 ± SEM 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 3.5: (continued)

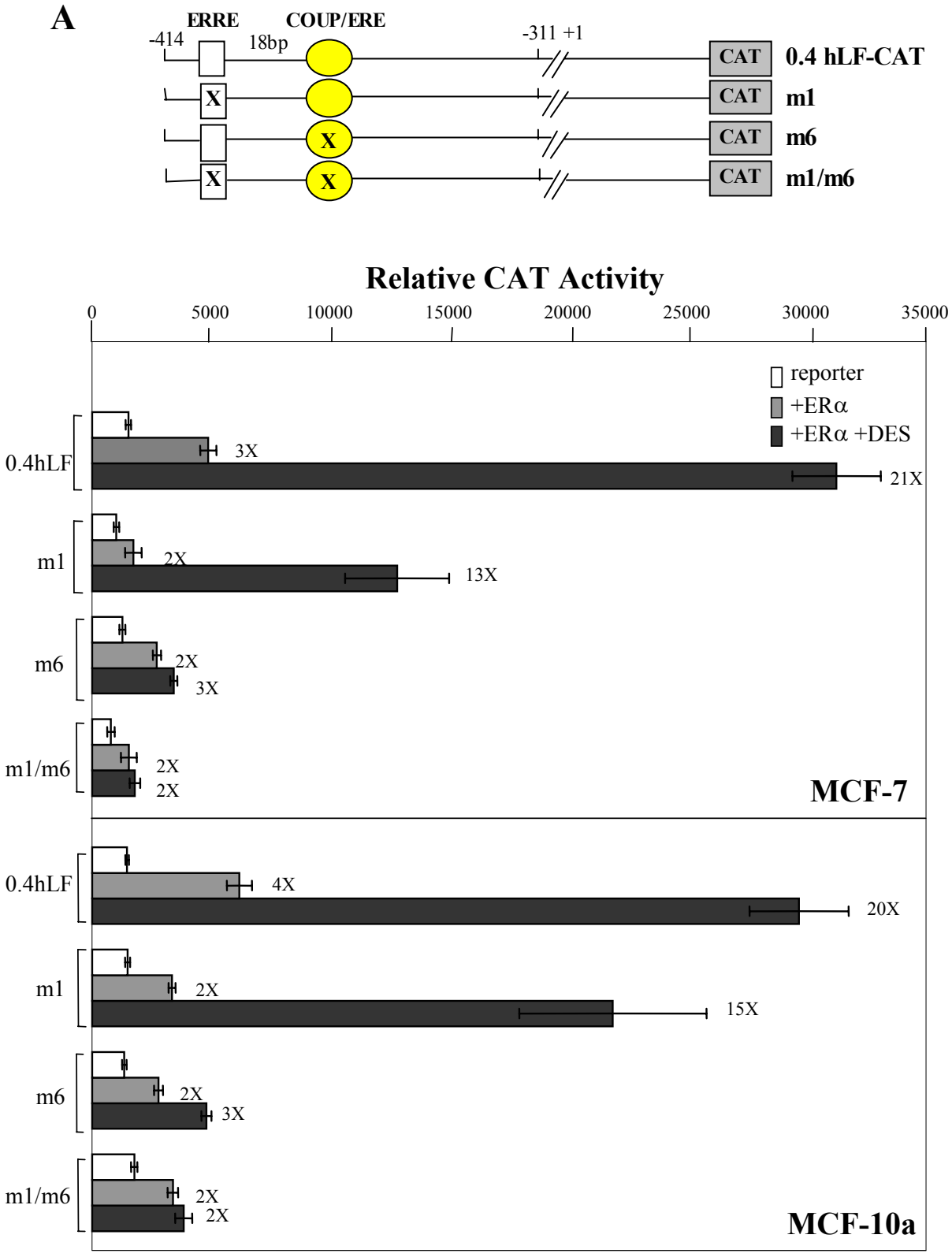
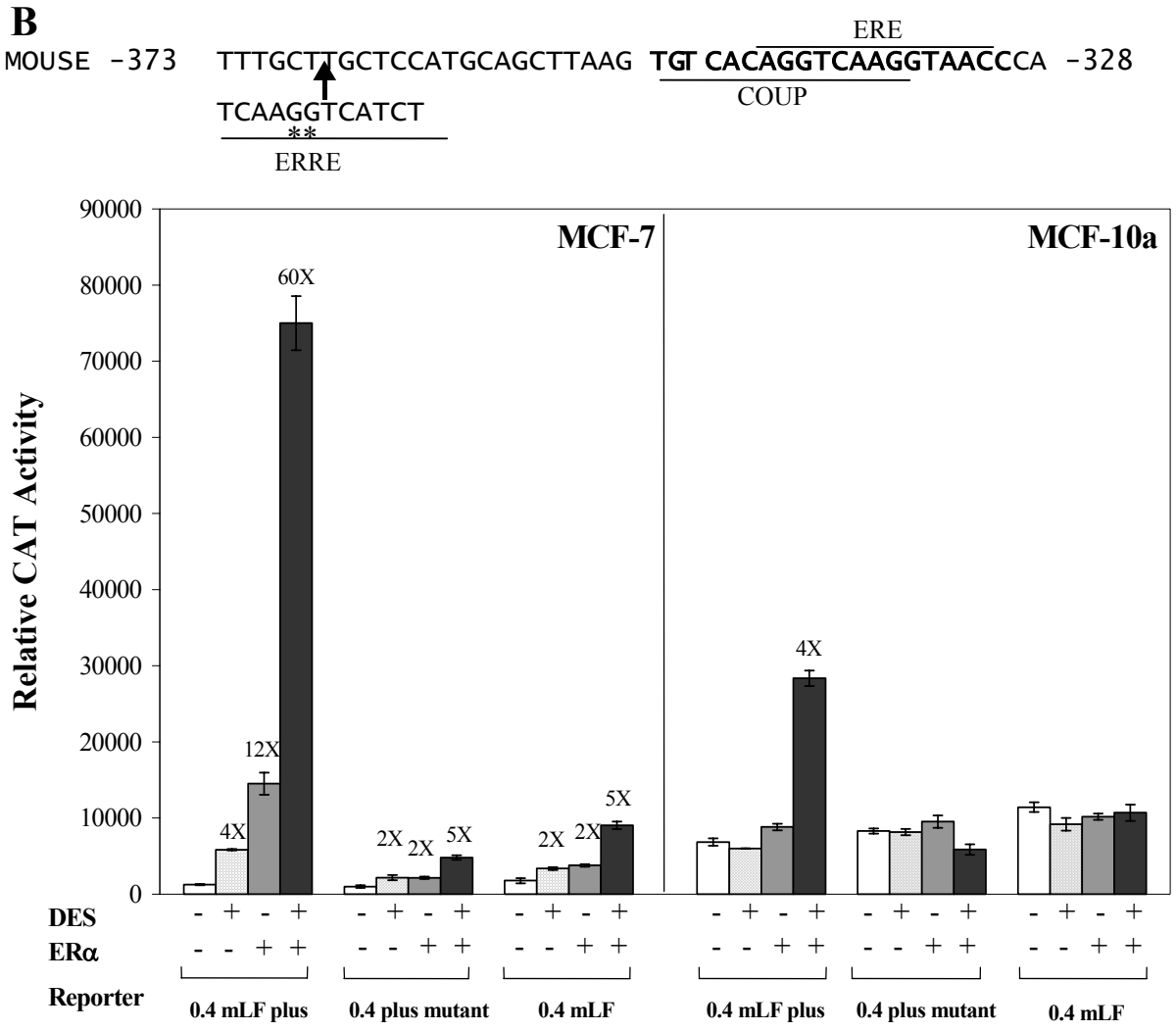


Figure 3.5: (continued)



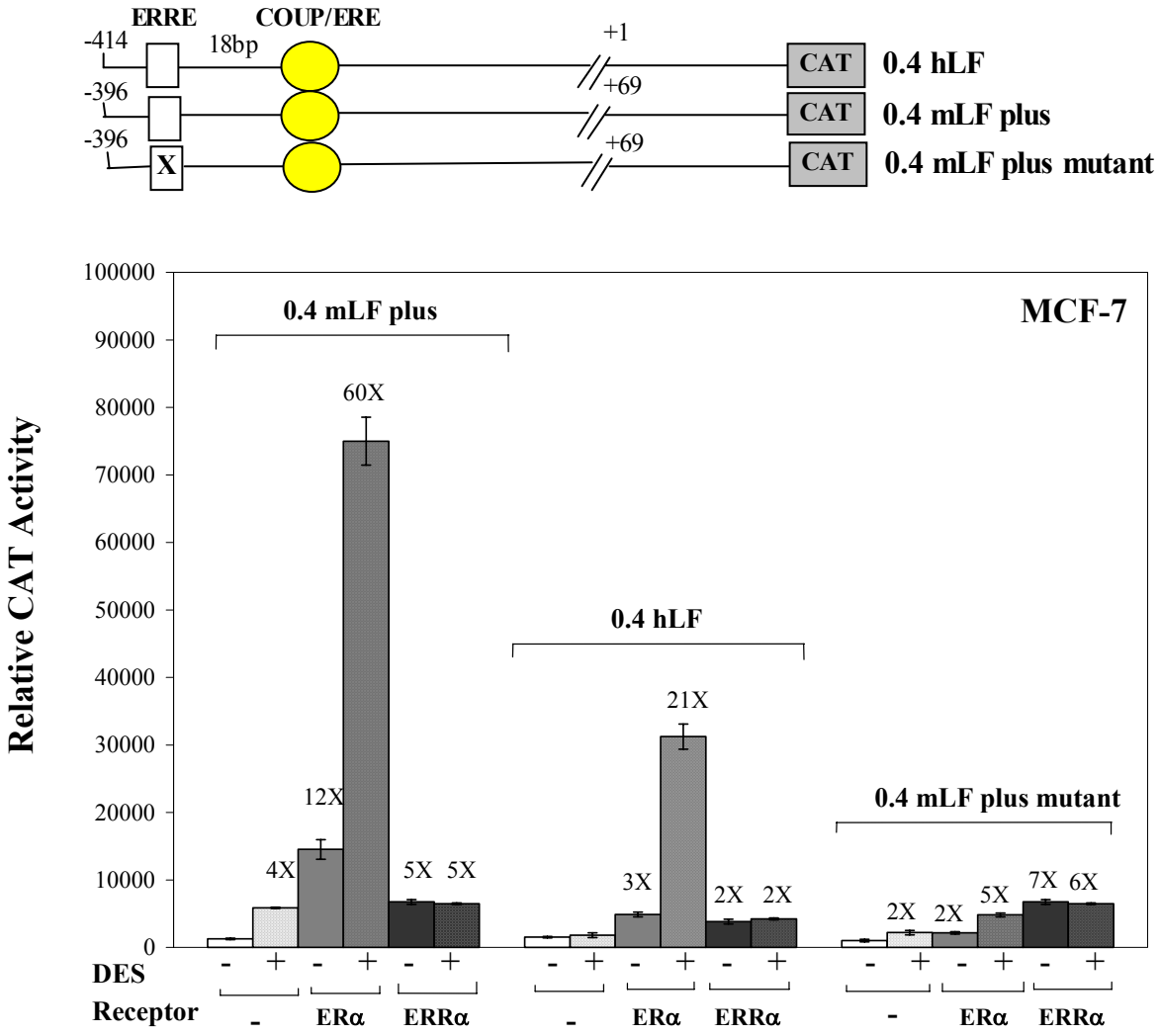


Figure 3.6: The ERM does not synergize with the ERRE to enhance ERRα mediated transactivation human and mouse lactoferrin genes

Top, Schematic presentation of the 0.4 hLF and 0.4 mLF plus reporters containing wild-type ERRE and ERE sequences and 0.4 mLF plus reporter containing mutated ERRE (denoted by an X) and wild-type ERE sequences. Nucleotide location of the 400 bp human and mouse LF reporters are indicated. There are 18 bp separating the ERRE and COUP/ERE elements. Bottom, Relative CAT activity normalized to β-gal activity is the value expressed as the mean ± SEM of three independent assays in duplicate. Fold activation in reference to control (transfection of reporter alone) is indicated above the error bars.

Figure 3.7: Electrophoretic mobility shift assay (EMSA) detection of specific interactions between MCF-7 nuclear proteins and the various lactoferrin ERMs and the consensus ERE

Antibodies to human ERα (H222), human COUP-TF (COUP) or human ERRα (PRE) are indicated. ^{32}P-labeled oligonucleotides of human lactoferrin (hLF), mouse lactoferrin (mLF), mouse lactoferrin plus (mLF plus) and vitA2 are presented in a separate graph. Arrows, the protein-DNA complexes. SS, supershifted bands. The free probe is present in every lane at the bottom.

Figure 3.7: (continued)

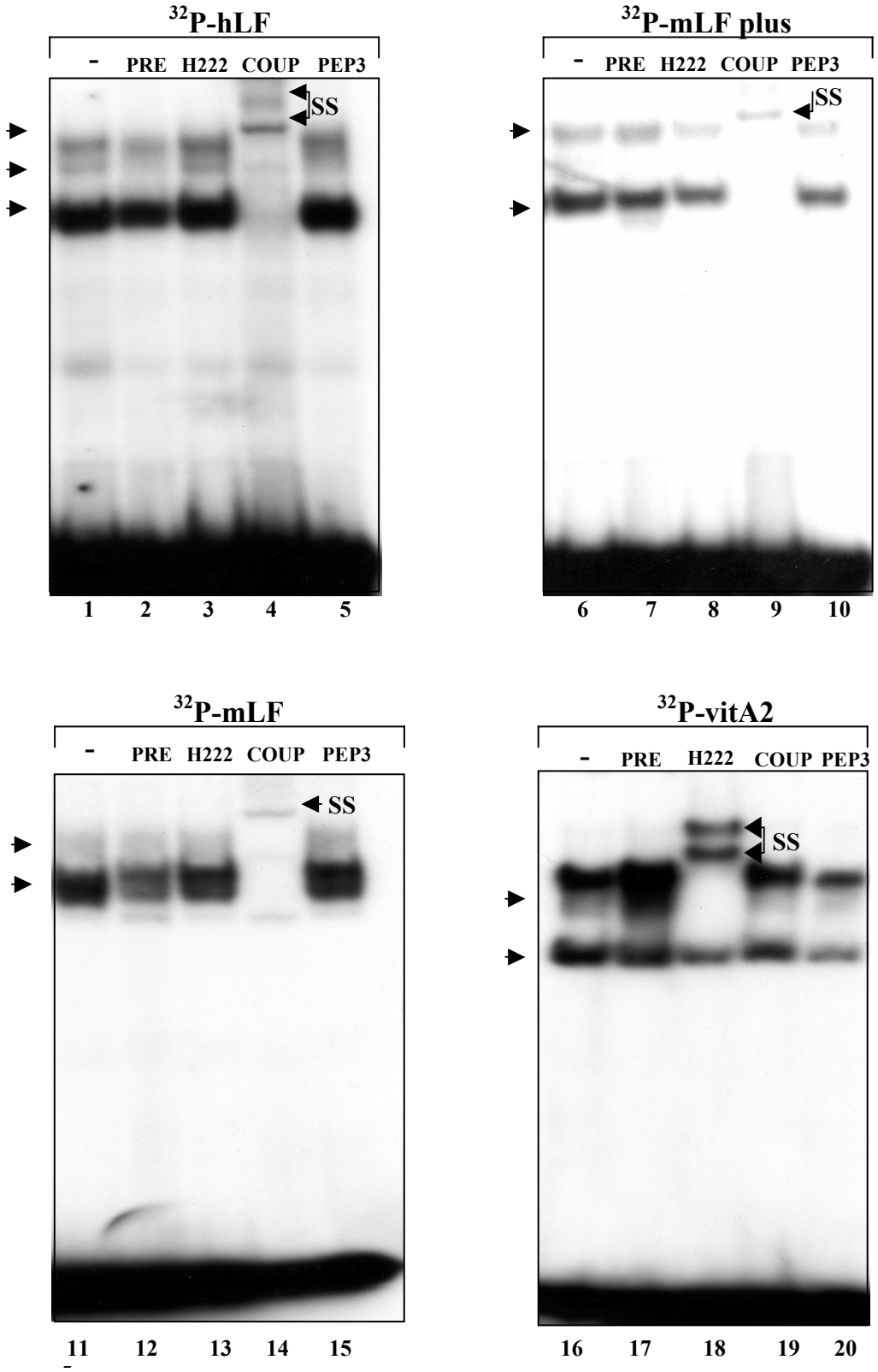
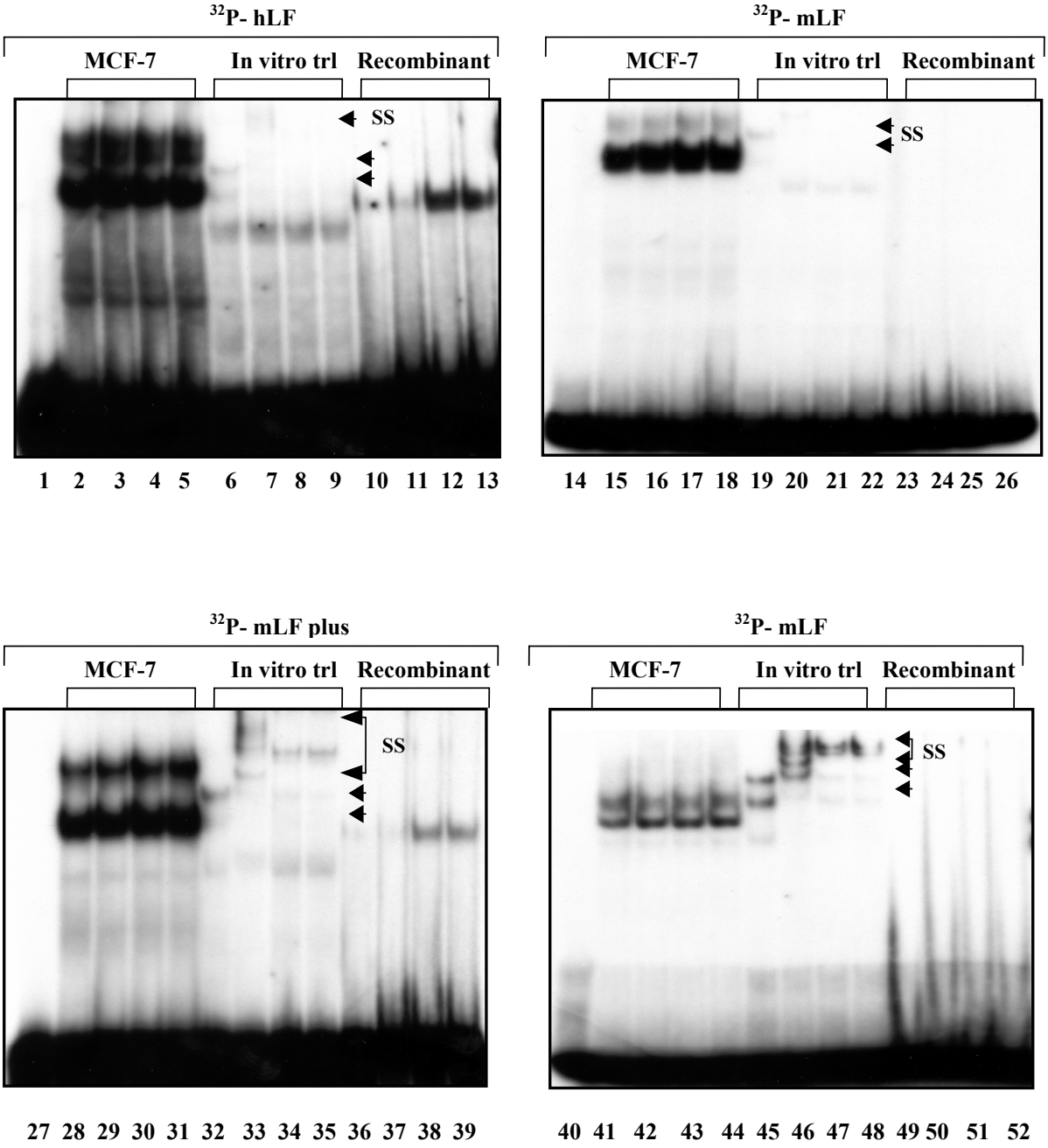


Figure 3.8: EMSA detection of specific interactions between ERα and the various lactoferrin ERMs and the consensus ERE

Human ERα from MCF-7 nuclear proteins extracts (MCF-7), *in vitro* translated protein (In vitro trl) and baculovirus-expressed (Recombinant) are indicated. Antibodies to human ERα (H222, lanes 3, 7, 11, 16, 20, 24, 29, 33, 37, 42, 46 and 50), (Ab-10, lanes 4, 8, 12, 17, 21, 25, 30, 34, 38, 43, 47 and 51) and (H-184, lanes 5, 9, 13, 18, 22, 26, 31, 35, 39, 44, and 52) are indicated. ^{32}P-labeled oligonucleotides of human lactoferrin (hLF), mouse lactoferrin (mLF), mouse lactoferrin plus (mLF plus) and vitA2 are presented in a separate graph. Arrows, the protein-DNA complexes. SS, supershifted bands. The free probe is present in every lane at the bottom.

Figure 3.8: (continued)



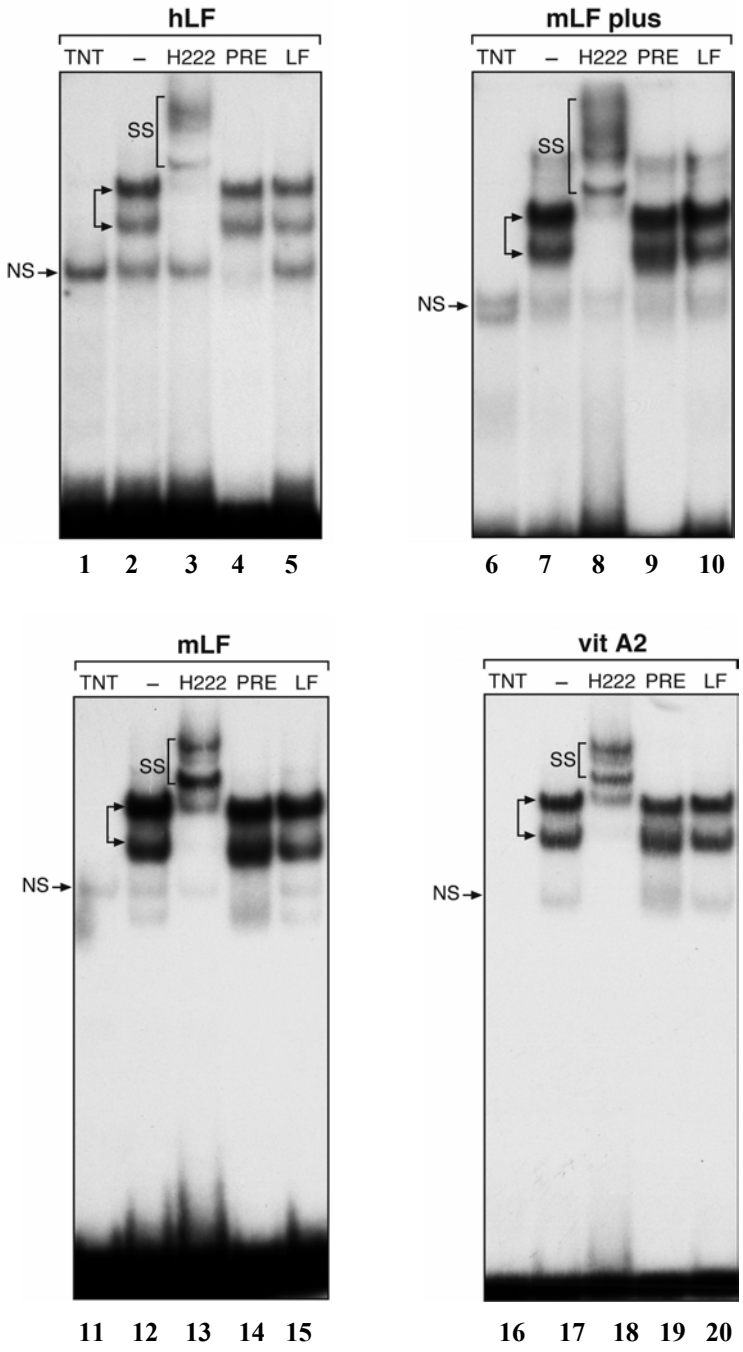


Figure 3.9: Electrophoretic mobility shift assay (EMSA) detection of ERα specifically binding to the various lactoferrin ERMs and the consensus vit A2 ERE
Antibodies to human estrogen receptor (H222), human lactoferrin (LF) or pre-immune serum (PRE) are indicated. ^{32}P-labeled oligonucleotides of human lactoferrin (hLF), mouse lactoferrin (mLF), mouse lactoferrin plus (mLF plus) and vitA2 are presented in a separate graph. Double arrows, the ERα-DNA complexes. SS, supershifted bands. NS, non-specific bands. The free probe is present in every lane at the bottom.

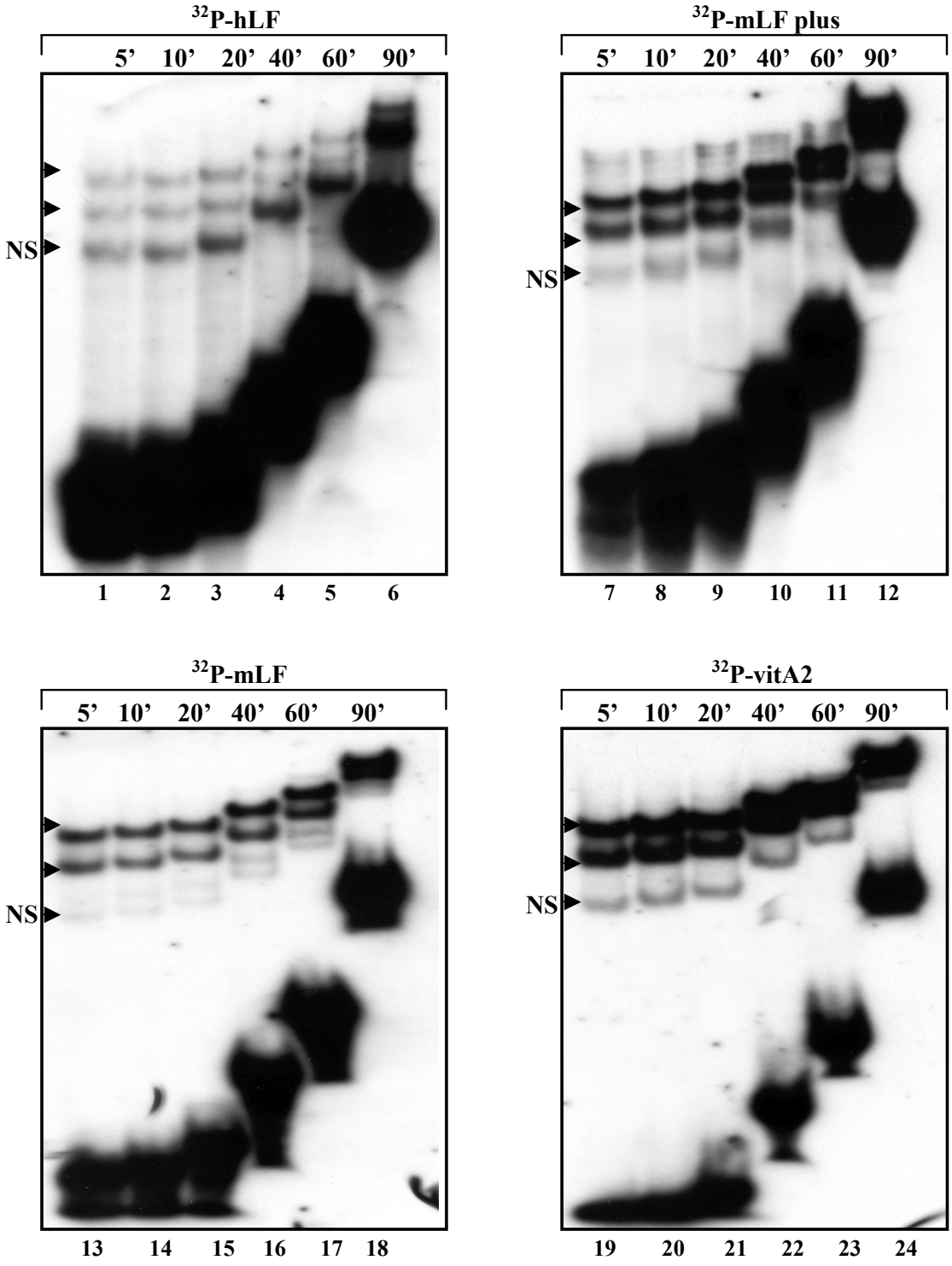


Figure 3.10: Liganded ERα binds quickly and stably to the various ERMs
Liganded *in vitro* translated hERα was incubated with the various ^{32}P-labeled ERMs that are presented in separate graphs. The ERα-DNA complexes were resolved at various time points as indicated. Arrows, the ERα-DNA complexes. NS, non-specific bands. The free probe is present in every lane at the bottom

A

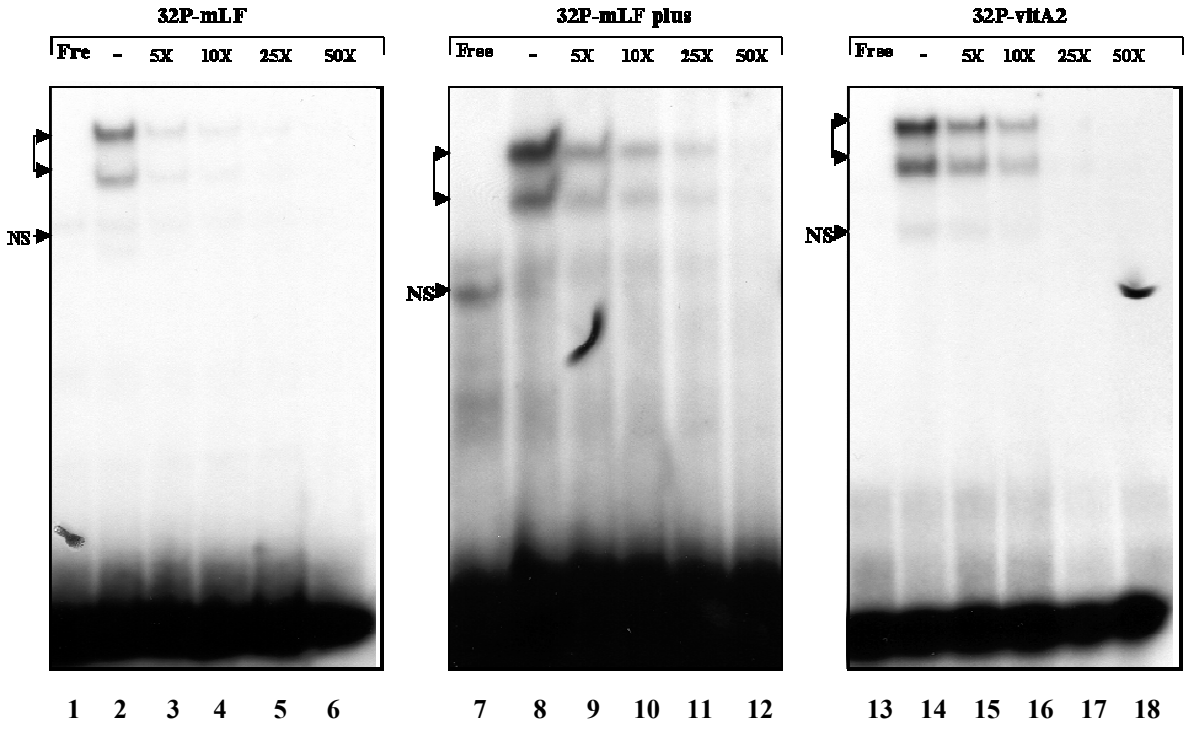
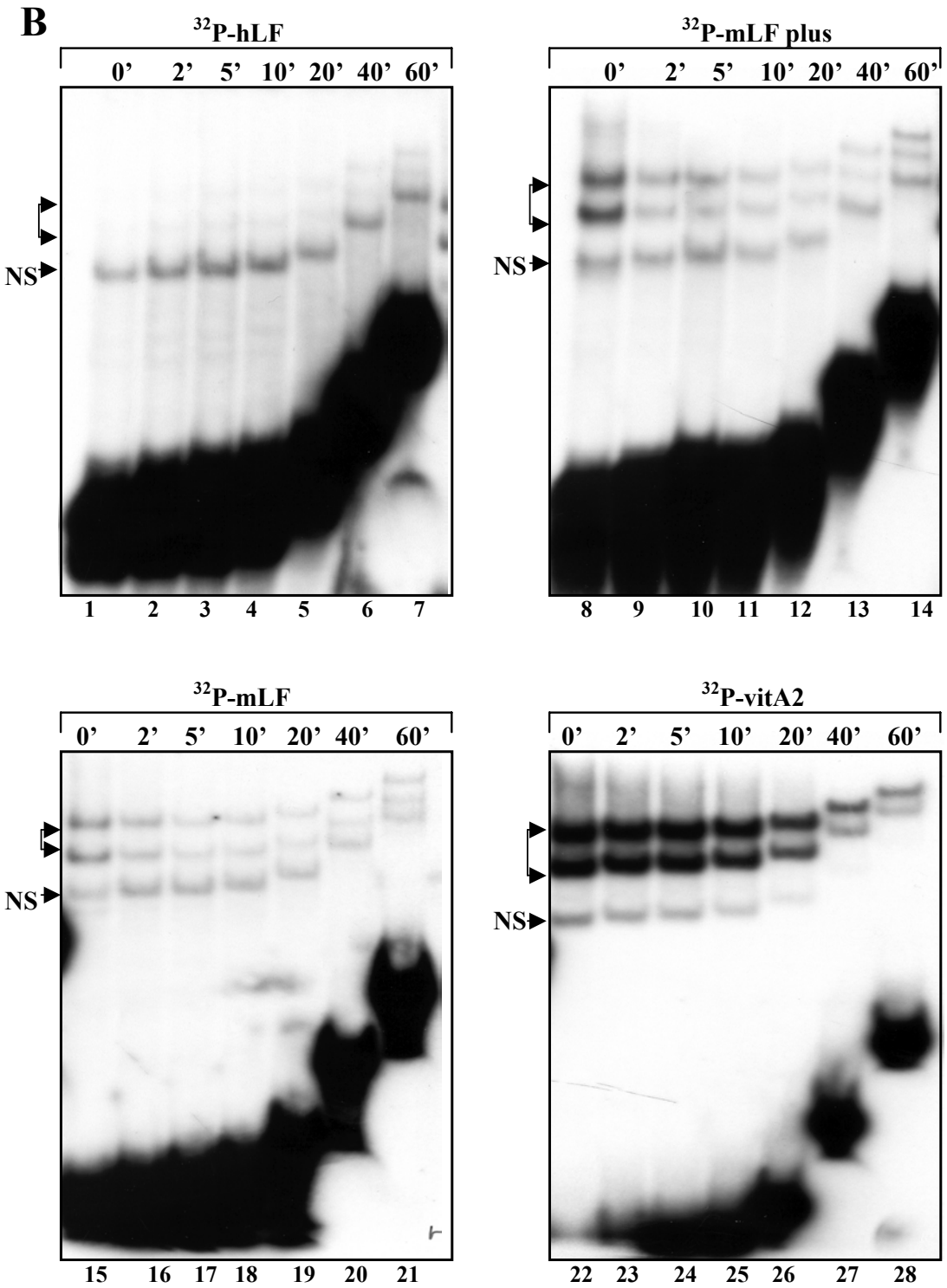


Figure 3.11: ERα binds to the various ERMs with different levels of stability
In vitro translated ERα were incubated with the various labeled ERMs as described in the Materials and Methods Section. A. The ERα-DNA complexes were incubated with the indicated molar ratio of cold self-competitor. B. The ERα-DNA complexes were resolved at various times after the addition of 100X cold self-competitor. Arrows, the ERα-DNA complexes. NS, non-specific bands. The free probe is present in every lane at the bottom.

Figure 3.11: (continued)



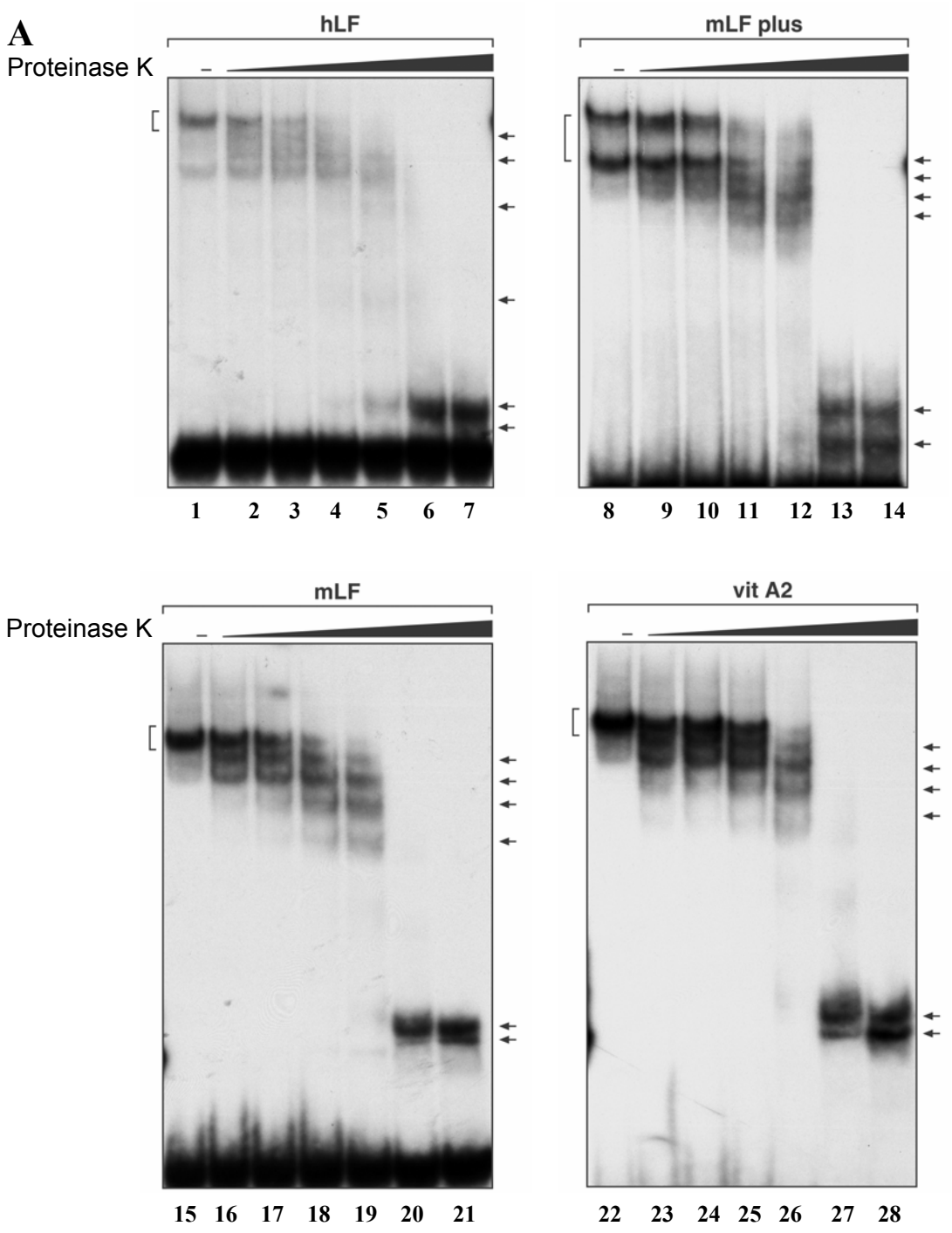


Figure 3.12: Limited protease digestion patterns of ERM-bound ERα show structural differences in the receptor bound to the different ERMs

^{32}P-labeled DNA fragments containing either hLF, mLF, mLF-plus or vitA2 ERE were incubated with *in vitro* translation mixture and liganded baculovirus-expressed ERα (1.5 pmoles). A. Limited protease digestion using proteinase K, 0, 1.25, 2.5, 5, 10, 20, or 40 ng. B. Limited protease digestion using α-chymotrypsin, 0, 25, 30, 35, or 40 ng. C. Limited protease digestion using trypsin, 0, 1.25, 2.5, 5, 10, 20, or 40 ng. Brackets, the ERα-DNA complexes. Arrows, the ERα-DNA digestion products.

Figure 3.12 (continued)

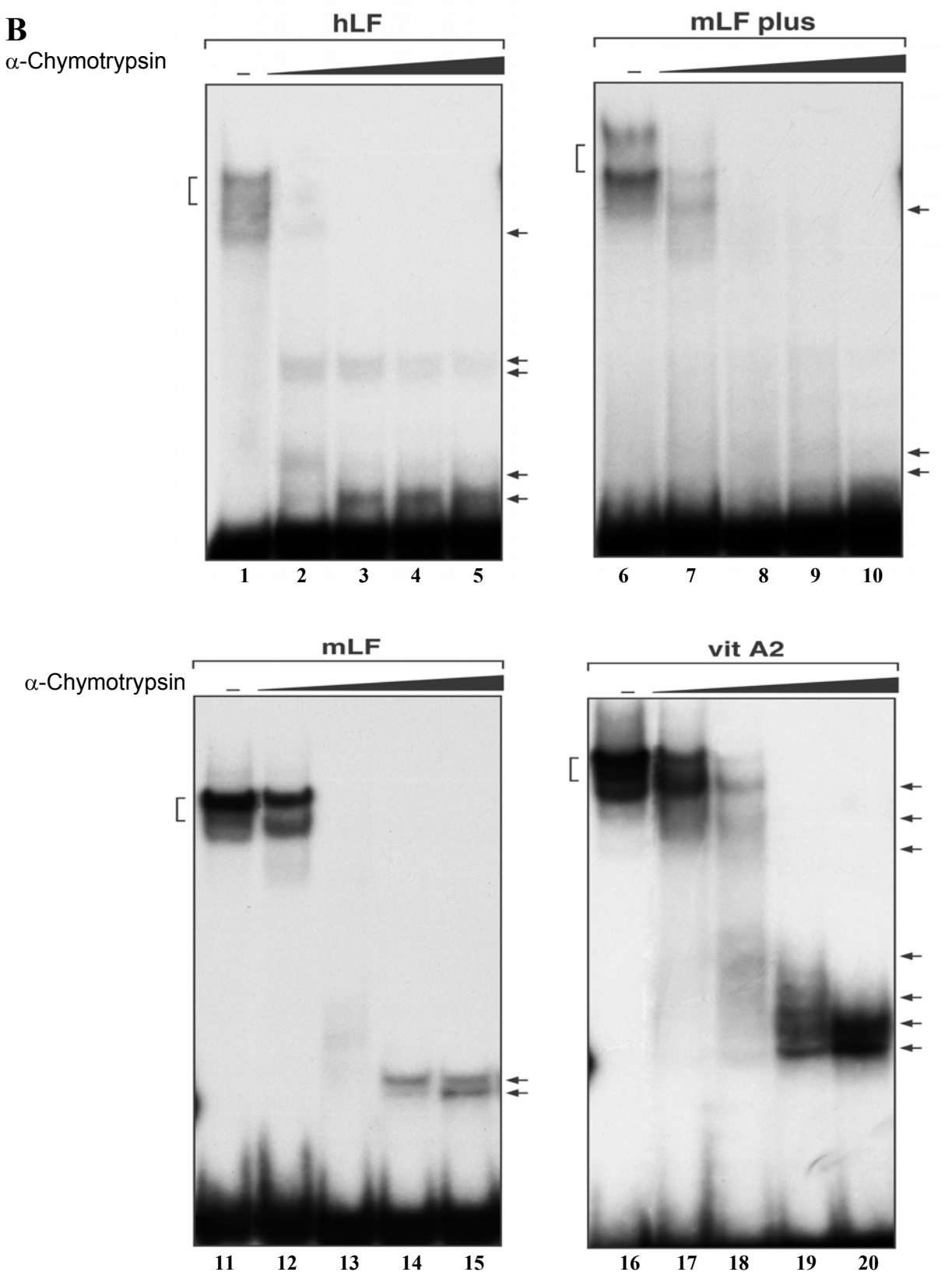
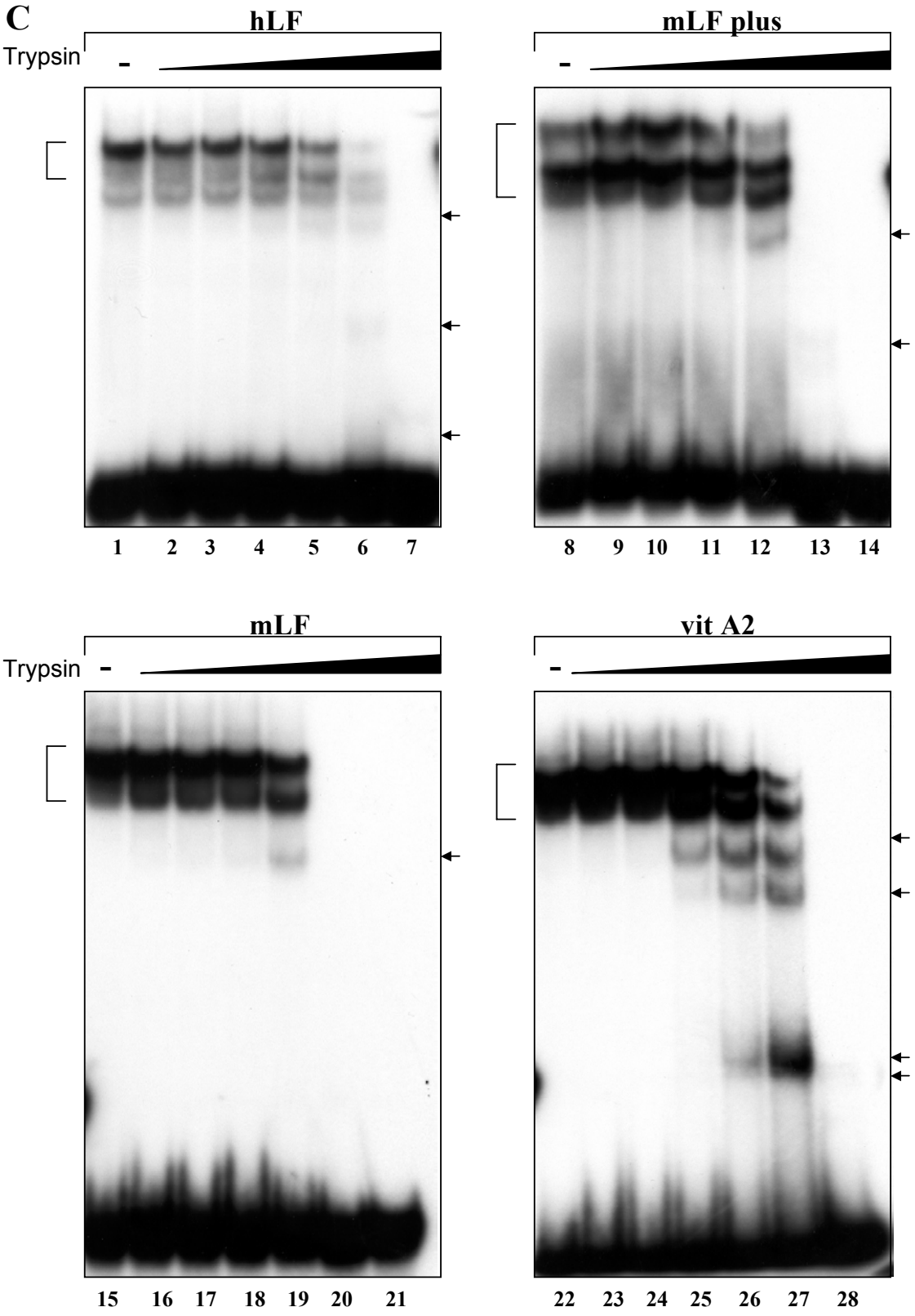


Figure 3.12 (continued)



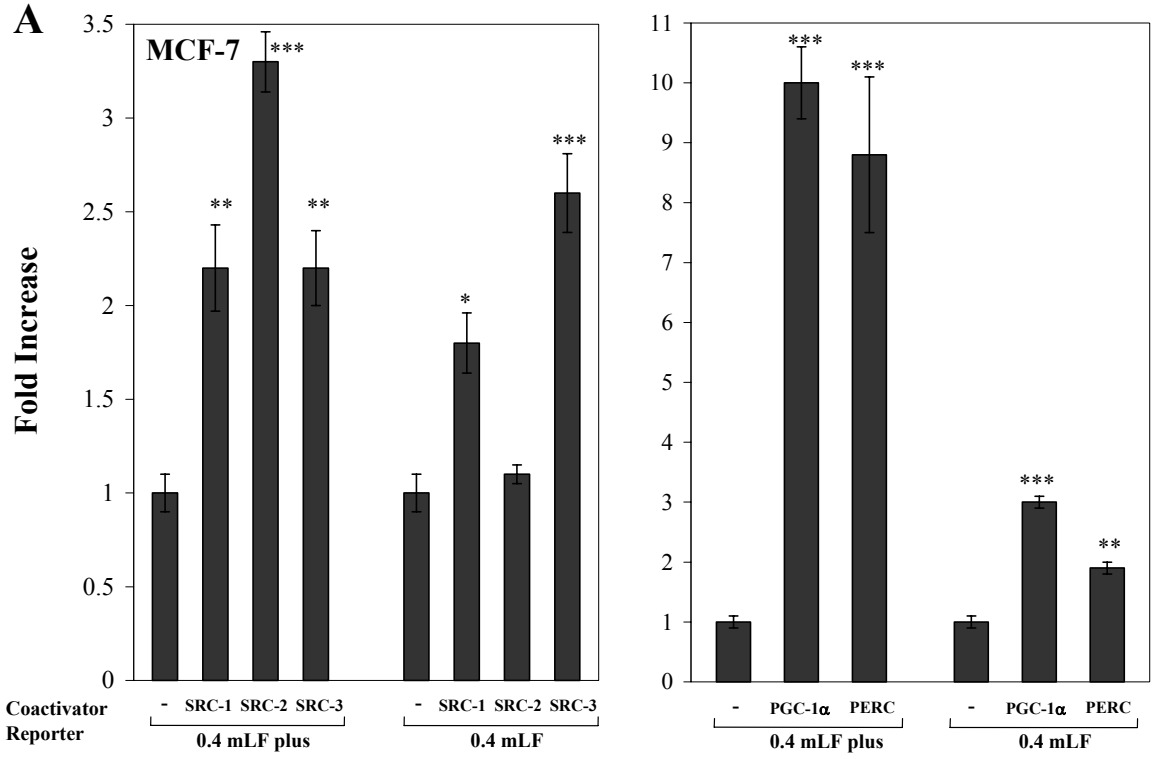
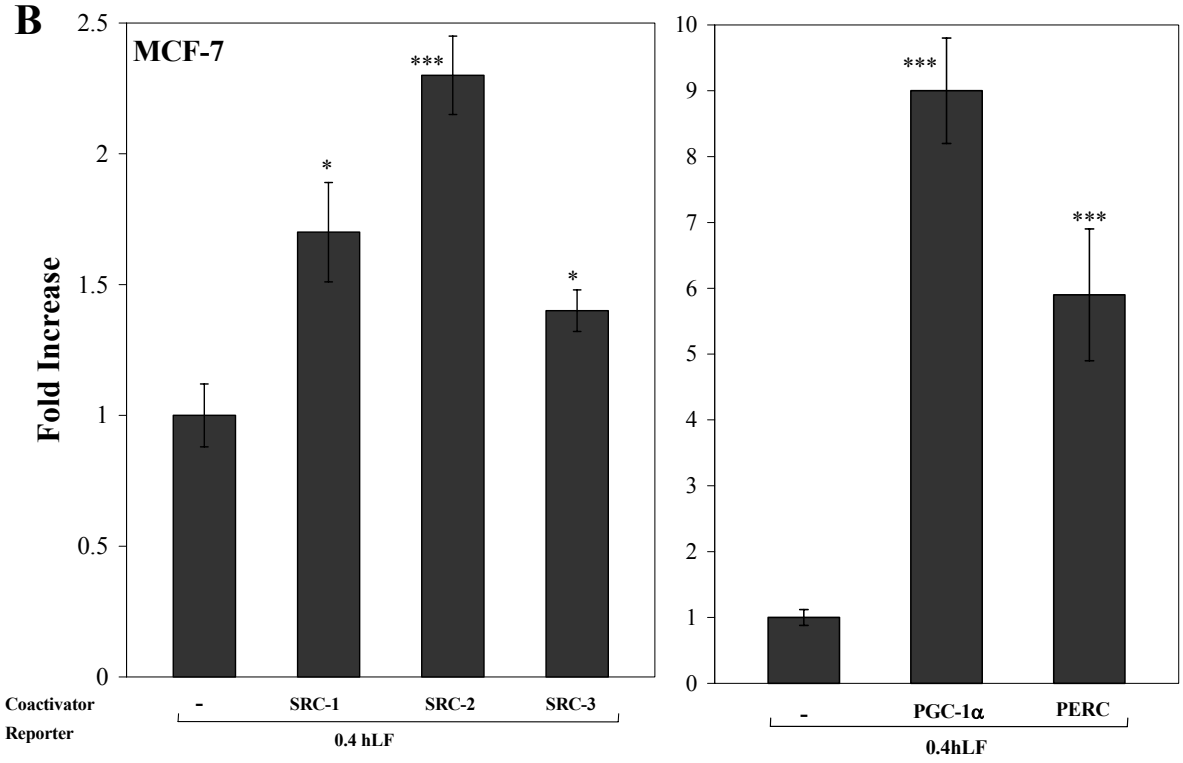


Figure 3.13: Effect of p160 and PGC families of coactivators interacting with ERα bound to mouse lactoferrin, mouse lactoferrin plus, and human lactoferrin gene promoters on reporter activity

A. Effect of p160 family (left panel) and PGC family (right panel) coactivators on ERα-mediated transactivation of the mouse lactoferrin reporters. B. Effect of p160 family (left panel) and PGC family (right panel) coactivators on ERα-mediated transactivation of the human lactoferrin reporter. The fold CAT activity is reported as the relative CAT activity of co-transfections with reporter, liganded hERα and coactivator divided by the relative CAT activity of co-transfections with the reporter and liganded hERα. Values are expressed as the mean ± SEM of three independent assays in duplicate. Fold activation in reference to control (transfection of reporter alone) is indicated above the error bars. (*** =$p<0.001$, ** =$p<0.01$, * =$p<0.05$).

Figure 3.13: (continued)



Appendix

Reduced Conformers of Bovine α-Lactalbumin Proteins Modulate Mammary Epithelial Cell Proliferation

I. Abstract

α-Lactalbumin (α-LA) is a milk-specific protein that functions as part of the lactose synthase complex during the synthesis of lactose in the mammary gland. However, other novel yet not so well-defined functions have been attributed to α-lactalbumin. In particular, native α-lactalbumin proteins have been shown to modulate cell proliferation in cell culture-based assays. In an attempt to investigate whether the structure of α-lactalbumin determines its function to modulate proliferation, we determined the purity and secondary and tertiary structures of different bovine α-lactalbumin proteins either purchased commercially or expressed in *E. coli*. One recombinant protein has a structure similar to the native protein and the other is a mutant that was generated by site-directed mutagenesis of the aspartate^{87} residue into an alanine. This protein does not retain the ability to bind Ca^{2+}. When tested in cell proliferation assays, neither of these proteins modulated cell growth. Only after the proteins were treated with a reducing agent that disrupts the native disulfide bonds did we detect changes in cell proliferation. These results suggested that the structure of bovine α-lactalbumin, as determined by correct disulfide bond formation, is a determinant of its ability to modulate cell proliferation.

II. Introduction

α-Lactalbumin is a small, acidic, water-soluble milk-whey protein that functions as the modifier protein in the lactose synthase complex in mammary epithelial cells during lactation (Brew *et al.* 1968). During the synthesis of lactose in milk, α-lactalbumin binds β1-4 galactosyltransferase in the Golgi apparatus to form a heterodimer known as the lactose synthase complex (Brodbeck *et al.* 1967). In the absence of α-lactalbumin, β1-4 galactosyltransferase catalyzes the transfer of uridine diphosphate- galactose to N-acetylglucosamine residues found on glycolipids and glycoproteins. α-Lactalbumin lowers the Km of glucose for β1-4 galactosyltransferase about three orders of magnitude (from ~1M to ~1mM) so that at physiological concentrations, glucose is now the preferred substrate (Brew and Hill 1975) and in the presence of Mn^{2+}, β1-4 galactosyltransferase then catalyzes the transfer of UDP-galactose to glucose for the synthesis of lactose during lactation (Brew *et al.* 1968). In 1994, α-lactalbumin deficient mice were created and their milks were analyzed (Stinnakre *et al.* 1994). The mutant milk is 40 fold more viscous than wild-type milk and this viscosity prevents the offspring from removing milk from the lactating gland, even after administering increasing doses of oxytocin. This viscosity is a result of the lack of lactose, which is the major osmotic regulator in milk. As such, the absence of a-lactalbumin as a modifier protein in the lactose synthase system results in lactose free milk.

The crystal structures of this calcium metalloprotein from several species have identified the structural elements that contribute to the native conformation of the protein (Acharya *et al.* 1989 and 1991, Pike *et al.* 1996). These include a large α-domain comprised mainly of three major α-helices, a flexible region, and two short 3_{10} helices, a small β-domain consisting of one antiparallel β-sheet and one short 3_{10} helix, four disulfide bridges

and a calcium-binding loop. Its tertiary structure is stabilized by the four disulfide bonds (residues 6-120, 61-77, 73-91 and 28-111) and a calcium ion which is coordinated through seven oxygen ligands, aspartate residues 82, 84, 87, 88, lysine 79 and two water molecules (Vanaman *et al.* 1970, Stuart *et al.* 1986, Acharya *et al.* 1989 and 1991). During proper folding of α-lactalbumin, a disulfide bond forms between cysteine residues 73 and 91, creating the calcium- binding loop. Amino acid side chains in helix 3 (86-98) of the loop interact with residues in the 2 domains and cysteine residues 61 and 77 form a cysteine bridge that connect the α-helical and β-sheet domains. The mature protein binds calcium in a 1:1 molar ratio (Lönnerdal and Glazier 1985) and such binding decreases the Stokes' radius of the protein, so that it becomes more compact as it folds into its native tertiary conformation (Rao and Brew 1989). In 1997, Anderson *et al.* studied the folding process of recombinant bovine α-lactalbumin proteins folded under various redox conditions and proposed a partial folding model based on the contributions from the calcium-binding loop. By singularly mutating each residue within the calcium-binding loop into an alanine, they determined its role in both the structural stability and functional ability of α-lactalbumin. Based on the observed calcium dissociation constants and spectroscopic data, the authors speculated that residues aspartate 87 and aspartate 88 first coordinate the calcium ion into the calcium-binding loop since mutation of this residue creates proteins with less than 3.5% activity in the lactose synthase assay versus the wild-type recombinant protein and non-detectable calcium binding affinities. Next, lysine 79 and aspartate 84 coordinate the calcium ion and finally aspartate 82 and the two water molecules.

α-Lactalbumin is a model protein for studying protein folding because it goes through an intermediate state known as the molten globule during its progression between unfolded

and folded states. The molten globule state of α-lactalbumin is a partially folded, compact, ordered secondary structure lacking a well-defined tertiary structure (Dolgikh *et al.* 1981). α-Lactalbumin forms a molten globule state during folding, denaturation and under a plethora of conditions, including low pH, high temperatures, and in the absence of Ca^{2+}. The acid state of α-lactalbumin at low pH is the classical molten globule state. Under acidic conditions, protons compete with Ca^{2+} for binding to the same oxygen residues the coordinate the formation of the calcium-binding loop. α-Lactalbumin denatures at low pH, high temperature and increased pressures and this transition depends on the metal ion concentration as Ca^{2+} binding increases the stability of the protein. At acidic conditions that occur in the stomach and intestines, most of the α-lactalbumin is digested and supplies the neonate with essential amino acids. But this protein also has several fatty acid binding sites to which the most abundant fatty acids in animals, oleate, stearate and palmitate, bind (Cawthern *et al.* 1997). The binding of α-lactalbumin to such lipophilic molecules changes the protein conformation from native to a molten-globule like state with increased exposure of hydrophobic residues that ultimately protects the protein from proteolytic digestion (Hirai *et al.* 1992).

In addition to the molten globule transitional state, α-lactalbumin has been observed to exist in several stable partially folded, non-native states. Before being transported by secretory vesicles and secreted into milk, α-lactalbumin is a peripheral protein attached to the hydrophilic tail of β1-4 galactosyltransferase integrated in the Golgi apparatus in mammary epithelial cells. Aggregates have been reported (Håkansson *et al.* 1995, Svensson *et al.* 1999 and 2000), although the factors that regulate such aggregation in milk are poorly understood. While investigating the effects of human milk on bacterial adherence to a human lung cell

line, two α-lactablumin folding variants were isolated. Multimeric α-lactalbumin (MAL), which is composed of 25% multimers of α-lactalbumin and 80% monomer, was initially isolated and its structure characterized, as (Håkansson *et al.* 1995) followed by human α-lactalbumin made lethal to tumors (HAMLET), which is a complex of α-lactalbumin oligomers and oleic acid (Svensson *et al.* 1999 and 2000). Both MAL and HAMLET induced apoptosis in non-mammary tumor cell lines. It can be hypothesized that protein aggregation may be a consequence of the high concentration of α-lactalbumin in milk or the formation of mismatch disulfide bonds within the protein. Here, we have treated mammary epithelial cells with native α-lactalbumin protein purified from bovine milk and bacterial expressed recombinant proteins in the native state (wild-type) or non-native conformation (D87A) to determine effects on mammary gland cell proliferation. We also treated these same protein species with dithiothreitol (DTT), an agent that reduces disulfide bonds, to generate mismatched disulfide bonds before testing their effects on cell proliferation. We showed that only the reduced forms of the native and non-native α-lactalbumin proteins modulated cell proliferation.

III. Materials and Methods

Materials

Recombinant wild-type and D87A DNA sequences in pT7-7 f1 phagemids were a generous gift from Dr. Charles L. Brooks (Department of Veterinary Biosciences at The Ohio State University, Columbus, OH). Ca^{2+} loaded bovine α-lactalbumin (lot 99H7029, 97% pure) was purchased from Sigma Aldrich (St. Louis, MO) and used without further purification. Dithiotreitol (DTT) was purchased from Promega (Madison, WI). Tris-Glycine polyacrylamide gels, IEF gels and electrophoresis buffers were purchased from Invitrogen (Carlsbad, CA). XTT cell proliferation kit was purchased from Roche Molecular Biochemicals (Indianapolis, IN).

Expression, folding and purification of recombinant bovine α-lactalbumins

Wild-type and D87A bovine α-LA cDNAs in pT7-7 f1 vectors were a generous gift from Dr. Brooks in the Department of Veterinary Biosciences at The Ohio State University. Construction of these plasmids was previously described (Anderson *et al.* 1997). Yields for the wild-type recombinant α-lactalbumin proteins were between 12-20 mg per liter of broth and that for D87A proteins was 25 mg per liter of broth. These phagemids were transformed into competent *E. coli* BL21 (DE3) cells and resulting colonies were grown and expanded in LB medium (1 L) supplemented with ampicillin (100 μg/ml) at 37°C until the OD_{600} was approximately 0.3. IPTG (0.4 mM) was added to each 1L-expanded culture to induce recombinant protein expression for 4 additional hours. The cultures were centrifuged at 5000 x g, 4°C for 10 min. The pellet was resuspended in 50 ml Tris-HCl pH 7.5 (100 mM),

dithiothreitol (25 mM) and PMSF (1 mM) and the cells were subsequently broken by two passages through a hydraulic press at 5000 psi.

The cell suspension was centrifuged at 20,000 x g, 4°C for 30 min and the pellet containing the cell membranes and inclusion bodies was solubilized for 1 hr at room temperature in 500 ml solubilization buffer (4.5 M urea, 100 mM Tris-HCl pH 9.5, 1 mM $CaCl_2$, 5 mM 2-mercaptoethanol and 0.5 mM 2-hydroxyethyl disulfide). Calcium ions are necessary for α-lactalbumin to fold in its proper tertiary conformation, urea maintains the proteins in a denatured state, and the redox pairs prevent disulfide bond formation. The solubilized pellet was centrifuged at 20,000 x g, 4°C for 30 min and the supernatant, containing solubilized proteins expressed in the inclusion bodies, was transferred to dialysis membranes (molecular weight cut off 3,500) and dialyzed four times for 8 hr against 2 L dialysis buffer (10 mM Tris-HCl pH 7.5, 5 mM 2-mercaptoethanol, 0.5 mM 2-hydroxyethyl disulfide, and 5 mM $CaCl_2$) at 4°C to remove the urea and allow the proteins to refold. The proteins were precipitated with ammonium sulfate (80% saturation) and then centrifuged at 20,000 x g, 4°C for 30 min. The pellet was resuspended in 250 ml Tris-HCl pH 7.5 (10 mM), $CaCl_2$ (1 mM) for 1 hr at room temperature and the solubilized proteins were centrifuged at 20,000 x g, 4°C for 15 min. The supernatant was transferred to dialysis membranes and dialyzed twice for 12 hr against 2 L dialysis buffer (10 mM TEA pH 7.5, 1 mM $CaCl_2$) at 4°C to slowly remove the redox pair and allow the formation of disulfide bonds.

The recombinant proteins were loaded onto a pre-equilibrated DE52 cellulose beaded resin (pH 7.6) anion exchange column and eluted with a salt gradient (0.1 M to 0.5 M sodium chloride). The absorbencies at 250, 280, 300 and 340 nm were monitored during elution.

Protein fractions were collected as the absorbencies at 280 nm increased and that of the other wavelengths remained low. Typically 50 to 100 ml of protein was collected and dialyzed against ammonium bicarbonate pH 7.8 (5 mM) to remove the salt. The proteins were lyophilized and stored at -20°C in a desiccator. Yields for the wild-type recombinant bovine α-lactalbumin proteins were between 12 mg and 20 mg per liter of broth and that for D87A proteins was 25 mg per liter of broth.

PCR

To verify the presence of the constructed mutations, plasmid DNA was prepared according to the manufacturer's recommendations (Plasmid MidiPrep Kit, Qiagen) from the 1L-expanded culture of transformed *E. coli*. The bovine α-lactalbumin cDNA insert was amplified (40 cycles of 94°C for 25 sec, 55°C for 25 sec, and 72°C for 1 min followed by one cycle at 72°C for 10 min) using primers (forward 5'ACGGTTTCCCTCTAGAAA3' and reverse 5'CAGGCTGCGCAACTGTTG3') to sequences just outside of the gene insert in the pT7-7 plasmid. The PCR product was digested for 1 hr with the EcoRV, whose recognition site was generated in the gene through silent mutations (codons GAC and ATT changed to GAT and ATC which still encodes the amino acids aspartate and isoleucine, respectively), and run on a 1% agarose gel in 1X TAE.

Electrophoresis

The size and purity of the proteins were analyzed by 10% SDS electrophoresis under reducing and non-reducing conditions and IEF electrophoresis under non-reducing

conditions. For polyacrylamide gel electrophoresis, bovine α-lactalbumin proteins (10 μg) was solubilized in 10 mM Tris-HCl pH 7.4, 1 mM $CaCl_2$ and 2X sample buffer with or without 2-mercaptoethanol (5%) and boiled for 5 minutes. The protein was loaded onto 10% Tris-Glycine polyacrylamide gels and separated at 125 V for 1.5 hr. The gels were stained with coomassie blue for 30 min and destained overnight. For two-dimensional gel electrophoresis, bovine α-lactalbumin proteins (50 μg) was electrophoresed on IEF pH 3-10 gels at 100 V for 1hr at room temperature, then at 200 V for 1 hr at 4°C, and finally at 500 V for 30 min on ice. The gels were fixed for 30 min in 20% TCA, stained for 15 min, destained and washed with distilled water, and finally soaked in 20% ethanol for 20 min. The gel was cut into strips containing each lane of protein electrophoresed and each strip was soaked in a 1:1 mixture of 2X sample loading buffer and ethanol. The gel slice was then loaded onto a 4-20% gradient Tris-Glycine polyacrylamide gel and subjected to electrophoresis at 125 V for 1.5 hr under non-reducing conditions. The gel was stained with coomassie blue for 30 min and destained overnight.

Spectroscopy

UV-visible spectroscopic measurements of 10 μM α-lactalbumin in 10 mM Tris pH 8.0, 1 mM $CaCl_2$ were recorded from 240 nm to 350 nm at 22±2°C on a Shimadzu UV-VIS NIR Scanning spectrophotometer in a quartz cuvette with a 1 cm path length, medium scan speed, and 0.5 nm steps. Circular dichroism measurements were recorded on a JASCO Model-600 spectrapolarimeter at 25 ± 2°C in a 1 cm-path length quartz cuvette. Each spectrum is the smoothed average of 5 repeat scans measured with 1nm bandwidth. Ellipticities were recorded at 0.1 nm steps at a rate of 20 nm per min. Mean residue

ellipticities θ_m (deg x cm^2 x $dmol^{-1}$) were calculated from the recorded ellipticity θ in deg, as $\theta_m = \theta/(c \times n \times l)$ where c is the protein concentration (M), n is the number or residues in the protein and l is the path length (m). Concentrations of α-lactalbumin proteins in 10 mM Tris pH 8.0, 1 mM $CaCl_2$ were 1 to 3 μM far UV (200 nm to 250 nm) and 75 to 200 μM near UV (250 nm to 320 nm).

Generation of reduced α-lactalbumin proteins

Protein concentrations (10 mg/ml) were made in 10 mM Tris pH 8.0, 1 mM $CaCl_2$ in 2 ml total volumes. DTT (5 mM) was added to the proteins for 30 min at room temperature and then the proteins were immediately diluted with cell culture media to the appropriate concentrations used in the cell proliferation assays.

Cell Culture

MCF-7 (ATCC, Manasas, VA, #HTB-22), MCF-10a (#CRL-10317) and MDA-MB-231 (#HTB-26) cells were maintained 75 cm^2 tissue culture treated flasks in a humidified atmosphere at 37°C and 5% CO_2. MCF-10a cells were cultured in a 1:1 mixture of Ham's F12: Dulbecco's modified Eagle's medium supplemented 10 ng/ml insulin, 500 ng/ml hydrocortisone, 20ng/ml EGF, 100 U/ml penicillin, 0.1 μg/ml streptomycin, and 5% FBS. MCF-7 cells were cultured in Eagle's minimal essential medium (EMEM) supplemented with 10 ng/ml insulin, 100 U/ml penicillin, 0.1 μg/ml streptomycin, and 10% FBS. MDA-MB-231 cells were cultured in EMEM supplemented with 100 U/ml penicillin, 0.1 μg/ml streptomycin, and 10% FBS.

Cell Proliferation Assay

Cells (1×10^4) were plated in 100 μl volume per well in 96-well tissue culture treated plates. After 24 hr, the medium was replaced with fresh medium fresh containing logarithmic bovine α-lactalbumin concentrations (1 ng/ml to 1 mg/ml). After an additional 24 hr incubation period, the number of metabolically active cells was determined according to the manufacturer's recommendations (XTT Cell Proliferation Kit, Roche Molecular Biochemicals). The cells were incubated with 50 μl of a 49:1 mixture of the XTT labeling reagent and PMS for 4 hr and the absorbance was read at 490 nm using a microtiter plate reader. Percent inhibition of cell growth was calculated using the formula %I= [(abs control-abs exp)/ abs control]*100.

IV. Results

Determination of the purities of various bovine α-LA proteins

The bovine α-lactalbumin cDNA contained in the pT7-7(+) vector has an added N-terminal methionine for translational initiation using T7 polymerase (Anderson *et al.* 1997). For the mutant D87A protein, the aspartate[87] residue is changed to an alanine by site-directed mutagenesis and is verified by DNA sequencing (Fig. A.1A). Additionally, two silent mutations to residues aspartate[88] and isoleucine[89] create a novel EcoRV restriction site, which along with DNA sequencing, is used to verify this mutant (Fig. A.1B). Five separate wild-type lots and four separate D87A lots of recombinant bovine α-lactalbumin proteins were expressed in inclusion bodies in *E. coli* cells. Because of the complex disulfide structure of α-lactalbumin, we took particular care to slowly fold the recombinant proteins in the presence of calcium. We folded the proteins under denaturing conditions an appropriated redox pair (2-mercaptoethanol and 2-hydroxyethyl disulfide) to prevent disulfide bond formation until the final folding step.

We next characterized these proteins. The native protein, reported as 97% pure by the manufacturer, migrated as one major and one minor protein species on SDS-PAGE under non-reducing and reducing conditions (Fig. A.2A), whereas one major and two minor proteins were visible by two-dimensional gel electrophoresis (Fig. A.3A). The major band migrated at the molecular weight and isoelectric point (pI 5.1) as that reported for α-lactalbumin. The higher molecular weight minor bands have the same isoelectric point as α-lactalbumin and may be glycosylated or aggregated forms of the protein. The five separate wild-type proteins were homogenous under non-reducing and reducing gel electrophoresis (Fig. A.2B) as well as non-reducing two-dimensional gel electrophoresis (Fig. A.3B). All of

the four D87A proteins migrated as a multimers under reducing and non-reducing SDS-PAGE (Fig. A.2B) and non-reducing two-dimensional gel electrophoresis (Fig. A.3C).

Spectroscopic characterization of bovine α-LA protein structures

UV-visible absorption spectra are good measures of the structural integrity of a protein and comparison of the absorbance from aromatic residues (280 nm) with the absorbance from disulfide bonds (250 nm) give insight into the conformation of α-lactalbumin. Far UV circular dichroism measurements indicate the presence of secondary structural elements and near UV measurements reveals the tertiary structure of a protein. Of the four wild-type and five D87A mutant recombinant proteins that we expressed, folded and purified, only one representative spectrum is shown.

According to the literature, the native protein is folded properly as its A_{280nm}/A_{250nm} ratio is 2.24 and absorption between 300 nm and 340 nm is relatively low (Fig. A.4C) (Quarfoth and Jenness 1975). Near UV circular dichroism reveals that native protein has a well-defined tertiary structure (Fig. A.4B) and secondary helical structure contributions detected by an A_{208nm}/A_{222nm} ratio of 1.02 (Fig. A.4A). Properly folded wild-type bovine α-lactalbumin proteins have a reported A_{280nm}/A_{250nm} ratio of 2.11 (Anderson *et al.* 1997). The changes in absorption ratio of the recombinant protein versus the native protein result from the presence of the N-terminal methionine, which affects the stability of the protein, but not its structure or function (Veprintsev *et al.* 1999). The wild-type lots 01, 03, 04 and 05 are correctly folded and have an A_{280nm}/A_{250nm} ratio of 2.09 (Fig. A.4C). These four wild-type lots have a slightly increased A_{208nm}/A_{222nm} ratio 1.28 (Fig. A.4A) and a well-defined tertiary structure (Fig. A.4B) and were combined and were used in subsequent experiments.

Surprisingly, wild-type recombinant lot 02 has increased disulfide bond absorption (A_{280nm}/A_{250nm} = 1.49) and increased absorption beyond 300 nm, which suggests that the protein is misfolded and aggregated (Fig. A.4C). Additionally, this lot has a high helical content compared to the native protein as indicated by its characteristic double minima at 208 nm and 222 nm (Fig. A.4A), but does not display alteration in its tertiary structure (Fig. A.4B). Therefore, this recombinant lot 02 lot was discarded and was not used for further analyses. The D87A proteins also have increased disulfide bond absorption indicated by their decreased A_{280nm}/A_{250nm} ratio of 1.82 (Fig. A.4C). All D87A proteins reduced helical contents (Fig. A.4A) and as expected, lack a well-defined tertiary structure in the near-UV circular dichroism region (Fig. A.4B).

Biological effects of bovine α-LA on cell proliferation

The XTT cell proliferation assay indirectly measures cell proliferation by determining the amount of metabolically active cells present in a given sample set. In the presence of metabolically active cells and an intermediate electron acceptor (phenazine methosulphate), the tetrazolium compound XTT is reduced to a water-soluble formazan salt by mitochondrial dehydrogenase enzymes (Scudiero *et al.* 1988, Jost *et al.* 1992, Goodwin *et al.* 1995). Initially, we tested the linearity of the XTT assay by plating increasing amounts of cells for 24 hr, and then performing the assay. There is a linear response for all cell lines; as the cell number increases, the absorbance increases (Fig. A.5A). Next, we tested the effects of native, wild-type, and D87A bovine α-lactalbumin proteins without DTT treatment on mammary epithelial cell proliferation and found that they did not effect mammary epithelial cell proliferation (Figs. A.5B, A.5C and A.5D, continuous lines).

We produced misfolded α-lactalbumin proteins containing a loss of secondary structure due to partial disulfide reduction in the presence of DTT (Kuwajima *et al*. 1990, Takeda *et al*. 1995), a reducing agent that creates disulfide bond mismatches, and therefore misfolded proteins. Although the exact conformation of every protein species within a given sample cannot be determined, we can successfully repeat this process for subsequent experiments. Reduction of the α-lactalbumin proteins produced bimodal effects on cell proliferation. All three reduced proteins, native-reduced (Fig. A.5B, broken line), wild-type-reduced (Fig. A.5C, broken line), and D87A-reduced (Fig. A.5D, broken line), began to stimulate cell growth at the higher concentrations near 10 μg/ml. However, the D87A-reduced proteins drastically reduced cell proliferation at the highest concentration (1 mg/ml, Fig. A.5D, broken line). As we prepared our different concentrations of reduced proteins from serial dilutions, we generated proteins with differing concentrations of DTT. Therefore, it is possible that the observed effects on cell proliferation result from the presence of the reducing agent. This is particularly important for the 1 mg/ml concentrations which contained the highest concentrations of DTT (0.5 mM) as well. To test the validity of the data from cells treated with the reduced α-lactalbumin proteins, we treated the MCF-7 cells with DTT concentration ranging from 0.5 mM to 0.5 nM. All concentration of DTT tested did not affect cell proliferation, thus the observed changes in cell proliferation resulted from the reduced conformations of the α-lactalbumin proteins.

V. Discussion

α-Lactalbumin is highly expressed during lactation and functions in a complex with β1,4-galactosyltransferase (GalT) (Brodbeck *et al.* 1967) for the synthesis of lactose, the major osmotic pressure regulator in milk (Brew *et al.* 1968). In addition to binding GalT, an integral membrane protein, α-lactalbumin readily bound to lipid components of membranes (Hanssens and Cauwelaert 1978, Bańuelos and Muga 1996, Köhler *et al.* 2001), fatty acids found in milk (Cawthern *et al.* 1997, Svensson *et al.* 2000) and to itself (Håkansson *et al.* 1995). Hydrophobic interactions between α-lactalbumin and β1-4 galactosyltransferase (GalT) primarily contributed to the formation of the lactose synthase complex. All of these interactions altered the structure of α-lactalbumin and possibly its function. Likewise, folding variants of human α- lactalbumin that were shown to inhibit proliferation of tumor cells and induce apoptosis also have an increased propensity to aggregate (Håkansson *et al.* 1995 and 1999, Svensson *et al.* 2000). Several *in vitro* studies showed that native α-lactalbumin from various species including human, cow, camel and goat decreased proliferation of normal and transformed mammary epithelial cell lines (Thompson *et al.* 1992, Rejman *et al.* 1992, Alston-Mills *et al.* 1998). In the study by Thompson *et al.* (1992), physiological concentrations of human, cow, goat and camel α-lactalbumin (10 ng/ml) inhibited the proliferation of human mammary epithelial cell lines MCF-7 (40%) and AIN4 (80%). The study by Rejman *et al.* (1992) used a higher concentration of commercially prepared bovine α-lactalbumin (625 μg/ml) to significantly decrease (33%) the proliferation of a bovine mammary epithelial cell line, MAC-T. Furthermore, hydrolysis of commercial bovine α-lactalbumin reduces the ability of the protein to modulate cell proliferation in MCF-7 and MCF-10a cell lines (Alston-Mills *et al.* 1998), highlighting the importance of an intact

protein. Whether its native conformation is essential for its function has not been confirmed but increased protein hydrophobicity may contribute to the ability of the non-native α-lactalbumin proteins to modulate cell proliferation.

There is increasing interest in characterizing the secondary structure of non-native states (Smith *et al.* 1999, Gast *et al.* 1999). Generally, the ellipticity defining the secondary structure of a protein was smaller in an intermediate state than in the final native protein (Kuwajima *et al.* 1993). Native α-LA has ~30% helical content (Acharya *et al.* 1989). Interestingly, α-lactalbumin constructs with greater helical contents have a higher affinity for hydrophobic molecules than the native protein (Demarst *et al.* 1999 and 2001). When complexed with small hydrophobic molecules, α-lactalbumin was protected against pepsin and trypsin digestion (Hirai *et al.* 1992). This observation may have some significance in the neonates' digestive tract. In addition, increased exposure of hydrophobic residues is an inherent property of non-native and molten-globule states and the orientation of such residues makes significant contributions to the mobility, flexibility and fold of α-lactalbumin (Grobler *et al.* 1994, Pike *et al.* 1996). An altered conformation with substantial secondary structure and increased hydrophobicity was required for α-lactalbumin to bind membranes and negatively charged vesicles (Bańuelos and Muga 1996) and to inhibit cell proliferation (Håkansson *et al.* 1999, Svensson *et al.* 2000). We hypothesized that like the human α-LA variants, these bovine α-LA variants have an increased hydrophobic nature. Treatment of α-lactalbumin proteins with DTT results in partial disulfide reduction and increased hydrophobicity (Li *et al*. 2001) and the bovine α-lactalbumin derivative that maintains only the cysteine 61-77 and cysteine 73-91 disulfide bonds also had an increased hydrophobic character (Ewbank and Creighton 1993b). Moreover, it is know that altered disulfide bond

arrangements can increase negative ellipticity at 222 nm in the far-UV region (Fig. A.4A), thus the presence of multimeric proteins in D87A preparations may result from incorrect disulfide bond formation within or between α-lactalbumin proteins (Figs. A.2B and A.3C).

We originally chose to use the D87A mutant protein in our cell proliferation assays because it was known to have a collapsed calcium-binding loop that contributed to its non-native structure (Anderson *et al*. 1997, Figs. A.4A, B and C) and the wild-type protein would serve as a control. Additionally, we chose to perform these assays with normal (MCF-10a), ER positive carcinoma (MCF-7) and ER negative carcinoma (MDA-MB-231) mammary gland cell lines because they collectively represent the composition of breast tumors. We were surprised to find that the non-reduced D87A protein, which lacks a detectable calcium-binding affinity and a defined tertiary structure, behave similar to the native and wild-type proteins in that it did not modulate cell proliferation (Fig. A.5D, continuous line). Considerable attention has been dedicated to characterizing the significance of the non-native α-lactalbumin states since aggregates of other non-native proteins have been associated with diseases. In the progression of scrapies, the amyloid prion protein changes into a molten-globule like conformation and irreversibly altered its secondary structure from the native α-helix-rich form to a disease causing β-sheet-rich structure (Safar *et al.* 1993 and 1994). The ultimate consequence of this change in secondary structure was the progression towards a diseased state. In an attempt to understand the aggregation of α-synuclein, a major constituent of Lewy bodies in Parkinson's disease, Kim and colleagues employed circular dichroism spectroscopy to provide evidence that α-synuclein underwent a conformational change in the presence of zinc ions and such changes promoted protein aggregation more readily than the native structure (Kim *et al.* 2000). Similar to these documented protein

aggregates, α-lactalbumin is a dynamic protein that tends to assume alternate conformations under physiological conditions and these non-native states may have significant biological functions.

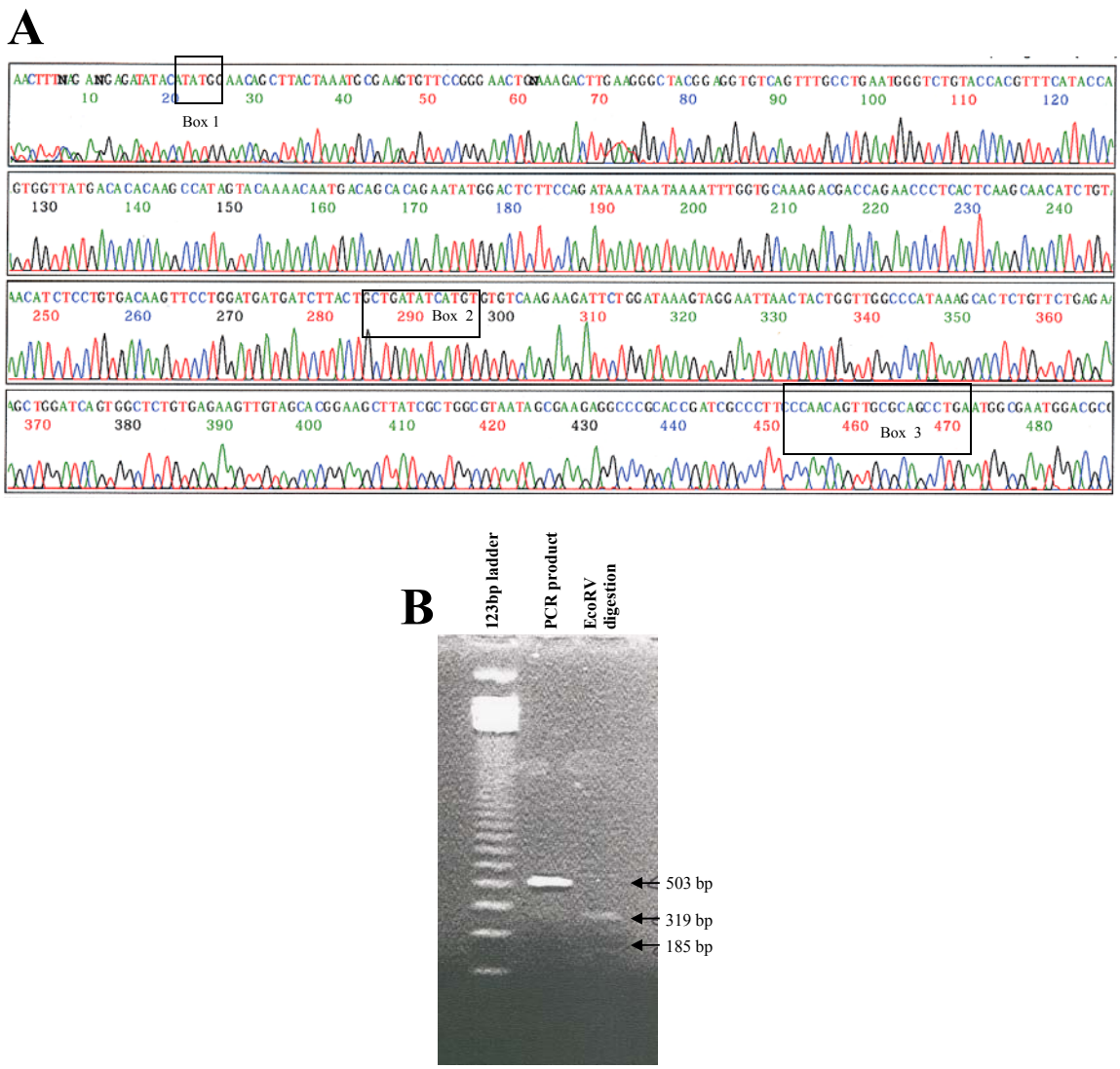


Figure A.1: Verification of the D87A mutant bovine α-lactalbumin gene sequence
A. Box 1, the first translated amino acid is methionine. Box 2, D87A gene has a point mutation in the calcium binding loop at base pair 286 (Box 2, GAT to GCT) which changes the translated amino acid from aspartate to alanine. Box 3, D87A gene creates two silent point mutations at base pairs 290 and 293 (Box 3, GACATT) that create a novel EcoRV restriction site (GATATC) but does not alter the translation of the wild-type aspartate and isoleucine amino acids. B. The presence of the novel EcoRV restriction site in the D87A PCR product is verified. The 503 bp PCR product is digested with EcoRV to give 319 bp and 185 bp products.

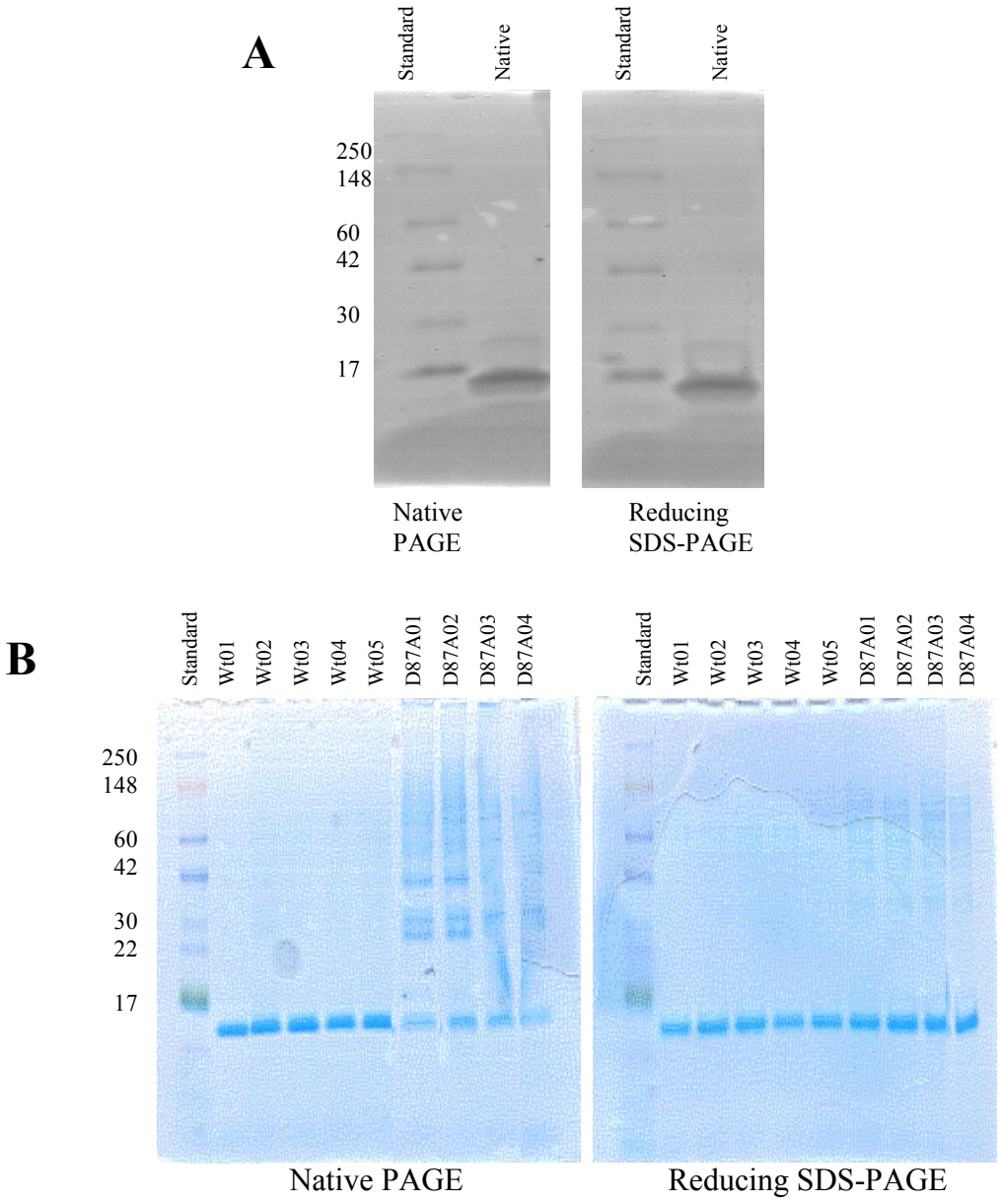


Figure A.2: One-Dimensional gel electrophoresis shows that the native and recombinant α-lactalbumin proteins are pure

A. Native and B. Recombinant α-LA proteins were separated on 16% Tris-Glycine gels under non-reducing and reducing conditions to detect the major protein species at a relative molecular weight of 14 kDa.

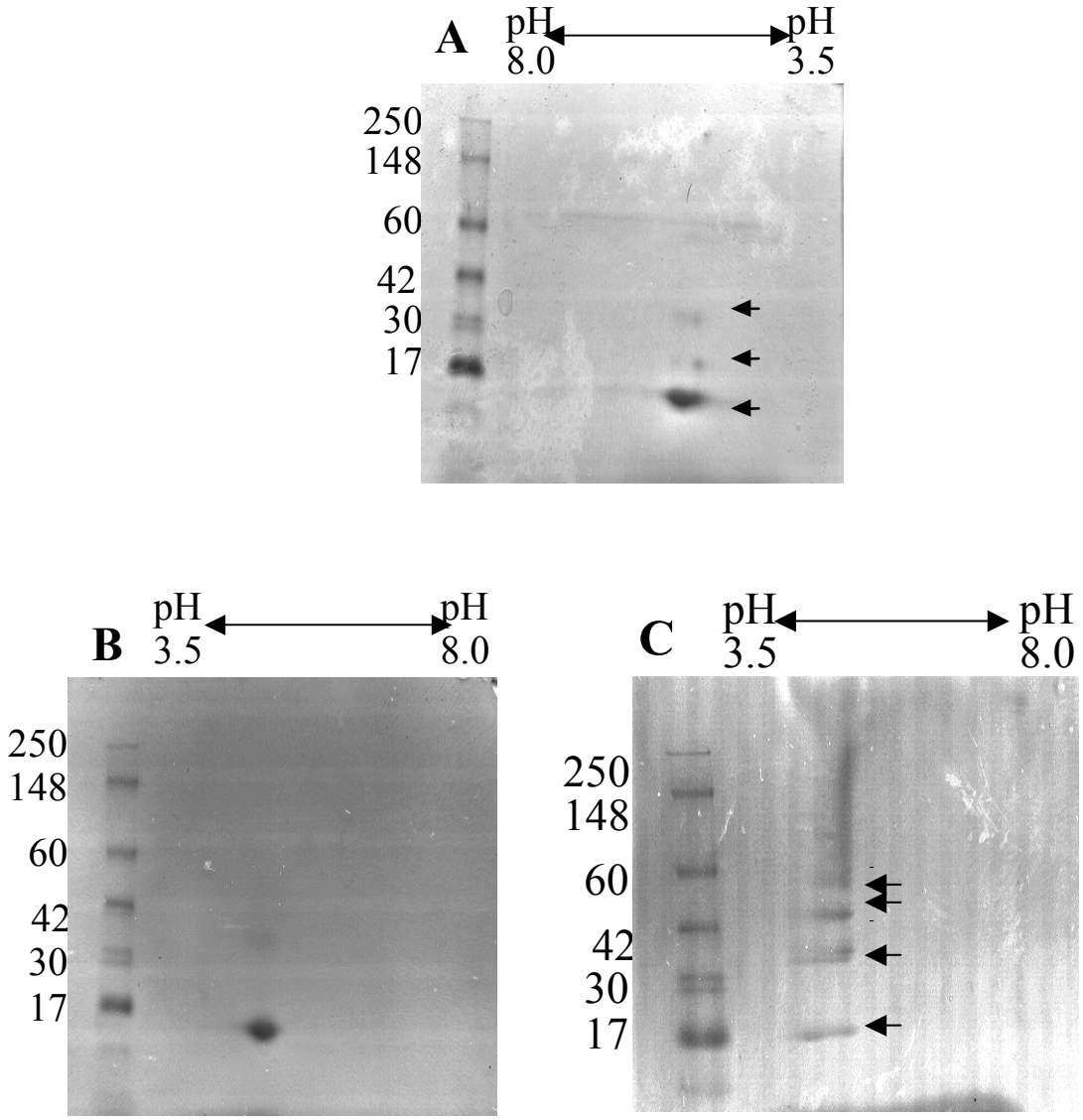


Figure A.3: Two-Dimensional gel electrophoresis to access the purity of the native and recombinant α-lactalbumin proteins

Detection of the major protein species at pI 5.1 and 14 kDa approximate molecular weight in native (A), wild-type (B), and D87A (C) protein preparations. Arrows, α-lactalbumin multimeric proteins. One representative gel is presented.

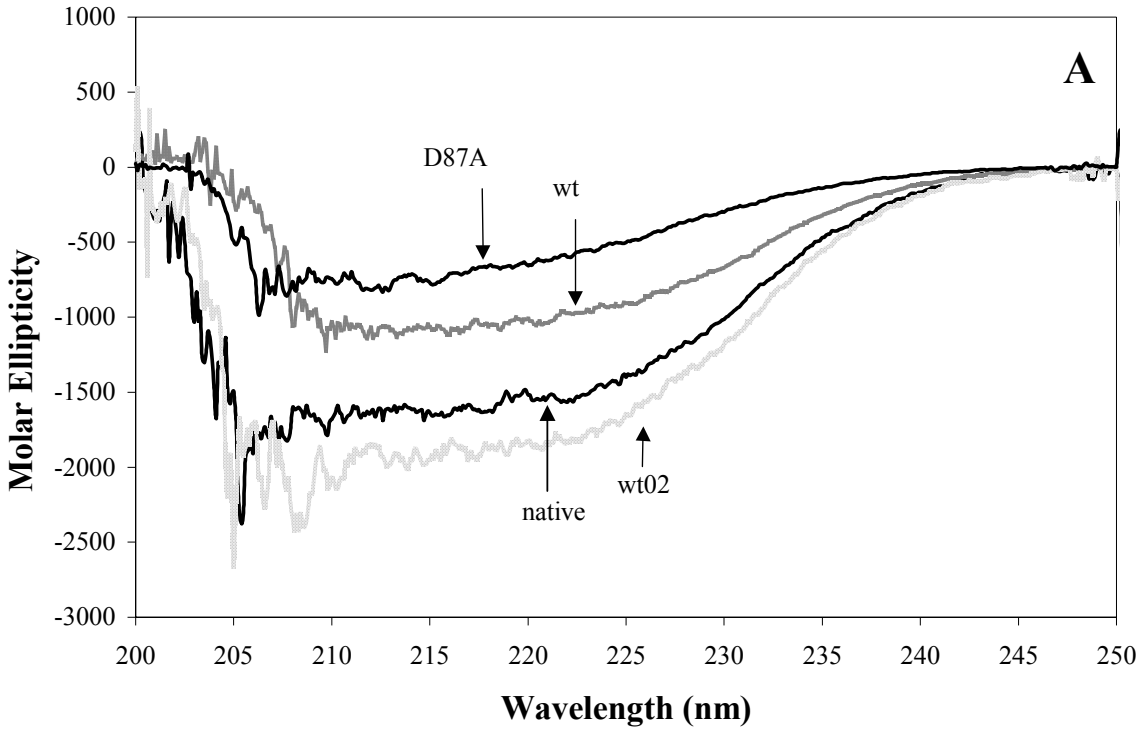
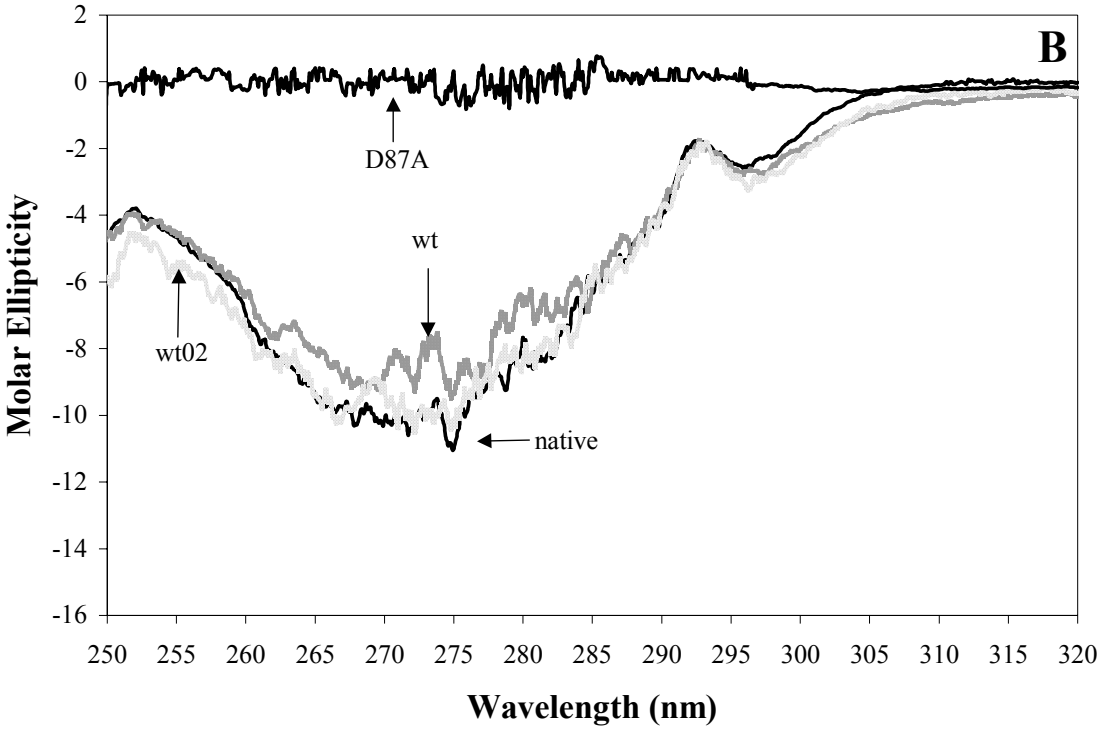
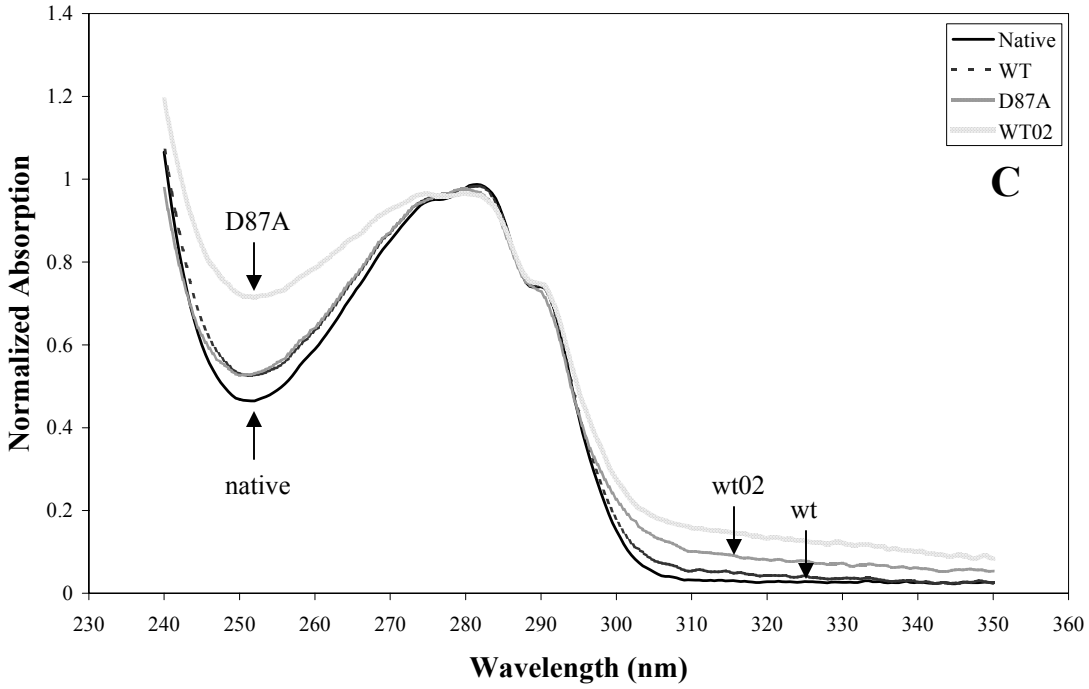


Figure A.4: Spectroscopic analyses of native and recombinant α-lactalbumin secondary and tertiary structures

A. Far-UV circular dichroism of proteins (1-3 μM) from 200 nm to 250 nm. B. Near-UV spectroscopic circular dichroism of proteins (75-200 μM) from 250 nm to 320 nm. C. UV-visible spectra (240 nm to 350 nm) are normalized to the absorbance at 280nm. α-LA proteins were prepared in 10mM Tris, 1mM $CaCl_2$.

Figure A.4: (continued)





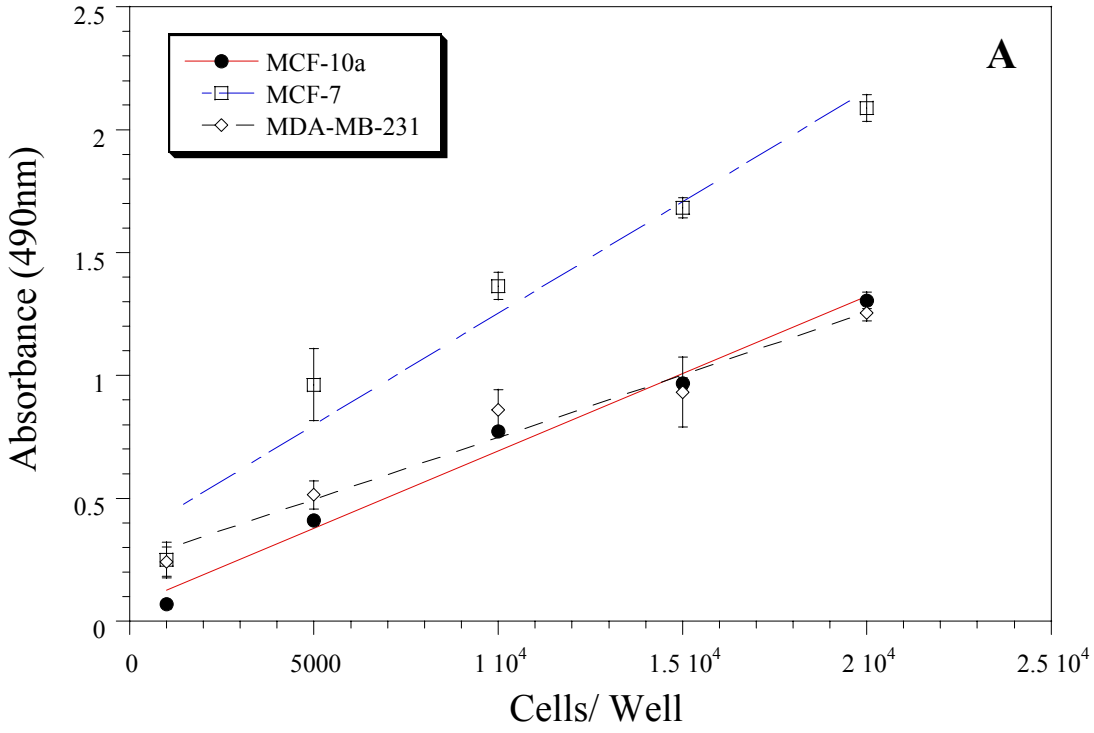
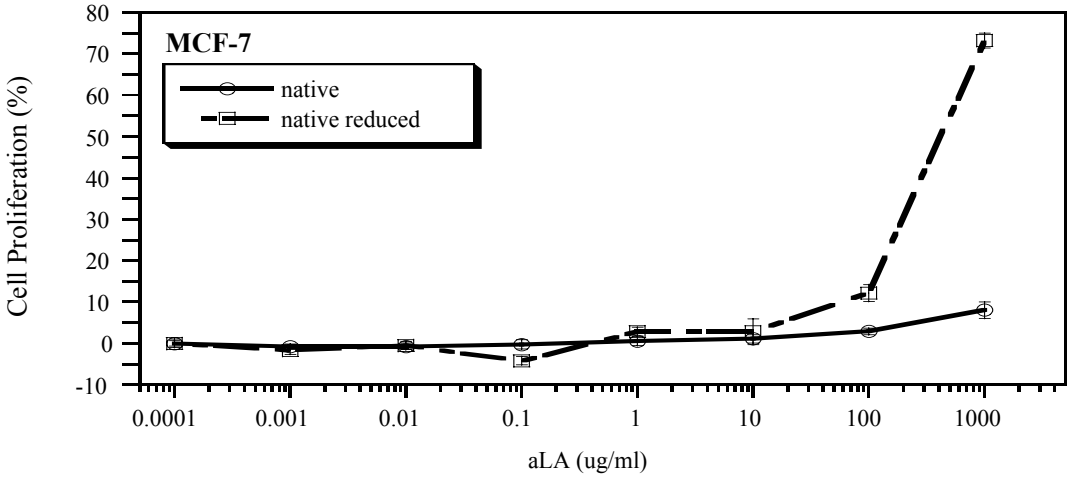


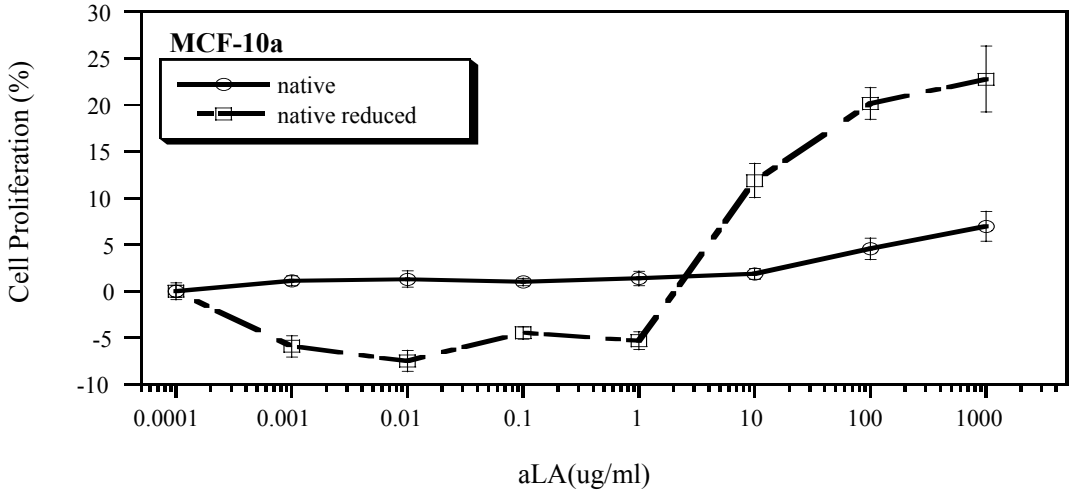
Figure A.5: α-Lactalbumin proteins exposed to a reducing agent exhibit bi-modal effects on mammary epithelial cell proliferation

A. The indicated number of cells for each cell line was plated in 96-well plates and allowed to adhere for 24 hours, after which the XTT Cell Proliferation Assay was performed. Closed circle and continuous line, MCF-7. Open square and long and short broken lines, MCF-7. Open diamond and short broken lines, MDA-MB-231. The effects of native (B), recombinant wild-type (C), and recombinant D87A mutant α-LA proteins in their natural conformations or after 10 min treatment with a reducing agent (DTT, 10 mM) were tested. Percent inhibition was calculated using the equation %I= [(abs control-abs treatment)/(abs control)] x 100. Data are an average of 3 independent assays consisting of 5 replicates per protein concentration per assay and are expressed as mean percent inhibition ± SEM.

Figure A.5: (continued)

B





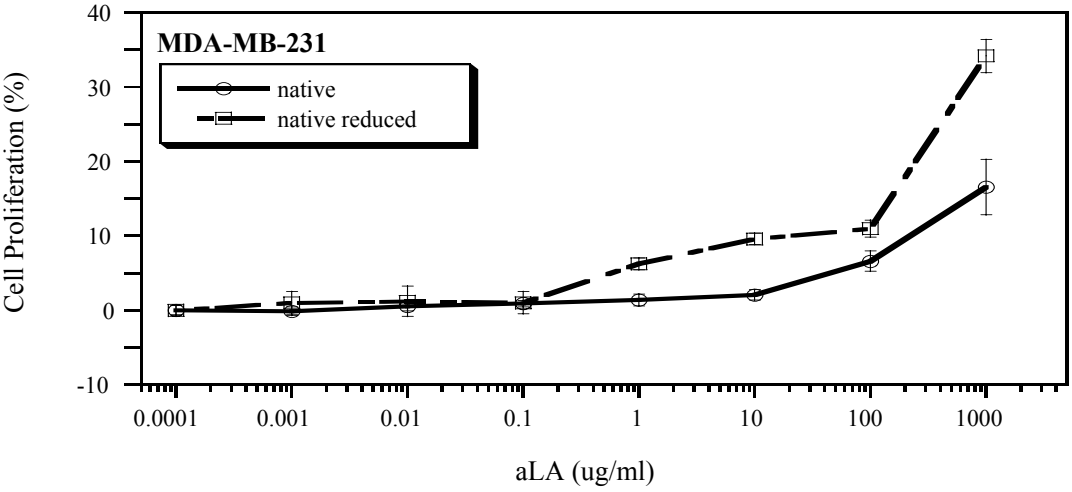
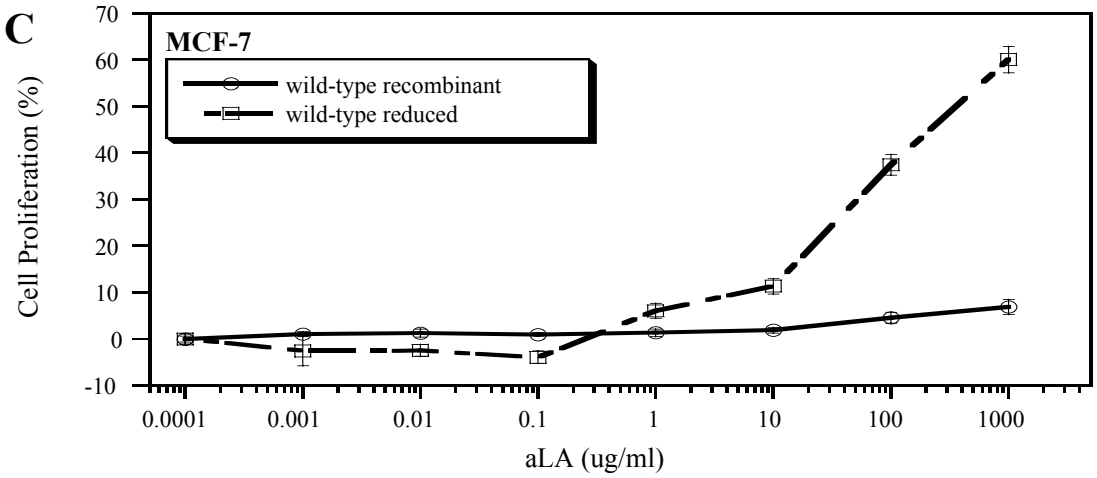
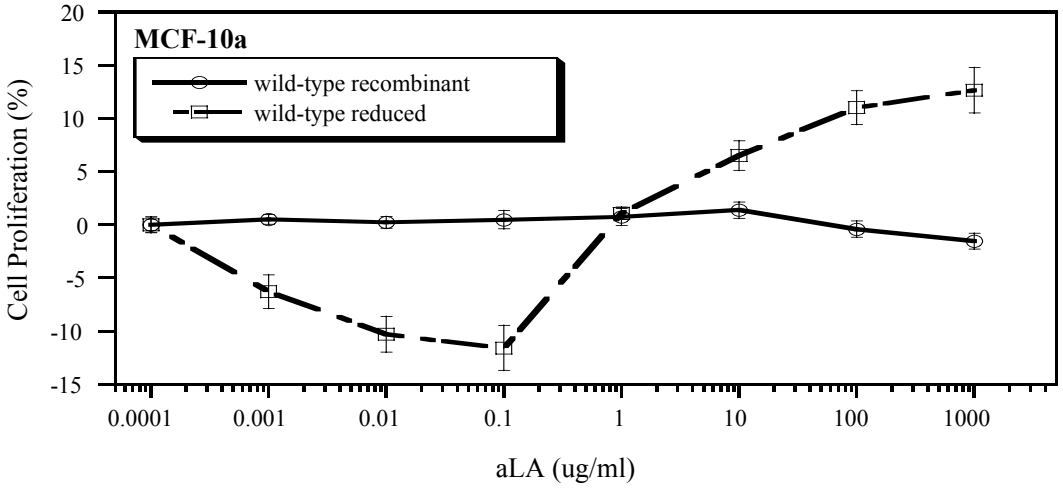


Figure A.5: (continued)

C





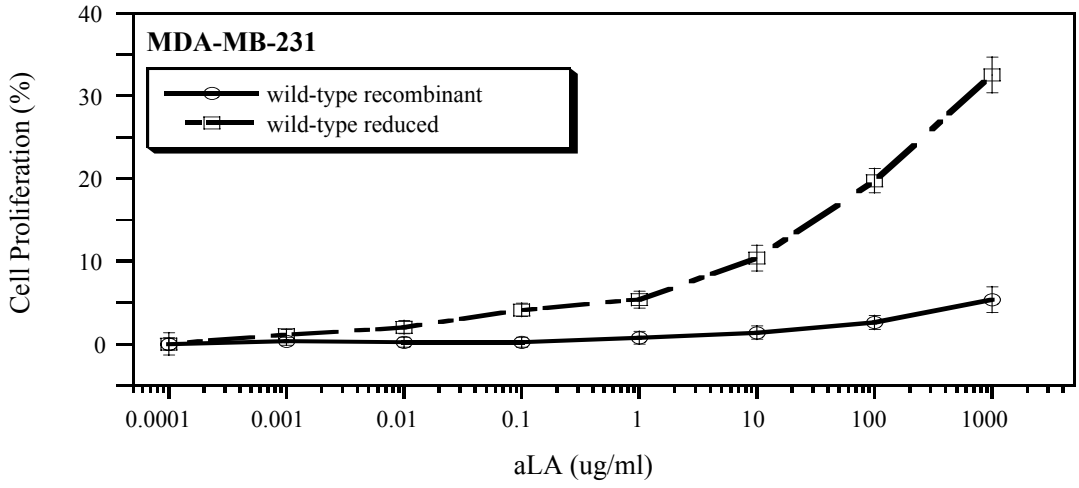
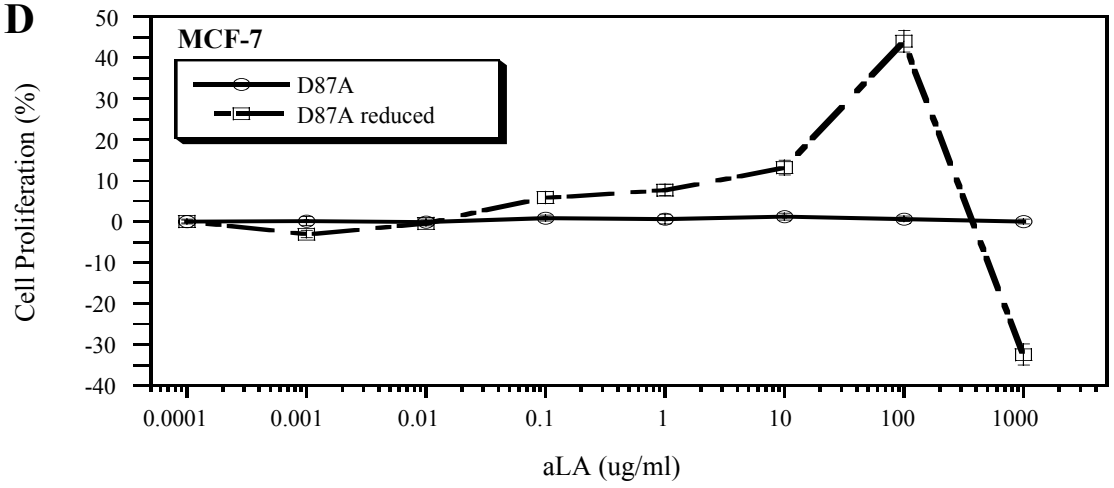
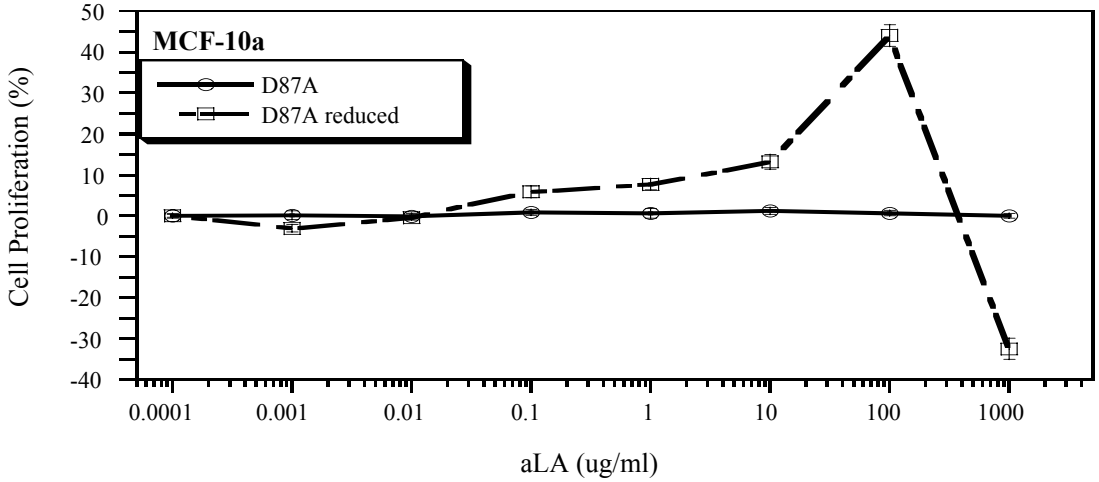
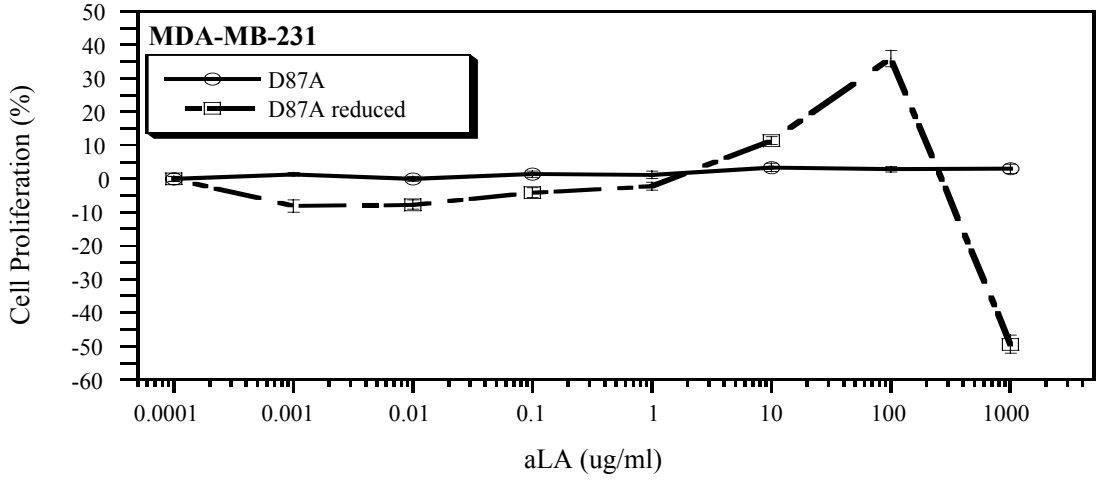


Figure A.5: (continued)

D







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