

**MECHANISMS OF TRANSCRIPTIONAL ACTIVATION OF
ESTROGEN RESPONSIVE GENES IN BREAST CANCER CELLS**

A Dissertation

by

CHIEN-CHENG CHEN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2006

Major Subject: Toxicology

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ABSTRACT

Mechanisms of Transcriptional Activation of Estrogen Responsive Genes in
Breast Cancer Cells. (August 2006)

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Chair of Advisory Committee: Dr. Stephen H. Safe

Estrogen receptor (ER) acts as a ligand-activated transcription factor that regulates the expression of genes. The genomic mechanisms of ER action include ligand-induced dimerization of ER which binds estrogen responsive elements (EREs) in the promoters of target genes. There are also nongenomic mechanisms of ER action which are associated with membrane bound or cytosol ER-dependent activation of various protein-kinase cascades which also influence expression of target genes.

Egr-1 is an immediate-early gene induced by 17β -estradiol (E2) in the rodent uterus and breast cancer cells. Deletion analysis of the *Egr-1* promoter identified a minimal E2-responsive region that contained serum response element (SRE3) which bound Elk-1 and serum response factor (SRF) in gel mobility shift assays. Hormone-responsiveness of *Egr-1* in MCF-7 cells was specifically inhibited by PD98059, a MAPKK inhibitor, but not by LY294002, an inhibitor of PI3-K. These results contrasted with the hormone-dependent

activation of the SRE in the c-fos promoter, which was inhibited by both PD98059 and LY294002, suggesting that Egr-1, like c-fos, is activated through non-genomic pathways of estrogen action but through activation of different kinases.

COUP-TFs are orphan nuclear receptors expressed in a variety of tissues where they regulate biological functions and organogenesis. In this study, we investigated coactivation of ER α by COUP-TF1 in cell lines transiently cotransfected with the pERE₃ construct. COUP-TFI coactivated ER α -mediated transactivation, but unlike many other coactivators, COUP-TFI also enhanced transactivation of ER α when cells were cotransfected with the TAF1-ER α mutant or the 19c-ER α mutant. These data indicate that helix 12 of ER α is not required for coactivation by COUP-TFI when AF-1 of ER α is intact. However, when the AF-1 of ER α is deleted, the intact AF-2 function is required for coactivation by COUP-TFI. Analysis of multiple COUP-TFI deletion mutants showed that the DNA-binding domain and C-terminal region of COUP-TFI were important for coactivation of ER α . Point mutations of the DNA-binding domain of COUP-TFI resulted in loss of interactions with ER α , suggesting that the DNA-binding domain of COUP-TFI is important for its coactivation activity facilitating interactions with ER α . These results demonstrate that COUP-TFI coactivated ER α through a non-classical LXXLL-independent pathway.

DEDICATION

To my wife, my son and coming daughter

My parents,

My parents in law,

For their love, support, patience, and friendship

ACKNOWLEDGEMENTS

First of all, I would like to thank my mentor, Dr. Stephen Safe, for giving me the opportunity to do meaningful research, for his guidance throughout my graduate career. I also wish to thank the other members of my committee: Dr. Burghardt, Dr. Phillips, and Dr. Porter. I appreciate all members of the Safe lab for their friendship and collaboration. I also thank Lorna Safe, Kim Daniel, and Kathy Mooney for their administrative help.

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CHAPTER I

INTRODUCTION

1.1 Cancer

1.1.1 Breast Cancer

Cancer, or neoplasia, is defined as “a group of diseases characterized by uncontrolled growth and spread of abnormal cells. If the spread is not controlled, it can result in death” (2). The word cancer is derived from the Latin for crab, because of the way it protrudes out from a central body like “the arms of a crab”. Even though cancer is often regarded as a single condition, it consists of more than 100 different diseases depending on its tissue of origin. Compared to the physiology of normal cells, cancerous cells exhibit deregulated homeostasis, uncontrolled growth, and invasiveness that are caused by cellular genetic or epigenetic alterations.

Cancer is the second leading cause of death after heart disease in the U.S. About 1.3 million new cases of cancer will be diagnosed in 2005 and approximately 570,000 people will die from this disease. Approximately, 1 out of 4 deaths are due to cancer. The 5-year relative survival rate of all cancers combined after first diagnosis is approximately 64%, whether in remission, under

This dissertation follows the style of Molecular and Cellular Biology.

treatment, or disease-free (2). Excluding cancers of the skin, breast cancer is the most commonly occurring cancer among women, accounting for nearly 1 in 3 cancers diagnosed in US women. The estimated annual incidence of breast cancer worldwide is about one million cases with ~200,000 cases in United States (27% of all cancers in women) and ~320,000 cases in Europe (31% of all cancers in women) (243). Over the last two decades, the annual incidence rate in the U.S. has been increasing steadily (94). Nevertheless, in the last 10-15 years, breast cancer mortality has declined by 2.3% per year due to multiple factors, including improvements in cancer screening and novel and more effective treatment regimens (319).

1.1.2 Structure and Development of the Mammary Gland

The human breast, in common with the mammary glands of other species, contains both epithelial and mesenchymal components. The adult human mammary gland comprises a number of “tree-like” glandular structures formed by dichotomous branching of each of several ducts arising from the nipple. The major functional units of the mammary gland are the lobular structures comprising several small blindended ductules situated at the end of the terminal ducts and known as terminal ductal lobular units (TDLUs). The entire ductal system is lined by a continuous layer of luminal epithelial cells that are, in turn, surrounded by a layer of myoepithelial cells as shown in Figure 1-1. These myoepithelial cells are in direct contact with the basement membrane. The TDLUs are surrounded by delimiting fibroblasts and embedded in a specialized

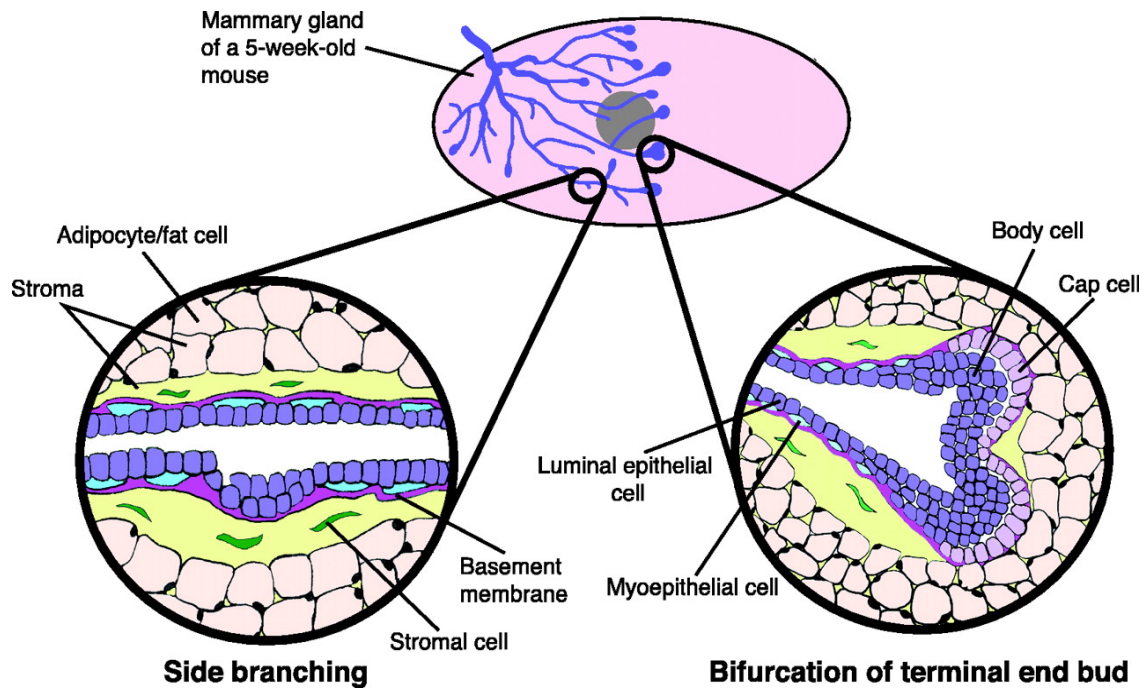


Figure 1-1 The two distinct mechanisms of branching morphogenesis in the pubertal mouse mammary gland (366).

intralobular stroma. The luminal epithelial cells are the major proliferating cell type, whereas cell division or expression of antigens associated with proliferation is exceedingly rare in the myoepithelial cell type (251). The mammary glands of most mammalian species, including humans, are not fully developed and functional at birth. Unlike other organs such as the liver that are fully formed at birth, breast tissue in newborns consists of only a few tiny ducts extending a small distance from the nipple. Between birth and puberty, the growth of this structure is isometric in relation to the rest of the body, but at puberty, under the influence of ovarian and pituitary hormones, the gland undergoes the first phase of allometric growth. In early puberty, the primitive ductal structures begin to rapidly divide and multiply to form a treelike structure composed of many ducts. Once ovulatory menstrual cycles have begun, there is a cyclical increase in proliferation associated with the luteal phase, and the TDLUs become more elaborate in terms of the number of alveoli they contain with each successive ovulatory cycle (287). This progressive development of the epithelium continues to the age of approximately 35 years. The second phase of allometric growth in the mammary gland occurs during pregnancy. During early pregnancy, there is another burst of activity in which the ductal trees expand further and the number of ductules within the TDLUs increases greatly. These ductules differentiate to synthesize and secrete milk in late pregnancy and subsequent lactation. Once weaning has occurred, the mammary gland involutes; the secretory luminal

epithelial cells apoptose, the alveoli collapse and both epithelial and stromal components are remodeled to resemble the prepregnant state.

The evidence from histological studies has shown that most human breast tumors are derived from TDLUs and have morphological characteristics of luminal epithelial cells (4). Significantly, more than 90% of breast tumors synthesize cytokeratins distinctive of the luminal phenotype, and greater than 70% synthesize steroid hormone receptors, indicating that the luminal epithelial cell population must be regarded as the primary target for the oncogenic events leading to tumor formation (295).

Breast tumorigenesis is thought to result from a 'benign to malignant' progression, in which the accumulation of genetic changes allows evolution from normal breast epithelium through benign and atypical proliferative lesions to carcinoma *in situ* and frankly invasive tumors (15). The lesions associated with the greatest risk of invasive breast cancer are, in order of increasing risk: hyperplasia of usual type, atypical ductal hyperplasia, lobular carcinoma *in situ* and ductal carcinoma *in situ* (DCIS). These premalignant lesions, with the exception of high grade DCIS, are frequently dependent on estrogen for their growth, as judged by the presence of the estrogen receptor (ER). ER-negative tumors often overexpress the genes encoding growth factor receptors, such as epidermal growth factor (EGFR or erbB1) and erbB2/HER2, and these are often overexpressed in DCIS of high nuclear grade (128). Premalignant lesions synthesizing ER might account for the success of the antiestrogens tamoxifen

and raloxifene in breast cancer prevention, because it is their progression to invasive breast cancer that might be inhibited (73).

1.1.3 Risk Factors for Breast Cancer

Based on epidemiological studies conducted in different populations, several well-established risk factors for breast cancer have been identified and these include: age, geographic location and socioeconomic status, reproductive events (menarche, menopause, pregnancy, breastfeeding), exogenous hormones (hormone replacement therapy and oral contraceptives), lifestyle risk factors (alcohol, diet, obesity and physical activity), mammographic density, history of benign breast disease, ionizing radiation, bone density, height, IGF-1 and prolactin levels, chemopreventive agents, as well as genetic factors (high- and low penetrance breast cancer susceptibility genes) (See Table 1-1).

Significant differences (5-10 fold) in the incidence and mortality rates of breast cancer have been observed between low- (Far East, Africa and South America) and high-risk (North America and Northern Europe) areas (244). These differences become even more profound after menopause (see section on age). For example, the overall breast cancer incidence in the Japan is 32.7 per 100,000 and 19.5 per 100,000 in the eastern Africa area; however the incidence in the U.S. is 91.4 per 100,000, and the country with the highest incidence is the Netherlands (91.6/100,000).

Table 1-1 Summary of breast cancer risk factors (92).

Breast Cancer Risk Factors		Magnitude of Risk
Factors that increase breast cancer risk		
Well-confirmed factors	Increasing age	++
	Geographical region (USA and western countries)	++
	Family history of breast cancer	++
	Mutations in BRCA1 and BRCA2 genes	++
	Mutations in other high-penetrance genes (p53, ATM, NBS1, LKB1)	++
	Ionizing radiation exposure (in childhood)	++
	History of benign breast disease	++
	Late age of menopause	++
	Early age of menarche	++
	Nulliparity and older age at first birth	++
	High mammography breast density	++
	High insulin-like growth factor I (IGF-I) levels	++
	Hormonal replacement therapy	+
	Oral contraceptives recent use	+
	Obesity in postmenopausal women	+
	Tall stature	+
	Alcohol consumption (~1 drink/day)	+
	High prolactin levels	+
Probable factors	High saturated fat and well-done meat intake	+
	Polymorphisms in low-penetrance genes	+
	High socioeconomic status	+
Factors that decrease breast cancer risk		+
Well-confirmed factors	Geographical region (Asia and Africa)	--
	Early age of first full-term pregnancy	--
	Higher parity	--
	Breast feeding (longer duration)	--
	Obesity in premenopausal women	-
	Fruit and vegetables consumption	-
	Physical activity	-
	Chemopreventive agents	-
Probable factors	Non-steroidal anti-inflammatory drugs	-
	Polymorphisms in low-penetrance genes	-

++ (moderate to high increase in risk) -- (moderate to high decrease in risk);

+ (low to moderate increase in risk) - (low to moderate decrease in risk)

The large variation of breast cancer incidence among or within different regions of the world may be attributed to genetic differences among populations and/or differences in lifestyle, including diet and environmental exposures. Studies on migrants have demonstrated that breast cancer incidence increases in people who move from a region with low breast cancer incidence (i.e. Asian countries) to other locations with higher breast cancer incidence (i.e. U.S.), as early as 10 years spent in the adopted country. This underlines the crucial contribution of the environmental and lifestyle factors to breast cancer risk (243, 388)

1.1.3.1 Lifestyle Risk Factors

Among lifestyle risk factors, alcohol has been identified in numerous epidemiological studies as an important risk factor for breast cancer (312). Specific diets rich in well-done meats (387) or fat (351) are associated with a slightly increased risk for developing breast cancer, in some studies while a high intake of fruits and vegetables (187) or omega-3 PUFAs (289) decreases breast cancer risk. Obesity represents a high breast cancer risk factor for postmenopausal women, whereas in premenopausal women it is protective (140).

Alcohol Intake

Numerous epidemiological studies have found a positive association between alcohol intake and the risk of developing breast cancer in both pre and

postmenopausal women with an overall risk of 1.6. The risk increases linearly in a dose dependent manner up to an intake of 60 g (approx. 2-5 drinks) /day. For every 10 g-increment (approx. 0.75-1 drink) increase in daily consumption of alcohol the risk increases by 9% (312, 313).

Diet

The human diet contains a great variety of natural and chemical carcinogens and anti-carcinogens. Some of these compounds may act through the generation of free oxygen radicals, which can lead to DNA damage, or other deleterious components. Accordingly, well-done meat consumption has been associated with increased breast cancer risk due to the formation of carcinogens during the cooking process (323, 387).

Physical Activity

A recent meta-analysis of 19 case-control and four cohort studies investigating the relationship between physical activity and breast cancer risk has shown a consistent 20% reduction associated with physical activity performed in adolescence and young adulthood (12-24 years old). For each one-hour increase in recreational physical activity per week during adolescence, the breast cancer risk drops with 3%. Physical activity may reduce the risk by delaying the onset of menarche and modifying bioavailable hormone levels (119, 182).

1.1.3.2 Genetic Risk Factors

Family history of breast cancer is a well-established major risk factor, especially in combination with mutations in high-penetrance breast cancer susceptibility genes, such as BRCA1 and BRCA2, p53, PTEN, ATM, NBS1 or LKB1, which are responsible for a high proportion of the hereditary breast cancers .

BRCA1/ BRCA2

Mutations in BRCA1 and BRCA2 (Breast Cancer 1 and 2), two of the most commonly implicated genes in hereditary breast cancer, are responsible for approximately 80-90% of all hereditary breast cancers, whereas they are rarely observed in sporadic breast cancer patients. Women who carry mutations in BRCA1 or BRCA2 have a considerably increased lifetime risk of breast cancer (~80%), that is roughly ten times greater than that of the general population. BRCA1 and BRCA2 are tumor suppressor genes whose primary functions are the maintenance of genomic integrity including DNA repair and recombination, cell cycle control, and transcriptional regulation (79). Germline mutations in BRCA1 are associated with approximately 42% of breast cancer families and 81% of families with both ovarian and breast cancer (107). Germline mutations in BRCA2 are linked to approximately 76% of breast cancer families in which both females and males are affected.

p53

p53 was the first tumor suppressor gene linked to hereditary breast cancer. p53 also plays an important role in maintaining genomic stability in response to DNA damage by inducing transient G1 cell cycle arrest or by triggering apoptosis. Women with germline mutation in p53 have an 18-fold higher risk for developing breast cancer before age of 45 compared to the general population, and the risk declines with age (110).

There are also low penetrance genes (but present in a high percentage of individuals) that enhance breast cancer risk in combination with exogenous (e.g. diet, pollution) and endogenous (e.g. hormones) factors (281). These genes include phase I metabolic enzymes which metabolically activate carcinogens (e.g. the cytochrome P450 family proteins) and phase II enzymes which inactivate carcinogens (e.g. N-acetyl transferase and GST family proteins). Polymorphisms in both phase I and II enzymes involved in xenobiotic and endobiotic metabolism therefore may modulate the relative risk of breast cancer for an individual (234).

1.1.3.3 Reproductive Risk Factors

Breast cancer incidence is very low (less than 10 new cases per 100,000 women) before age 25 and increases up to 100-fold by age 45 (141). This pattern suggests the involvement of reproductive hormones in the etiology of breast cancer (259), as hormone-independent cancers would not significantly increase during the active reproductive period. The number and timing of different

reproductive events in a woman's life modulates the risk of breast tumorigenesis. Several reproductive factors such as early age at menarche (before age 12), late age at menopause (after age 55), nulliparity and late age at first full term pregnancy increase breast cancer risk, whereas other factors including early age at first full-term pregnancy, higher parity and prolonged lactation are protective against breast cancer (92).

The duration of lifetime exposure to ovarian hormones is closely related to breast cancer risk. Early age at menarche (less than 12 years of age versus more than 14 years of age) has been associated with an increase in breast cancer risk on the order of 10-20% magnitude (28, 41) and a 1-year delay in the onset of menarche is associated with a 5% reduction in risk for developing breast cancer in later life (142). Similarly, delayed menopause maximizing the number of ovulatory cycles lead to an increased breast cancer risk and each 1-year delay in the onset of menopause is associated with a 3% increase in risk (142). In contrast, surgically induced menopause before the age of 35 results in a decrease of breast cancer risk. These women have only 40% of the risk of women experiencing natural menopause. Mechanistically, it has been demonstrated that mammary epithelial cells proliferation, which is linked to breast cancer development, can be correlated with serum ovarian hormonal levels. Proliferation rates are low in the follicular phase of the menstrual cycle, when estradiol and progesterone levels are also low, whereas during the luteal phase proliferation rates are twofold higher and correlate with the significantly increased

ovarian hormone levels (259). The higher cellular proliferative activity confers a higher susceptibility of the mammary gland to be transformed by chemical carcinogens (286). After menopause, ovarian hormone levels drop and this correlates with a substantial decrease in mammary epithelial cell proliferation (29). Numerous prospective epidemiological studies also provide strong evidence for this mechanism. Accordingly, postmenopausal women who develop breast cancer have on average 15% higher levels of circulating estradiol than other postmenopausal women (29).

Epidemiological studies have also firmly established associations between risk for breast cancer and other reproductive factors, including nulliparity (having no children) or low parity, late age at first birth, and breast feeding (160). After a transient increase in risk for breast cancer, peaking at about 5 years after giving birth (196), having at least one child is associated with a decrease in the long-term risk of developing breast cancer compared with risk among the nulliparous, and this protective effect increases with number of children. Each birth reduces the relative risk of breast cancer by an average of 7% (1). The reduction in risk per birth is greater for births at young ages, such that women who have their first birth before the age of 20 years have a 30% lower risk than women with a first birth after the age of 35 years (99). The protective effects of pregnancy against breast cancer is explained by the induction of complete differentiation of the breast that may markedly reduce the susceptibility of the fully differentiated mammary gland to carcinogens due to, at least in part, by

decreasing proliferative activity of parous epithelium (285). Another hypothesis is that the decrease risk may also be due to the altered hormonal environment during pregnancy, and these include specific molecular changes induced by estrogen and progesterone and decreased circulating growth hormones (288).

Breast feeding is also protective against breast cancer and this effect might be due to the suppression of ovulation, reducing exposure to ovarian hormones (29).

1.1.4 Estrogens and Breast Tumorigenesis

1.1.4.1 Synthesis and Catabolism of Estrogens

Estrogens are a class of steroid hormones important for normal sexual development and are essential for the normal functioning of the female reproductive organs such as the ovaries and uterus which are required for childbearing and hormone synthesis. Estrogens help control a woman's menstrual cycle and are important for the normal development of the breast. Estrogens are also required for maintenance of healthy bones and for cardiovascular health.

Both estrogens and their androgen precursors are biosynthetically derived from cholesterol. In premenopausal women, the ovaries, which are under the cyclic control of pituitary gonadotropins, are the predominant source of serum estrogen, and only a small proportion of serum estrogen comes from peripheral organs. In contrast, the low levels of estrogen produced in postmenopausal

women comes predominantly from aromatization of adrenal and ovarian androgens in extragonadal tissues such as the liver, muscle, and fat tissues (293).

Estrogens are catabolized mainly by hydroxylation that result in the formation of 2-hydroxyestrone and 2-hydroxyestradiol, 4-hydroxyestrone and 4-hydroxyestradiol, and 16 α -hydroxyestrone and 16 α -hydroxyestradiol (catechol estrogens). Estrogens are also metabolized by subsequent methylation to form methoxyl estrogens (239) and methylation of 2- and 4-hydroxyestrogen by catechol *O*-methyltransferase is also observed (115) (see Figure 1-2).

Catechol estrogens bind the estrogen receptor and have weak estrogenic activity in animals. In addition, catechol estrogens are capable of continuous metabolic redox cycling, which yields quinone intermediates as metabolites. Because of the formation of free radicals in this process and the covalent binding of these intermediates to DNA, it has been proposed that estrogens have genotoxic activity (194). After synthesis, estrogens are secreted into the blood stream where it binds with sex-hormone-binding globulin and albumin. Free estrogens diffuse into target tissues to exert their specific biological effects.

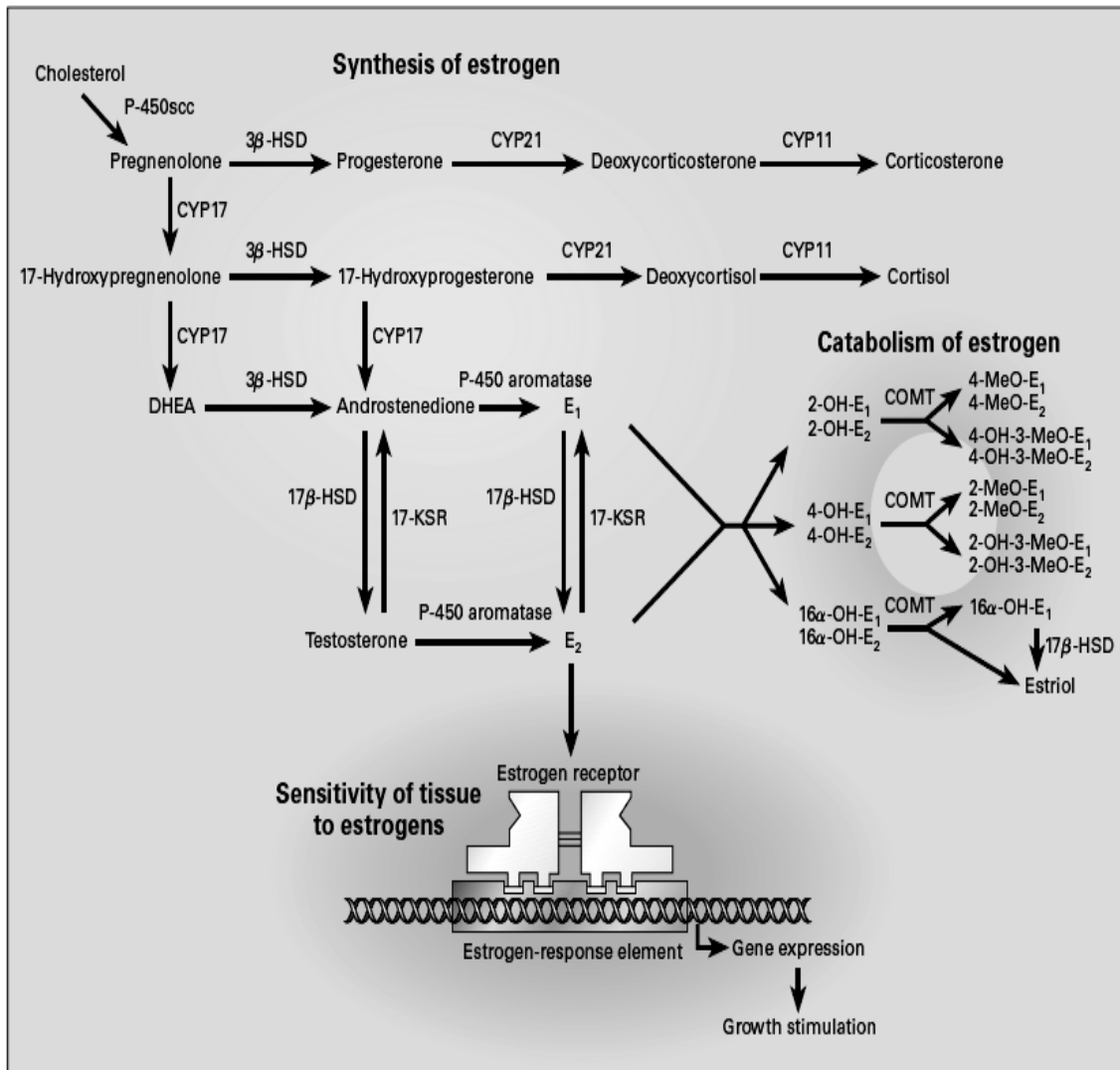


Figure 1-2 Pathways of estrogen synthesis and catabolism (68).

1.1.4.2 Estrogen Exposure and Breast Cancer Risk

The clinical and epidemiological evidence for an obligate role of estrogen in human mammary gland development and tumorigenesis is considerable. Estrogens are crucial for the normal development of the human mammary gland. There is complete failure of breast development in the absence of intact ovarian function, and estradiol-replacement therapy is necessary to induce breast development (83). The obligate role for estradiol in mammary gland development is also supported by the studies using estrogen receptor α (ER α) knockout mice (33). The mammary glands in ER α knockout mice comprise rudimentary ducts confined to the nipple area, which cannot undergo further development with estradiol treatment.

In breast cancer, there is evidence that estrogen stimulates the growth of both premalignant and invasive tumors. More than 100 years ago Beatson first recorded the successful treatment of breast cancer by removal of the ovaries (224). Recent studies have shown that women who undergo ovariectomy early in life have a very low incidence of breast cancer (284). Similarly, rats and mice whose ovaries have been removed develop few if any breast tumors. Men, who do not have ovaries and have low blood levels of estrogen, have low breast cancer rates compared to women (333).

An association between the risk of breast cancer and persistently elevated blood levels of estrogen has been found consistently in many studies. Several

endocrine-associated risk factors are regularly associated with an increased relative risk of breast cancer in postmenopausal women (68, 380). One of these factors is obesity, which is probably related to an increased production of estrogen by aromatase activity in breast adipose tissue (159). Another factor is an elevated blood level of endogenous estrogen (relative risk, 2.00 to 2.58) (158). An increased relative risk is also associated with higher-than-normal blood levels of androgens which can be directly converted by aromatase to estrone and estradiol, respectively. Elevated urinary levels of estrogens and androgens are also associated with an increased risk of breast cancer in postmenopausal women (237). All this evidence supports the hypothesis that cumulative, excessive exposure to endogenous estrogen over a woman's life span contributes to and may be a causal factor in breast cancer.

1.1.4.3 Mechanism of Estrogen-dependent Carcinogenesis

Experimental, clinical, and epidemiologic data suggest that estrogens contribute to development of mammary cancer, but the mechanisms of this process are not well understood. Studies in rodents have demonstrated that estrogens or their catechol metabolites are carcinogens in various tissues, including the kidney, liver, uterus, and mammary glands. Figure 1-3 outlines two different but complementary pathways that may contribute to the carcinogenicity of estrogen (375).

Several mechanisms have been proposed to explain carcinogenicity of estrogens in breast cancer (53, 194). One of them is that the ER-mediated activity of E2 is related to induction of genes critical for regulating the cell cycle and stimulating cell proliferation. With each cycle of new DNA synthesis during mitosis, the chances for error in DNA replication without adequate repair are increased. As the proliferative process continues, mutations can accumulate and disrupt critical genes required for cellular proliferation, DNA repair, angiogenesis, or apoptosis, and these modifications can lead to neoplastic transformation (53, 103). Once the breast cancer initiation has taken place, these hormone-responsive transformed cells cannot repair any spontaneous or induced DNA damage with impaired function. The rapid proliferation activated by estrogens might promote the growth of transformed cells, leading to the development of detectable breast tumors. Estrogens can also stimulate production of autocrine and paracrine growth factors from the epithelium and stroma in the breast that can further contribute to breast cancer progression (315).

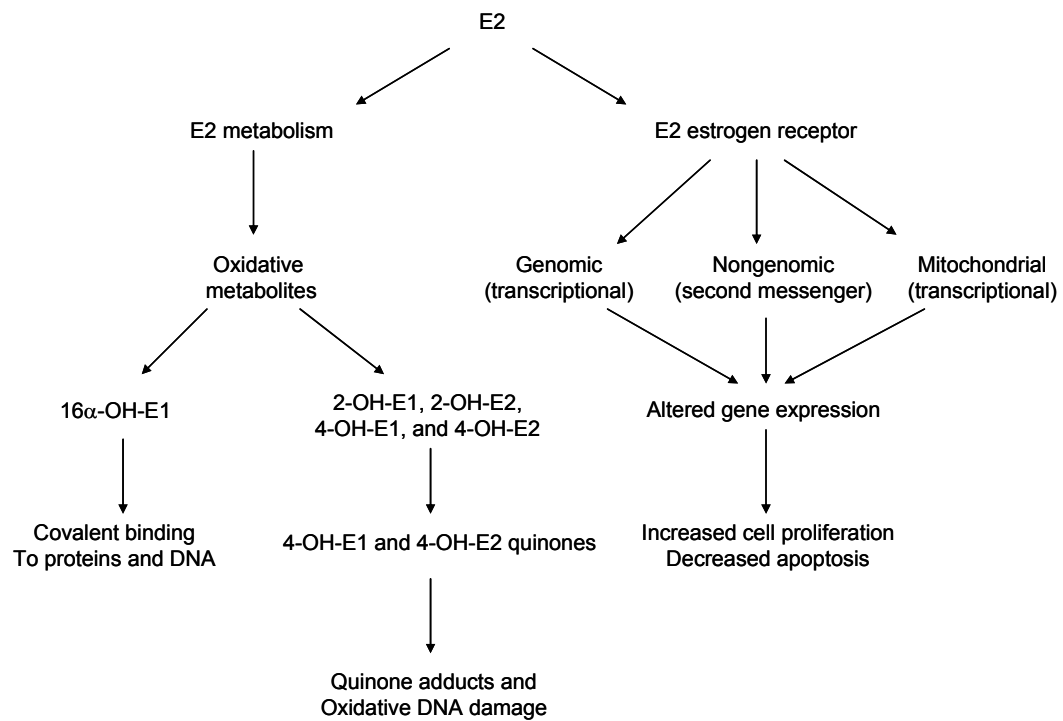


Figure 1-3 Pathways for estrogen carcinogenesis (375).

Another mechanism involves oxidative metabolites of estrogens. The estradiol-3,4-quinone, which can form unstable adducts with adenine and guanine in DNA, results in destabilization of the glycosyl bond that links purine bases to the DNA backbone. Consequently, adenine and guanine, which are covalently bound to the estradiol quinone, are released from the DNA backbone and a naked, apurinic site is left behind in the DNA. Through the process of error-prone DNA repair, this site can form point mutations and serve as potential initiators of neoplastic transformation. In addition, reduction of estrogen quinones back to hydroquinones and catechols provides an opportunity for redox cycling which produces reactive oxygen species (53, 194) and probably accounts for the oxidative damage to lipids and DNA that is associated with estrogen treatment (185, 194).

Estrogen has crucial roles in the proliferation of cancer cells in reproductive organs such as the breast and uterus. Estrogen-stimulated growth requires the ER which is a ligand-dependent transcription factor. It has been shown that about two-thirds of human breast tumors express higher levels of ER than normal breast tissues where ER levels are quite low. E2 and its intracellular receptor (ER) play a critical role in the formation and subsequent growth of mammary tumors and the molecular mechanisms of these responses are important for understanding the development and treatment of this disease.

1.2 Nuclear Receptor Superfamily

1.2.1 Introduction

Small lipophilic steroid hormones play an important role in the growth, differentiation, development and homeostasis of human tissues/cells. Most responses induced by these molecules are mediated through binding to nuclear receptors (NRs) that control gene expression (360). NRs exhibit a modular structure consisting of a C-terminal ligand dependent transcriptional activation domain (AF-2), a central DNA binding domain, and an N-terminal ligand-independent transcriptional activation domain (AF-1) Since the cloning of the first nuclear receptor for the glucocorticoid, more than 60 genes encoding nuclear receptors have been identified in vertebrates, arthropods and nematodes (344). Based on sequence similarity and evolutionary relatedness, the NR superfamily is divided into seven subfamilies, and within subfamilies there are further divisions into groups. In general, receptors within a group share at least 80-90% identity within their DNA binding domains, and at least 40-60% identity within their ligand binding domains (223).

1.2.2 Structures and Functions of NR Domains

Nuclear receptors share a common modular structure with autonomous functional domains that can be interchanged between related receptors without loss of function (180). A typical nuclear receptor consists of a variable amino-terminal region (A/B domain), a conserved DNA-binding domain (DBD) or

region C, a linker region D, and a conserved E region that contains the ligand-binding domain (LBD). Some receptors contain an additional F domain in the C-terminal region which exhibits a highly variable sequence and whose structure and function are not well defined. Figure 1-4 is a schematic representation of a typical nuclear receptor.

The A/B region is variable in both size and sequence and interacts with coactivators and/or other transcription factors in a cell- and promoter specific manner (34, 357). The A/B region in many receptors contains one constitutively active transcriptional activation function, referred to as AF-1 which contributes to ligand-independent activation of the receptor (337). The A/B domain is also a target for phosphorylation in many receptors including ER and PPAR γ and this may result in activation or repression of its transcriptional activity (152, 155).

The DBD or the C-region has the most conserved amino acid sequence among the members of the NR superfamily and is required for the recognition and binding of specific target sequences on DNA. The DBD has two highly conserved zinc-finger motifs spanning ~60-70 amino acids: C-X₂-C-X₁₃-C-X₂-C and C-X₅-C-X₉-C-X₂-C that are common to the entire family with the exception of two divergent members: DAX-1 and SHP (301, 381). In addition, the DBD has a COOH-terminal extension (CTE) that contains the so-called T and A boxes critical for specificity and polarity of NRs in DNA binding (134, 214).

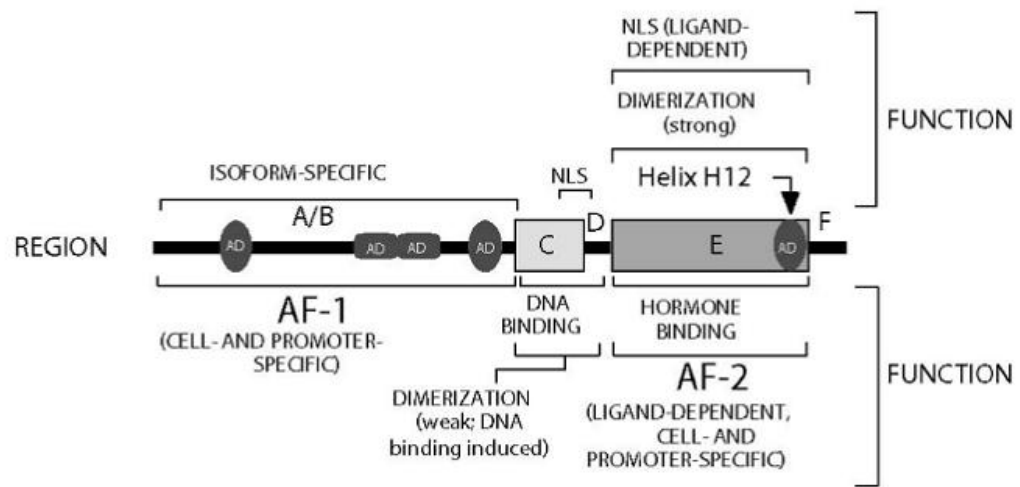


Figure 1-4 Structural and functional organization of NRs (114).

Each zinc-finger contains four highly conserved cysteine molecules that coordinate the binding of a zinc atom. Amino acids required for sequence specificity in DNA binding are present at the base of the N-terminal finger in a region termed the “P box”, and residues of second zinc finger that form the so-called “D box” are involved in dimerization (227, 382). The core DBD contains two α -helices: the first one is known as the recognition helix and binds the major groove of DNA making contacts with specific bases; the second helix spans the COOH terminus of the second zinc finger and forms a right angle with the recognition helix (17). The DBD may also contain a nuclear localization and nuclear export signals (31, 133).

The D domain or hinge region of nuclear receptors is variable in length and amino acid sequence. Its flexibility can provide DBD rotation along the LBD by 180°. This is important for the interaction of receptor dimers with asymmetric hormone response elements (HREs) representing direct repeats and HREs representing inverted repeats. This region also forms a surface for interaction of receptors with coregulators (264) and it may contain a nuclear localization signal (310).

The LBD or the E domain is moderately conserved among members of the NR superfamily. It contains an additional transactivation domain, AF-2, which is strictly ligand dependent and is a target for interaction with several coactivator and corepressor complexes (23, 209, 230). The crystal structure of the LBD has been determined for several nuclear receptors and has provided insights

regarding the mechanisms involved in ligand binding and transactivation (39). The overall structure of the LBD is similar for several NRs and is composed 12 helices, H1-H12, arranged together in an antiparallel, three-layered sandwich which may include two to four β -strands. Helices H1-H11 form the hydrophobic ligand-binding pocket whose entrance is guarded by H12 (40, 348). Agonist ligand binding induces a conformational change in many NRs resulting in alternate positioning of H12. This promotes recruitment of coactivators that interact with their short LXXLL-like motifs (where L is leucine and X is any amino acid) called NR-boxes. LXXLL-like motifs are present in many coactivators and are common motifs required for interacting with the LBD of NRs. The residues of the ligand-dependent AF-2 are located in H12 (74, 209). The structural data, together with transcriptional activation data, imply that the positioning of helix 12 is crucial for receptor activation. In addition the LBD also contains nuclear localization signals, a dimerization domain, and in some cases, repression domains (124, 153, 207).

The F domain is not found in all receptors and this domain may be involved in additional discrimination between receptor agonists and antagonists. For example, the F domain of ER α is essential for E2-dependent gene transactivation through ER α /Sp1 pathway, but F domain of ER α is not essential SERM-mediated activation of ER α /Sp1 in breast cancer cells (163).

1.2.3 NR-mediated Transaction

NRs regulate transcription by binding to specific DNA sequences in target genes known as response elements (REs). These elements are located in regulatory sequences normally present in the 5'-flanking region of target genes. Although REs are often found relatively close to the core promoter, in some cases they are present in enhancer regions several kilobases upstream of the transcriptional initiation site. The analysis of a large number of naturally occurring as well as synthetic REs revealed that a sequence of 6 base pairs of DNA sequence constitutes the core recognition motif, also referred to as "half-sites". Although some monomeric receptors bind to a single hexameric motif, most receptors bind as homo- or heterodimers to REs composed typically of two core hexameric motifs. For dimeric REs, the half-sites can be configured as palindromes (Pal), inverted palindromes (IPs), or direct repeats (DRs). Diversity among REs is also achieved by the varying number of neutral base pairs separating the half-site repeats. This is the key identity factor contributing to the binding specificity of different retinoid X receptor (RXR) heterodimer pairs. It provides the geometry that is needed for two subunits to interact specifically. The insertion of even one extra base pair in the inter-half-site spacing displaces the interacting subunits by nearly 3.4 Å and re-orient them by ~35°. This leads to the disruption of supportive protein-protein and protein-DNA interactions (268). Sequence composition of the spacer nucleotides has been shown to play a less critical role in the recognition of REs (268, 386). According to the inter-half-site

spacing, these elements are systematic named as shown in Table 1-2. (204, 347, 386).

Table 1-2 Space rules for nuclear receptor response elements (255).

Spacer NTs	Systematic name	Acronym	Receptor Complex
1	DR1	RARE, PPARE	RXR-RXR, PPAR-RXR, RAR-RXR,
2	DR2	RARE	RAR-RXR
3	DR3	VDRE	RXR-VDR
4	DR4	TRE	RXR-TR
5	DR5	RARE	RXR-RAR

RXR: retinoid X receptor, **PPAR**: peroxisome proliferator–activated receptor, **RAR**: retinoic acid receptor, **VDR**: vitamin D receptor, **TR**: thyroid hormone receptor; **RARE**: retinoic acid receptor response element, **PPARE**: peroxisome proliferator–activated receptor response element, **VDRE**: vitamin D receptor response element, **TRE**: thyroid hormone receptor response element.

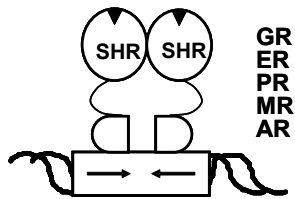
The NR superfamily can be broadly divided into four classes based on their dimerization and DNA binding properties as shown in Figure 1-5 (205, 322). Class I receptors include the known steroid hormone receptors, which function as ligand induced homodimers and bind to DNA half-sites organized as inverted repeats. Steroid hormone receptors typically bind to palindromes containing AGAACA sequence separated by three nucleotides, with the exception of the estrogen receptor that recognize the consensus AGGTCA motif with the same configuration.

Class II receptors such as thyroid hormone receptor (TR), retinoic acid receptor (RAR), and vitamin D receptor (VDR), heterodimerize with RXR and characteristically bind direct repeats. The classic retinoic acid response element (RARE) which was found in the RAR β 2 gene promoter is a 5 bp-spaced direct repeat (DR5) containing the AGTTCA motif. In addition, response elements with a DR5 containing the AGGTCA motif also act as RAREs as well as direct AGGTCA repeats spaced by 1 bp (DR1) or 2 bp (DR2) (76, 127). RAR-RXR heterodimers bind to, and activate transcription from these three RAREs, provided target cells express both RARs and RXRs. Only a few natural vitamin D response elements (VDREs) are known; several of them contain DR3 elements.

Studies with “optimized” synthetic response elements assembled from AGGTCA motifs have confirmed that DR3 elements bind VDR-RXR heterodimers, and that the cognate ligands, vitamin D, and 9C-RA, activate the corresponding promoters (78, 122). The thyroid hormone response element (TRE) consensus sequence is AGGTCA and TR α binds both AGGTCA and AGGACA motifs (63, 64). TRs have a strong preference for DR4, nevertheless, TRs bind other direct repeats including DR5, DR2, or DR0 (44, 121). TRs can also bind to inverted palindromes with a preferred spacing of six nucleotides (102).

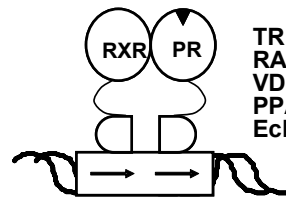
Class III receptors such as hepatocyte nuclear factor 4 (HNF4), chicken ovalbumin upstream promoter-transcription factors (COUP-TFs), RXR, Germ-cell nuclear factor (GCNF), testicular receptors 2 (TR2), and Tailless homolog (TLX) bind primarily to direct repeats as homodimers. Class IV receptors such as steroidogenic factor 1 (SF-1), Rev-erb, estrogen-receptor-related receptor (ERR), Nerve growth factor induced protein I-B (NGFI-B) and RAR related orphan receptor (ROR) typically bind to extended core sites as monomers. Most of the orphan receptors fall into class III and IV categories.

**Class I
Steroid Hormone Receptors**



GR
ER
PR
MR
AR

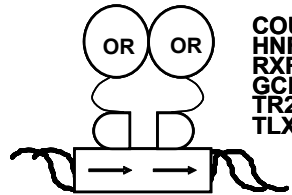
**Class II
RXR Heterodimers**



TR
RAR
VDR
PPARs
EcR

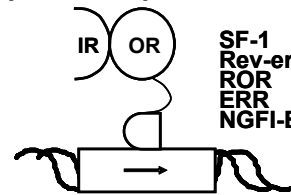
FXR
CAR
PXR/SXR
LXR

**Class III
Dimeric Orphan Receptors**



COUP
HNF4
RXR
GCNF
TR2
TLX

**Class IV
Monomeric/Tethered
Orphan Receptors**



SF-1
Rev-erb
ROR
ERR
NGFI-B

Figure 1-5 Modes of action of NRs (205).

1.2.4 Subclasses of Nuclear Receptor by its Ligands

Lipophilic molecules that bind and activate nuclear receptors are referred to generically as “ligands” for nuclear receptors. Unlike polypeptide hormones that function via cell surface receptors, ligands for nuclear receptors are not directly encoded in the genome. All nuclear receptor ligands are small (molecular weight < 1000 daltons [d]) and lipophilic, enabling them to enter cells. Another common feature of nuclear receptor ligands is that all are derived from dietary, environmental, and metabolic precursors. In this sense, the function of these ligands and their receptors is to translate cues from the external and internal environments into changes in gene expression. Their critical role in maintaining homeostasis in multicellular organisms is highlighted by the fact that nuclear receptors are found in all vertebrates as well as insects but not in single-cell organisms such as yeast (98). NRs have been divided into three categories based on their function and source of ligand (Table 1-3) (3). The "Endocrine"

class of NRs binds endocrine-derived ligands and includes the steroid hormone, retinoic acid, thyroid hormone, and vitamin D receptors. The "Adopted Orphan Receptors" bind dietary lipid-derived ligands and regulate lipid metabolism. "Orphan Receptors" are the third category of NRs and the identity of endogenous and exogenous ligands for orphan receptors are unknown or poorly defined.

Ligand-dependent activation of NRs can be variable; however, receptor ligands typically induce formation of a DNA-bound homodimer/heterodimer which subsequently recruits other nuclear coactivator and coregulatory proteins. This complex of nuclear factors associated with NRs is required for association with the basal transcription machinery and subsequent activation of gene expression. This process is highly complex and may involve different classes of coactivators/corepressors and other proteins which modify chromatin structure through acetylation or methylation (histone acetyltransferases and methyltransferases). We are going to further discuss the mechanisms of transcriptional regulation by NRs in the next section.

Table 1-3 Nuclear receptor ligands and their receptors (3).

Endocrine Endocrine lipld sensors		Adapted Orphan Dietary & endogenous lipid sensors		Orphan Endogenous ligands uncertain	
GR	glucocorticoids	RXR	9-cis RA, DHA	ERR	synthetc steroids
MR	mineralocorticoids	PPAR	fatty acids	HNF-4	fatty acids?
PR	progesterones	LXR	oxysterols	ROR	fatty acids, sterols?
AR	androgens	FXR	bile acids	SF-1	phospholipids?
ER	estrogens	PAR	xenobiotics	LRH-1	phospholipids?
		CAR	xenobiotics	GCNF	?
RAR	retinoic acids			PNR	?
TR	thyroid hormones			TLX	?
VDR	vitamin D, LCA			TR2,4	?
				NGFI-B	?
				COUP-TF	?
				RVR	?
				DAX-1	?
				SHP	?

1.3 Mechanism of Transcriptional Regulation by NRs

1.3.1 Chromatin Structure and Gene Expression

The genomic DNA in all eukaryotic cells is condensed and packaged by histone and nonhistone proteins into a dynamic ordered structure termed chromatin. The basic unit of chromatin is the nucleosome which contains approximately 146 base pairs (bp) of DNA wrapped in a lefthanded superhelix around an octamer of core histone proteins containing two molecules each of the following histones: H2A, H2B, H3, and H4 (201). Each core histone has a hydrophilic amino-terminal tail containing specific sites for post-translational modifications. In addition to core histones, linker histones (H1) can serve to lock the incoming and outgoing DNA helix to the outside of the core histone octamer, further stabilizing the nucleosome particle as shown in Figure 1-6.

In the genome, each nucleosome is separated by a stretch of linker DNA varying in length from 10 to 60 bp. This form of DNA packaging is considered the primary functional unit of chromatin. In vivo, arrays of nucleosomes are packaged into canonical '30-nm' fibers and then further condensed into a higher level of chromatin structure characterized by 80- to 100-nm chromonema fibers (132). Specific nucleosome-nucleosome interactions are essential for the condensation of nucleosome arrays into higher ordered chromatin structures (120). The packaging of genomic DNA into higher ordered chromatin presents an obstacle

for regulated gene expression by presumably restricting access of RNA polymerase II and of the basal transcription machinery (131).

One of the predominant mechanisms used by NRs to activate or repress target-gene transcription is the recruitment of coregulatory factors capable of covalently modifying the amino terminal ends of histones. These modifications, including acetylation and deacetylation, methylation, and in some cases phosphorylation, are thought to alter chromatin structure and facilitate the subsequent recruitment of other effector proteins. Proteins such as coactivators which possess specific enzyme activities such as histone acetyltransferases (HATs) and histone/protein methyltransferases (HMTs) are recruited by NRs in the presence of cognate ligand where they facilitate activation of target genes. In contrast, corepressor complexes containing SMRT / NCoR proteins can associate with NRs in the absence of ligand, or in the presence of specific antagonists, and facilitate transcriptional repression of target genes. Importantly, NR corepressor complexes contain HDAC activity that apparently reverses the effects of HAT action mediated by NR coactivators. A proposed model for the interaction of coactivators and corepressors with NRs is summarized in Figure 1-7.

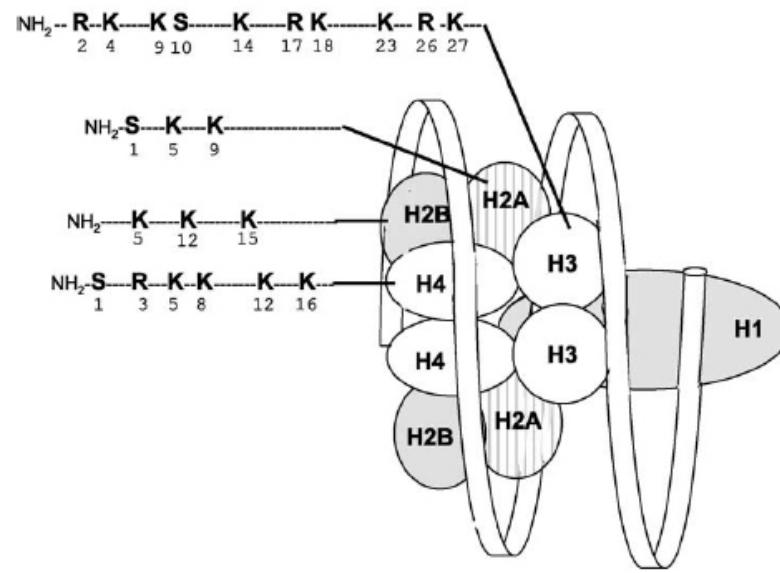


Figure 1-6 Structure and assembly of the nucleosome (340)

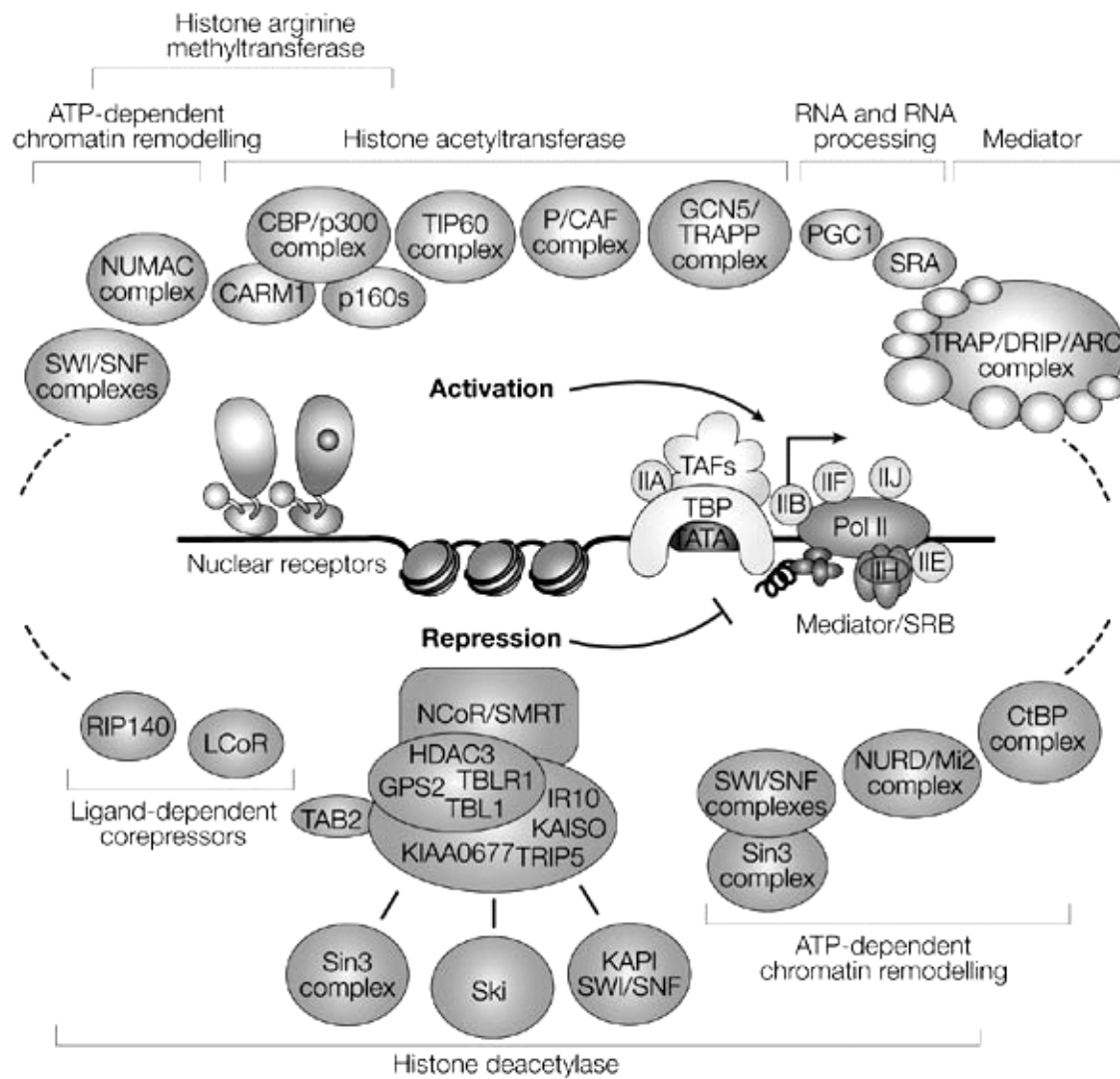


Figure 1-7 Coactivator and corepressor complexes for regulation of nuclear receptor-mediated transcription (250).

1.3.2 NR Mediated Gene Activation

Transcriptional activation by NRs involves recruitment of distinct classes of coactivators and other transcription related factors to promoters in the chromatin environment of the nucleus (Fig. 1.7). NRs may use several mechanisms to increase transcription of specific NR-dependent genes. First, NRs may directly interact with and recruit general transcription factors that are components of the preinitiation complex (PIC) to increase the rate of initiation of transcription. Second, the receptors may interact with proteins which can alter chromatin structure and render the promoter DNA of the target gene more accessible to various transcription factors and to RNA polymerase II. Third, the receptor may interact with other cellular components that act to bridge interactions with members of the PIC to promote formation of a transcriptional-active complex.

1.3.2.1 NR and Transcription Preinitiation Complex (PIC) Formation

The initiation of mRNA synthesis by RNA pol II involves the direct or indirect binding of core promoter DNA elements such as the TATA box, DPE (downstream promoter element) and the Inr (initiator) by a collection of “basal” transcription factors (TFs) (45, 238). The binding of ligand-activated NRs to DNA response elements in the promoter or regulatory regions of a hormone-responsive gene stimulates the assembly of a stable basal factor/RNA Pol II transcription PIC at the promoter, with recognition of the TATA box and

other core promoter elements by a complex called TFIID (81). The role of liganded NRs in promoting the formation of a stable PIC is 2-fold: (i) promoting PIC assembly through direct contacts with components of the basal transcription machinery (including TFIIB and TFIID) and (ii) recruiting coactivators, which in turn facilitate promoter PIC assembly through direct contacts with components of the basal transcription machinery and by loosening chromatin structure at the promoter (176, 350).

TFIID is a complex of proteins containing the TATA-binding protein (TBP) and a collection of 10 to 12 polypeptides called TBP-associated factors (TAFs) (13). The TAFs in the TFIID complex are required for transcriptional activation by a number of different DNA-binding activators, including NRs. Several TAFs in TFIID, as well as TBP itself, make direct contacts with NRs as a part of the transcriptional regulatory process (211). For example, hTAFII30 in TFIID binds to ER α , an interaction critical for ER α -dependent transcription. Such interactions can help recruit or stabilize binding of TFIID at the promoter, a process that is enhanced by the interactions of some TAFs to the core promoter elements (13, 146). The role of TAFs in NR-dependent transcription is illustrated by the fact that TFIID, but not TBP alone, can act synergistically with other cofactor complexes, such as Mediator and SWI/SNF, to potentiate transcription by NRs. Together, the available data indicate that TAFs are required for full transcriptional activation by NRs (13, 202).

1.3.2.2 NR Interaction with Coactivators

Modulation of the assembly of preinitiation complexes by transcription factors involves not only direct actions but also indirect actions on components of the basal transcriptional machinery. When NRs are bound to their target promoters, like other transcription factors, they recruit coregulatory proteins termed coactivators or corepressors that activate or inhibit transcription. NR coregulators, interact with different NRs domains through their specific NR-interacting motifs such as LxxLL or the FxxLF. Many coregulators are most likely recruited at the promoter as part of preformed complexes (81, 212). When present on target gene promoters, transcriptional coregulators play different roles depending on their specific enzymatic activities (e.g., kinase, acetyl- or methyltransferase, or ubiquitin- or sumo-ligase activities) or due to their ability to recruit other regulatory proteins. Certain coregulators play a crucial role in remodeling chromatin structure by modifying histone tails and/or by promoting nucleosome remodeling, which in turn facilitates the access of other proteins to the promoter. Finally, transcriptional coregulators recruit and stabilize the basal transcriptional machinery at the promoter, including RNA polymerase II (pol II), leading to the formation of the transcriptional preinitiation and initiation complexes as shown in figure 1-7. Since their discovery in the mid-1990s, the number of transcriptional coregulators has rapidly increased to more than 150. The major group of coactivators enhances NR-dependent transcription by modification of the chromatin environment and alleviation of the repressive

effects of histone–DNA contacts. Coactivators in this group can be divided into two general classes: members of the switch/sucrose nonfermentable (SWI/SNF) family of proteins and members of the histone acetyltransferase (HAT) family.

ATP-dependent Chromatin Remodelers

The packaging of genomic DNA into nucleosomes restricts the receptor-dependent assembly of transcription complexes at the promoters of hormone regulated genes. Unlike many DNA-binding transcriptional regulators, NRs bind stably and with relatively high affinity to DNA even when their cognate HREs are assembled into chromatin (350). Thus, the relevant issue seems to be how receptors promote formation of an open chromatin architecture at the promoter. One way is through the ligand-dependent recruitment of chromatin remodeling complexes, which are multi-polypeptide enzymes categorized by the type of ATPase subunit that they contain, including yeast Snf2-like (e.g. SWI/SNF) or *Drosophila* ISWI-like (e.g. RSF, CHRAC, ACF) (223). Human SWI/SNF (hSWI/ SNF) represents a family of related complexes usually containing eight or nine subunits, with either hBrg1 or hBrm as the ySnf2-related ATPase subunit; however, the exact composition of these complexes can vary among cell types (164). Chromatin remodeling complexes use the energy stored in ATP to mobilize or structurally alter nucleosomes, allowing for greater access of the transcriptional machinery to promoter DNA, thus facilitating transcriptional activation (164, 278, 350).

The involvement of SWI – SNF complexes in NR dependent transcription was originally suggested by studies in yeast and mammalian cells which show a stimulatory effect of SWI – SNF components on NR-dependent activity (47, 377). In addition, cell-based approaches have also supported these results, including experiments showing a requirement for hBrg1-receptor interactions in estrogen receptor and glucocorticoid receptor gene regulatory activity and chromatin immunoprecipitation (ChIP) experiments showed the recruitment of hBrg1 to an estrogen-regulated promoter upon hormonal stimulation (84, 109).

ATP-dependent chromatin remodeling is required for NR-dependent transcription but it is not sufficient. Chromatin remodeling may set the stage for subsequent actions by coactivators with histone modifying activities, such as HATs (82, 192).

HAT-dependent Chromatin Remodeling

Histone acetyltransferase coactivators were identified initially on the basis of their interaction with the ligand binding domains of a variety of nuclear receptors in the presence of cognate receptor ligands, and subsequent studies showed these coactivators exhibited HAT activity (318). The most well characterized group of HAT coactivators is the p160 family, which contains multiple members that share a striking homology. These common structural features are represented by steroid receptor coactivator family (SRC-1a) in figure 1-8A. The SRC (steroid receptor coactivator) family is composed of three distinct

but structurally and functionally related members, which are named SRC-1 (NcoA-1), SRC-2 (TIF2/GRIP1/NcoA-2), and SRC-3 (p/CIP/RAC3/ACTR/AIB1/TRAM-1), respectively (211). Sequence analysis of SRC proteins has identified a basic helix-loop-helix (bHLH) and two Per-Arnt-Sim (PAS) domains in the amino-terminal region, a centrally located receptor-interacting domain (RID) and a C-terminal transcriptional activation domain (AD). The bHLH/PAS domain is highly conserved among the SRC members and it serves as a DNA binding and protein dimerization motif for interacting with many transcription factors. Detailed analysis revealed three conserved LXXLL motifs (NR box) in the RID, which appear to contribute to the specificity of coactivator-receptor interactions. HAT activity was identified in the C-terminal region of SRC members and there are also activation domains that interact with the CREB-binding protein (CBP). Members of the SRC family interact with steroid receptors, ER, PR and AR, and enhance their transcriptional activation in a ligand-dependent manner (184, 211).

p300 and CBP are highly related HATs that interact with SRC-1, but also bind independently to nuclear hormone receptors in a ligand-dependent manner as shown in Figure 1-8 B (54). CREB-binding protein (CBP) was initially characterized as a coactivator required for efficient transactivation of cAMP-response element-binding protein. p300 was first identified as a coactivator of the adenovirus E1A oncoprotein. CBP and p300 share many functional properties and protein functions as coactivators for multiple NRs as

well as p53 and NF- κ B; both possess intrinsic HAT activity and recruit HAT and p/CAF (CBP/p300-associated factor) (211). CBP/p300 also interacts with SRC family members and synergizes with SRC-1 in transactivation of ER and PR (314). This shows that a very large multicomponent HAT complex may be assembled in the vicinity of a ligand-bound receptor.

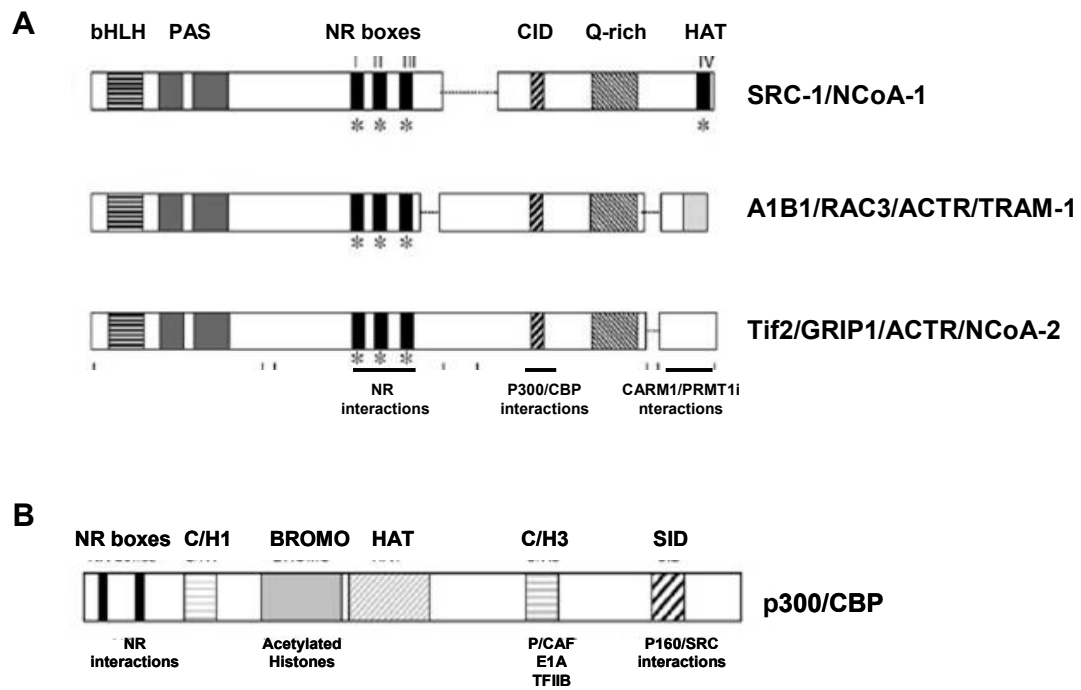


Figure 1-8 Functional domains of the p160/SRC family and p300/CBP (340).

The link between histone acetylation and transcriptional activation is well-established; however, the mechanism of histone acetylation-dependent activation of transcription is unclear. Although histone acetylation was initially thought to facilitate chromatin remodeling by loosening the association of the histone octamer with DNA through the neutralization of positive charges in the histone tails, more recent studies suggest that histone acetylation may require prior chromatin remodeling or may occur at a post-remodeling step (82, 192, 349). The results of one study suggest that post-remodeling histone acetylation by p300 may direct the transfer of histone H2A–H2B dimers from nucleosomes to a histone chaperone (144). Such an effect may help to establish and maintain an open chromatin configuration that favors transcription. The differences observed in different experimental systems for the order of chromatin remodeling and HAT activity have not been adequately explained, but may represent promoter-specific types of regulation (349). Recent results suggest another role for histone acetylation, namely to create binding sites on the amino-terminal tails of core histones for acetylated lysine binding domains, such as the bromodomain. A mechanism like this may allow for the recruitment of bromodomain-containing factors (e.g. the HAT TAFII250) to promoters that have nucleosomal histones with specific patterns of acetylation (148). Although HAT activity is critical for NR-dependent transcription, it is important to note that coactivators such as PCAF (233) and p300/CBP contribute other activities to the transcription process. For example, p300/CBP interacts with RNA pol II complexes (226) and possess a

glutamine-rich C-terminal region similar to the glutamine-rich activation domains found in some transcriptional activators, suggesting that p300/CBP may also function as classical coactivator by interacting with RNA pol II (175). Furthermore, both PCAF and p300/CBP can acetylate nonhistone, transcription-related factors, which in many cases alters the activity of those factors (174). For example, the acetylation of SRC3 by p300 disrupted the receptor–coactivator complex and decreased receptor-mediated gene activation (62). Estrogen receptor alpha is a target for p300-mediated acetylation, which may alter the transcriptional activity of the receptor (354). Thus some HATs, such as p300/CBP and PCAF, serve as multifunctional coactivators for NR-dependent transcription, contributing multiple activities to this process.

HMT-dependent Chromatin Remodeling

Recent studies indicate that proteins which have HMTs activity are also potential coactivators. Two PRMT (protein arginine methyltransferase) family members, CARM1 (coactivator-associated arginine methyltransferase) and PRMT1, that interact with the carboxyl-terminal region of SRC2 , enhanced nuclear receptor mediated transcriptional activation (61, 170). More recent studies have shown that the intrinsic methyltransferase activities of CARM1 and PRMT1 are required for enhancement activity. CARM1 methylates arginine in the tail of histone 3 and functions as a molecular switch that regulates the decision to express either genes induced by ligand-activated nuclear receptors or those activated by CREB transcription factor (300). CARM1 not only methylates H3 but

also an arginine residue in a domain of p300/CBP required for interaction with CREB, thus, inactivating the transcriptional activity of CREB. In this regard, CARM1 not only functions as a coactivator for nuclear receptor-mediated transcription but at the same time acts as a corepressor for CREB-mediated transcription (222, 373).

Mediator Complexes

Interactions between DNA-bound NRs and the RNA pol II transcriptional machinery help to promote formation of stable transcription PICs at the promoter. The multiprotein mediator complexes also known as the TRAP and DRIP complexes, are another class of NR-and RNA pol II-interacting co-activators. At least two individual subunits of Mediator can interact directly with NRs. Med220 binds to NR ligand-binding domains in a ligand-dependent manner via a receptor interaction domain that contains two NR boxes. This subunit is responsible for the association of the entire Mediator complex with a variety of NRs in vitro and is probably responsible for the recruitment of mediator complex to the promoters of NR-regulated genes. For example, Warnmark and co-workers showed that TRAP220 interacted preferentially with ER β compared to ER α and interactions were dependent on the two LXXLL NR box motifs (NR1 and NR2) in TRAP220 (356). However, recently Wu and co-workers in this laboratory showed that in ZR-75 breast cancer cells coactivation of ER α by DRIP205 involves multiple regions of DRIP205 and ER α , and interaction of these proteins do not require the NR box motifs of DRIP205 (368).

New coactivators are continually being discovered and these include some unexpected molecules such as steroid-receptor-RNA activator-1 (SRA1), an RNA transcript, that functions as a eukaryotic transcriptional coactivator for steroid hormone receptors (184). An actin-binding protein (mACTN2) not only serves as a coactivator for the androgen, estrogen and thyroid hormone receptors, but also acts synergistically with GRIP1 to enhance NR-mediated (139). In summary, it is clear from coactivator studies that transcriptional regulation cannot be considered solely as a chromatin-based process, but should be considered as a process that is coupled to many other cellular events that are carried out by several distinct groups of proteins and enzymatic activities.

1.3.3 NR Mediated Gene Repression

It is now well recognized that gene repression or gene silencing is as important as gene activation. Nuclear receptors represent a large family of ligand-regulated transcription factors and although DNA binding of steroid hormone receptors is ligand-dependent, other nuclear receptors are bound to DNA in the absence of their cognate ligand. For example, unliganded NRs such as thyroid hormone receptor (TR), retinoic acid receptor (RAR) and most orphan receptors are located in nucleus and are bound to their response elements. These unliganded DNA-bound receptors actively repress transcription of target genes by recruitment of co-repressors. Gene repression by NRs is an important and crucial function *in vivo* since aberrant silencing leads to disease and developmental abnormalities. This repression “turns off” target genes and

amplifies the magnitude of the subsequent activation by hormone or ligand. For example, if the level of gene transcription in the repressed state is 10% of basal activity levels in the absence of receptor, a 10-fold hormone-dependent induction of this gene above basal levels represents an overall 100-fold increase in expression compared to the repressed genes (136).

The ligand-dependent switch between the repressed and activated receptor conformations explains how hormones activate gene expression. However, many hormone-repressive target genes can be down-regulated by receptors after treatment with hormone. This is referred to as ligand-dependent negative regulation of transcription, or transrepression, and is different from the repression of basal transcription by unliganded receptors. The mechanism of negative regulation is not well understood. One mechanism involves nuclear receptor binding to DNA binding sites that reverse the paradigm of ligand-dependent activation (negative response elements), where the ligand-bound receptors recruit corepressors and HDAC activity to these binding sites (296).

1.3.3.1 NR Interaction with Corepressors

Various corepressors for NRs have also been identified. Corepressors such as silencing mediator of retinoid and thyroid hormone receptors (SMRT), nuclear receptor corepressor (NCoR) interact with NRs in a ligand-dependent manner and ligand binding leads to their dissociation from the receptors.

However, some corepressors such as LcoR (ligand-dependent nuclear-receptor corepressor), RIP140 (receptor-interacting protein-140) and REA (repressor of estrogen receptor activity) (77, 365), bind nuclear receptors in a ligand-dependent manner and competitively displace coactivators. These observations indicate the existence of specific regulatory mechanisms that use similar, but reverse, approaches for attenuating the function of agonist-bound receptors.

SMRT, NCoR

The SMRT and NCoR are related transcriptional corepressors isolated by virtue of their interaction with RAR and TR (65, 130). SMRT and NCoR bind unliganded TR or RAR, and their interactions are disrupted after binding of TR or RAR to their respective ligands. Subsequent studies show that SMRT and NCoR also interact with other NRs, including VDR, PPAR δ , and LXR, and with orphan NRs, such as Rev-ErbA, COUP-TF, ROR α , and DAX (212). SMRT or NCoR also interact with steroid hormone receptors, including ER, AR, and PR (86, 193), and this only takes place when steroid hormone receptors bind their corresponding antagonists. For example, tamoxifen, a known ER antagonist/agonist used for breast cancer treatment, enhances interactions between ER and NCoR. The interaction between SMRT or NCoR and NRs is dictated by two NR-interacting motifs located at the C-terminal ends of both proteins, with a consensus sequence of L/IXXI/VI, named the CoRNR motif (135). Sequence comparison of SMRT and NCoR indicates that they share a conserved domain called the

SANT domain (4). The N-terminal SANT domain of SMRT and NCoR is involved in associating with histone deacetylase 3 (HDAC3) and is required for activating its deacetylase activity (116, 383). The SANT domain is involved in histone binding. This observation was later confirmed by a study on the SANT2 domain of SMRT (378) which showed that the SANT2 domain can directly bind histones. Interestingly, SANT2 of SMRT prefers to bind unacetylated histones over acetylated forms; however, this property was not observed for the SANT1 domain of SMRT, indicating that each of these two SANT domains encodes distinctive properties. The preferential binding of the SMRT SANT2 domain to unacetylated histone tails suggests that it can block the binding of HATs to histones and it appears that the two SANT domains of SMRT and NCoR can synergize with each other to promote and maintain histone deacetylation.

Both SMRT and NCoR complexes are estimated to be 1.5–3 MDa in size (117, 191), suggesting that SMRT and NCoR associate with multiple protein components. Biochemical purification and characterization of these SMRT and NCoR associating proteins have identified HDAC3, transducing beta-like protein 1 (TBL1), and TBL1 related protein (TBLR1) as common components (117, 191, 378). Further characterization of TBL1 and TBLR1, which are related WD40 repeat proteins, has revealed their selective affinity for histones H2B and H4 (378), and this histone-binding activity is important for their transcriptional repression activity. These results reveal that TBL, TBLR1, and HDAC3 are

integral components of the SMRT and NCoR complexes and are critical for the transcriptional repression of NRs.

RIP140 and LCoR

Although agonist binding to NRs is primarily associated with recruitment of coactivators, recent studies show that agonist binding also results in binding of corepressor such as RIP140 and LCoR (52, 104). RIP140 was first identified by its interaction with TR, RAR, RXR, and PPAR as a corepressor in a ligand-dependent manner by using the GAL4 reporter system (186, 339). Interactions between RIP140 and NRs is mediated through a unique motif at the C-terminal region of the protein (148), although the constitutive binding appears to be mediated through repeated LXXLL motifs (101). Mutations in the NR-interacting motif in RIP140 decrease its ability to suppress an RA-responsive reporter gene, suggesting that RIP140 indeed functions as an NR corepressor. Moreover, RIP140 suppresses RA receptor-mediated induction by RA in a dose-dependent manner (148). Transcriptional repression by RIP140 has also been attributed to its interaction with HDAC1, HDAC3, and CtBP (352, 364).

LCoR is another NR corepressor that was first isolated as a ligand-dependent interacting factor of ER α LBD in a yeast two-hybrid screening (104). Interactions of LCoR with ER in yeast and in mammalian cell lines takes place in an E2-dependent manner and there is evidence that LCoR interacts with other nuclear receptors, including GR, PR, and VDR, in a ligand binding-dependent fashion. Furthermore, LCoR also interacts selectively with

HDAC3 and HDAC6 but not with HDAC1 or HDAC4. The interactions between RIP140 or LCoR and NRs are mediated through motifs similar to the LXXLL motif and it has been postulated that in addition to the active role of RIP140 and LCoR as gene repressors, they also compete with coactivators for binding the hydrophobic pocket in the LBD. This competitive property of RIP140 and LCoR is ligand-dependent. The combination of both properties could be the key reason for the rapid attenuation of transcription immediately after agonist-induced transactivation.

1.3.4 From Repression to Activation

The possibility of switching gene expression from 'off' to 'on' and vice versa in mammalian system includes several regulatory strategies that cooperate to impose precise control of gene expression.

1.3.4.1 Allosteric Regulation of NR Activity

Ligand binding is the crucial molecular event that switches the function of nuclear receptors from active repression to transcriptional activation for the heterodimeric receptors such as RAR or TR that are constitutively bound to DNA. The hormone binding induces a conformational change in the ligand-binding domain of the receptor, which results in reduced affinity for corepressors and, enhanced affinity for coactivators. Similarly, agonist binding to steroid receptors, such as ER, progesterone receptor (PR), GR or AR, also induces a specific conformation that favors coactivator binding, whereas antagonist binding

promotes the interaction with corepressors. However, some recent studies show that there are additional molecular events that also modulate the nuclear receptor switch from repression to activation.

1.3.4.2 Turnover and Transcription

The degradation of transcriptional activators such as NRs is often required for gene activation. The cyclic turnover of some NRs on regulated promoters correlates with proteasome-dependent degradation activity, chaperone activity and chromatin remodeling events (108, 225, 276). The significance of an association between transcriptional activation and proteolysis of the activator is unclear and is somehow counterintuitive, since one might expect the removal of activators to correlate with the negative control of gene transcription. However, the cyclic clearance of nuclear receptors may be crucial, because it allows a continuous reassessment of the 'state of the cell' — each cycle would overcome the default of transcriptional repression only if the activating stimulus was still present (291). However, there are also examples where proteasome inhibition is reported to enhance transcriptional activation, and this indicates that the role of protein degradation in transcriptional regulation could be cell, nuclear-receptor and even promoter specific (37, 100).

Reid et al. have reported that proteasome-mediated degradation and hER α -mediated transactivation are inherently linked and act to continuously turn over hER α on responsive promoters (276). In contrast, Fan et al. showed that the

proteasome-dependent degradation is not essential for ER transcriptional activity (100). In HeLa cells transfected with ER α , blocking either ubiquitination or proteasomal degradation markedly increased E2-induced expression of an ER-responsive reporter. In MCF-7 breast cancer cells, proteasome inhibition enhanced E2-induced expression of pS2 and cathepsin D, but decreased progesterone receptor (PR) expression. The results further indicate that promoter context must be considered when evaluating the relationship between ER α transcription and proteasome inhibition. In addition to ER, PPAR α , GR, RAR γ , RXR α , and TR are also regulated by the ubiquitin–proteasome system (38, 80). Ubiquitination of PPAR α was decreased in the presence of ligand, providing a mechanism for the ligand-dependent stabilization (32).

Recruitment of the ubiquitylation machinery and proteasome-dependent degradation of the coregulators is also required for transcriptional activation. In the case of the NCoR-containing corepressor complex, the 26S proteasome components are involved in promoting the release of the corepressors in response to ligand binding (249). TBL1 and TBLR1, two NCoEx (nuclear corepressor exchange factors) factors, are components of the NCoR and SMRT corepressor complexes and are required for the repression of specific transcription units (191, 335). TBL1/TBLR1 also serve as specific adaptors for the recruitment of the ubiquitin conjugating/20S proteasome complex to mediate exchange of NR corepressors for coactivators upon ligand binding. This implies that signals that promote gene induction must turn on parallel pathways to

activate the exchange machinery and release the repression checkpoint. Therefore, activation of the NCoEx factors could represent a control, which is imposed to maintain more robust transcriptional repression and to avoid undesirable gene expression. This level of regulation would increase the amplitude of transcriptional activation events by imposing a repression checkpoint. Furthermore, periodic cycles of NRs are important for continuous assessment of the hormonal state of the cell, then re-establishment of a repression checkpoint at each clearance phase could also provide a tighter control on gene activation. This means that the ubiquitylation and the release of the corepressors would be crucial, not only during the first activation step, but at each cycle of receptor assembly on the promoter.

1.3.4.3 Nuclear Integration of Signaling Pathways

NRs respond not only to hormonal stimulation, but they can also integrate information derived from a large variety of external stimuli. Several signaling pathways activated by various developmental or physiological signals exhibit crosstalk with nuclear receptor-mediated responses through both direct and indirect mechanisms. The transcriptional activity of nuclear receptors is modulated by the induction of post-translational modification of the receptor itself or of its coregulatory proteins. Phosphorylation, acetylation, sumoylation, ubiquitylation and methylation are among the modifications that modulate the functions of nuclear receptors and that potentially constitute an important cellular integration mechanism. It has been suggested that these modifications influence

cellular localization, enzymatic activity and stability of targeted proteins, and could also be important in modulating the timing of sequential recruitment of the different classes of coregulators to a single transcription unit. The transcriptional coactivators CBP and p300 are involved in numerous transcriptional events mediated by different *trans*-acting transcription factors. Both CBP and p300 are phosphoproteins and their phosphorylation status is under cell-cycle control (374). For example, p300 is phosphorylated by CDC2 and CDK2 kinases and negatively regulated by cyclin E–CDK2 (22). An interesting possibility is that the enzymatic activities of CBP and p300 are directly modulated as a result of the phosphorylation events that occur during cell-cycle progression. This was suggested by Ait-Si-Ali *et al.*, who reported that general HAT activity peaked during the G1/S transition, and that the HAT activity of CBP was enhanced by the C-terminal phosphorylation mediated by cyclin E–CDK2 (12, 165).

Other kinases, which include PKA, Ca²⁺/calmodulin-dependent kinase IV (CaMKIV) and MAPK, phosphorylate different CBP residues, thereby enhancing its transcriptional-activation activity (59, 147, 372). For example, phosphorylation by p44 MAPK has a positive effect on the enzymatic activity of CBP (11). In contrast, p300 phosphorylation by PKC represses transcriptional activity (379), which is consistent with the opposing activities of CBP and p300 on proliferation and the response to DNA damage.

Phosphorylation is not the only modification that is used to integrate signalling pathways and coactivator functions. For example, CARM1-dependent

methylation of a specific domain of CBP (the KIX domain) which interacts with the kinase-interacting domain (KID) of CREB, is important for inducing the dissociation of CBP from CREB, and for inhibiting CREB-dependent transcriptional activation (373). Furthermore, p300 is also ubiquitinated and degraded by the ubiquitin–proteasome pathway during F9 embryonal carcinoma cell differentiation. Interestingly, p300 shows different phosphorylation patterns in undifferentiated versus differentiated cells, and the changes in phosphorylation status that are promoted by PKA affect its HAT activity only during differentiation (42, 145).

Post-translational modifications of corepressors have also been reported. A recent paper showed that the direct phosphorylation of SMRT by IKK α is required for NF- κ B mediated transcription (126). The phosphorylation on SMRT resulted in the dissociation from HDAC3 and nuclear export of SMRT. Failure of IKK α to stimulate this response inhibits the recruitment of NF- κ B to promoters, blocking transcription and sensitizing cells to apoptosis (249). This is consistent with the observation that the ubiquitin-dependent dismissal and degradation of corepressors is required for the switch from gene repression to gene activation by nuclear receptors. Furthermore several kinases, including MAPKs, AKT/protein kinase B and casein kinase-2, modify NCoR and SMRT and to induce their relocalization from the nucleus to the cytoplasm (19, 129, 150).

1.4 Mechanism of ER Mediated Transcription Activity

1.4.1 Introduction

Estrogens are steroid hormones that regulate growth, differentiation, and function in a broad range of target tissues in the human body. The most potent and dominant estrogen in humans is 17β -estradiol, but lower levels of estrone and estriol are also present. The biological effects of estrogens as shown in Figure 1-9 are mediated through ER α and β , which are members of a large superfamily of nuclear receptors. These receptors act as ligand-activated transcription factors. The classical genomic mechanism of ER action involves estrogen binding to receptors in the nucleus, after which the receptors dimerize and bind to specific response elements known as estrogen responsive elements (EREs) located in the promoters of target genes (229). However, around one third of ER regulated genes in humans that do not contain ERE-like sequences (232), and the molecular mechanisms of transcriptional regulation by ER at alternative response elements are being extensively investigated. E2 induced transactivation of some genes involved ER-protein interactions where ER does not directly bind DNA but activates another DNA-bound transcription factor (113). This mechanism can be referred to as non-classical genomic pathway.

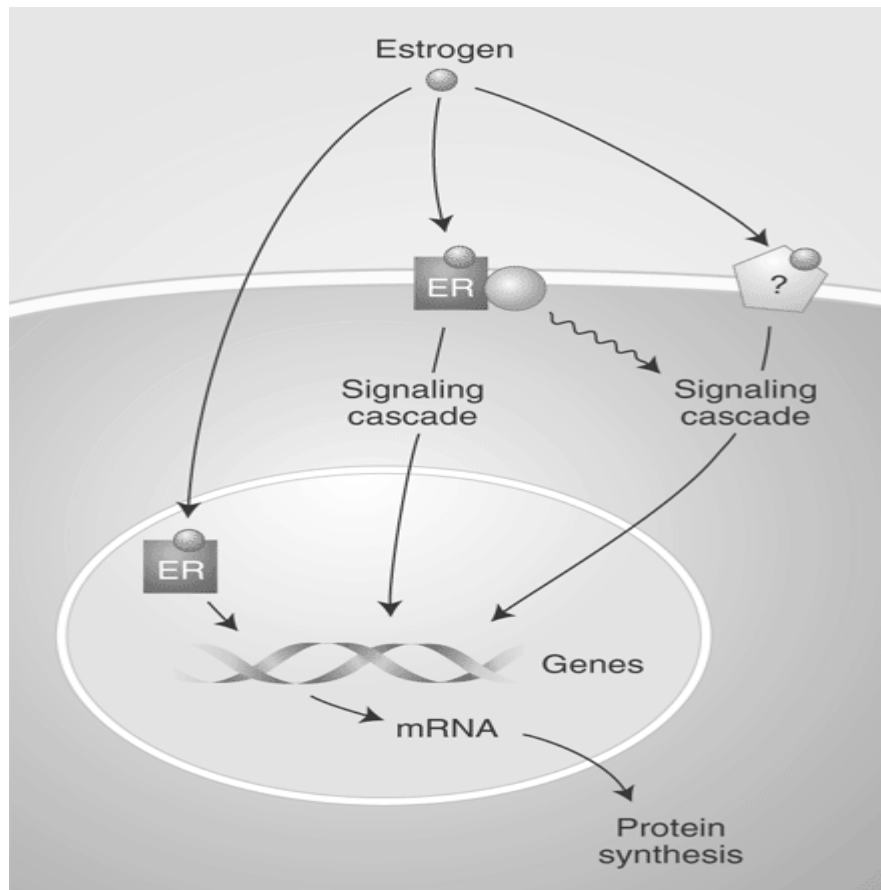


Figure 1-9 Models of estrogen action (125)

There are rapid biochemical and physiological responses that occur within seconds or minutes after estrogen administration that cannot be accounted for by changes in gene expression mediated by nuclear ER. These are known non-genomic actions of E2 and are believed to be mediated through membrane-associated ER. Non-genomic pathways are associated with activation of various protein-kinase cascades which indirectly influence gene expression, through phosphorylation of other transcription factors such as AP-1, serum response factor (SRF), and Elk-1 (75, 88, 154, 173).

1.4.2 Overview of Estrogen Receptor Structure

The biological effects of E2 are mediated by the ER and until 1996 it was assumed that a single ER was responsible for mediating the effects of E2, anti-estrogens and other selective ER modulators (SERMs). However, a second ER, designated as ER β , has been identified (the former ER is called ER α). And this has initiated an extensive reevaluation of the comparative functions of ER α and ER β in normal physiology and in diseases including cancer (179).

ER α and ER β are encoded by separate genes and belong to the steroid/thyroid hormone superfamily of nuclear receptors (118). The structures of the two ERs are compared in Figure 1-10.

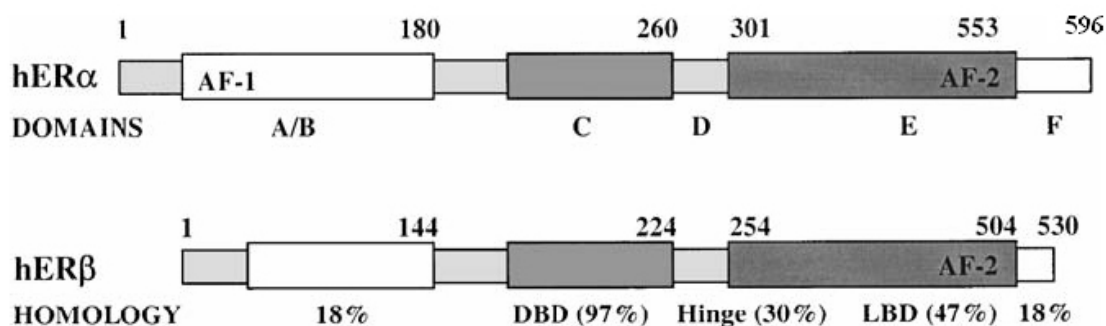


Figure 1-10 The structure homology between ER α and ER β (171)

Like other NRs, both ER subtypes consist of six defined structural domains. There is considerable variability in the A/B, hinge (D), and F domains of ER α and ER β . However, the DNA binding domains (DBDs) of ER α and ER β are highly homologous (97) and thus ER α and ER β bind various EREs with similar affinities. The ligand-binding domain (LBD) of ER α and ER β also share a high degree of homology and it is not surprising that many compounds bind both receptors with similar affinities (178) or have similar potencies in activation of ERE-reporter gene expression (24). However, the two receptors have distinctly different tissue distribution and levels in normal tissues and in tumors (71, 190). These data together with the different phenotypes of the ER α and ER β knock-out mice support the idea that the two ERs are not merely redundant but have distinct roles in estrogen and SERMs signaling (33).

The ER contains two distinct transcriptional activation domains; activation function 1 (AF1) at the N-terminal and activation function 2 (AF2) at the carboxy

terminal. The N-terminal A/B domain is the least conserved region with only 17% identity between human ER α and ER β (97). The N-terminal domain encodes a ligand-independent transactivation function (AF1) a region involved in protein-protein interactions and transcriptional activation of target gene expression. AF1 interacts with multiple proteins, including the p160 steroid receptor coactivator-1 (SRC1) and p300 (210, 236), general transcription factor TBP (362) and other coactivator proteins such as p68 RNA helicase (96), MMS19 (369) and RNA coactivator SRA (357). AF1 is also responsible for ligand-independent activation of ER through several different kinase signaling pathways (168). Serine 118 is the target for mitogen-activated protein kinase (MAPK)-dependent phosphorylation in response to growth factors (14, 149). Serines 104 and 106 are phosphorylated by the cyclin A-CDK2 complex (279).

The ER LBD is a wedge-shaped structure that consists of 12 α helices. The LBD forms the pocket for ligand binding, homo- and hetero-dimerization and a binding surface for coactivators and corepressors. The ligand-binding pocket is guarded by helix-12 (H12), which forms a movable lid over the pocket and contains residues that are crucial for AF2 function. Crystallographic analysis of the ER α LBD has established that ligand binding has a dramatic effect on receptor structure. Agonists such as 17 β -estradiol and diethylstilbestrol induce a receptor conformation in which the H12 is aligned over the hormone binding cavity, resulting in the formation of a specific binding site for coactivators (43, 305). Coactivators recruited to the AF2 of ER α contain a distinctive common

signature motif termed an NR-box, comprising the core consensus sequence LXXLL where L is leucine and X is any amino acid. This interaction site on ER α is a shallow, hydrophobic groove that is formed by residues from H3, H4, H5 and H12. The LXXLL motif functions as a hydrophobic docking module in a helical conformation and all the three leucines of the motif make contacts with the groove which is stabilized by a charge clamp. Introduction of mutations in either partner abrogates the interactions (305). The agonist-induced conformational change of the LBD is also necessary for the nuclear receptor transactivation function.

SERMs such as raloxifene and tamoxifen, bind across the cavity in a similar manner to agonists. However, their bulky side chains cannot be fully accommodated within the binding cavity. Instead, the side chains protrude from the binding cavity, resulting in the displacement of H12. This repositioning of H12 in the LBD disrupts interactions between the hydrophobic grooves in the LBD with coactivators (43, 305).

The crystal structures of ER β isoform bound to genistein, a partial agonist for ER β , and raloxifene, a pure antagonist for ER β , have also been determined (257). Genistein, an isoflavonoid phytoestrogen, displays 7-30 fold higher affinity for ER β over ER α . The orientation of H12 in genistein-bound ER β LBD is in a partially occupied antagonistic position compared to an agonist, and this explains the partial agonistic activity of genistein. However, in raloxifene-bound to the LBD

of ER β , the piperidine ring of the ligand protrudes from the cavity and prevents H12 from adopting its agonist position. This feature is responsible for pure antagonistic properties of raloxifene on ER β . The structure of the ER β LBD complexed with a pure antagonist, ICI 164,384, has been determined; and shows that binding of this antagonist completely destabilizes H12 and prevents it from adopting either agonist or SERM orientation (258).

1.4.3 Membrane-associated ER

The existence and function of membrane-associated ER (mER) was first reported in 1977 (256) and over the last 10 years there has been renewed interest on the structure and function of mER. There is evidence showing that the membrane and nuclear receptors are the same protein and this is based on immunohistochemistry staining of membrane ER, using a panel of antibodies directed against multiple epitopes of the nuclear ER (241). In addition, GH₃/B6/F10 rat pituitary tumor cells transfected with an antisense oligonucleotide to nuclear ER resulted in loss of mER (231). Also in ER-negative Chinese hamster ovary (CHO) cells, transfection with cDNAs of ER α or ER β resulted in a single transcript, specific binding activity of labeled E2, and expression of ER in both nuclear and membrane fractions (272). However, the definitive proof that endogenous membrane and nuclear ER are the same protein which requires separate isolation and sequencing of the two receptor pools has not been carried out. In contrast to most membrane receptors, ER has no intrinsic transmembrane

domain, and there is evidence that a third party protein such as Shc, p85 α of phosphatidylinositol 3-kinase (PI3K), caveolin and insulin-like growth factor-1 receptor (IGF-1R) maybe required for linking the cytoplasmic ER pool to the inner face of the plasma membrane (218, 271, 316).

Shc, an adapter protein, has no intrinsic kinase domain and mainly transduces signals dependent on protein–protein interactions (269). Three domains on Shc mediating protein–protein interactions are the phosphotyrosine binding (PTB) domain in the amino-terminal region, the Src homology 2 (SH2) domain in the carboxy-terminal region and a proline rich region called the collagen homology (CH) domain (246). When a receptor like IGF-1R is activated, Shc binds rapidly to IGF-1R through its PTB domains, leading to Shc itself being phosphorylated by receptor tyrosine kinase (247). The phosphorylated tyrosine residues on the PTB domain of Shc provide the docking sites for binding the SH2 domain of Grb2 (Growth factor receptor binding 2) and this complex recruits SoS (Son of Sevenless), a guanine nucleotide exchange protein (282), leading to activation of the Ras/Raf/MAPK pathway (35, 247). It has been reported that E2 rapidly induced Shc phosphorylation as well as Shc interaction with ER α in MCF-7 and long-term estrogen-deprived (LTED) MCF-7 cells. The N-terminal A/B domain of ER α was required and sufficient for interactions with the PTB and SH2 domains of Shc, although the full-length receptor was required for E2-mediated response in MCF-7 cells (317). Recently it is also shown that Shc, ER α and IGF-1R form a ternary complex in MCF-7 cells and down-regulation of

any of these proteins by small inhibitor RNAs (siRNAs) abrogates E2-induced activation of the MAPK pathway (316). These data suggested that Shc serves as a translocator for ER α by binding to the receptor and then carrying it to the Shc-binding sites of IGF1-R that are located on the cell membrane and transduce estrogen signals for activation of MAPK pathway.

The p85 α subunit of PI3K is also an adapter protein. The p85 α contains an N-terminal SH3 and a C-terminal SH2 domain separated by an N-terminal SH2 domain (183). p85 α can associate with IGF-1R directly or indirectly by binding IRS-1, a substrate of IGF-1R (16, 376), leading to the activation of the p110 catalytic subunit. It has been reported that ER α but not ER β interacts directly with the P85 α , and this association is required for E2-induced activation of PI3K pathway in endothelial and breast cancer cells (50, 311). Recently, the adaptor protein p130Cas was shown to transiently interact with ER α in a multi-molecular complex containing the c-Src kinase and the p85 α in T47D breast cancer cells (46). Transient overexpression of p130Cas in T47D cells increases E2-dependent Src kinase and ERK1/2 MAPK activities. Furthermore, downregulation of p130Cas by siRNA was sufficient to inhibit E2-induced ERK1/2 MAPK activity and cyclin D1 induction, suggesting that the adaptor protein p130Cas associates with the ER α transducing complex, regulating E2-dependent activation of c-Src kinase and downstream signaling pathways.

The cellular tyrosine kinase c-Src is involved in intracellular signaling and cell proliferation initiated by both growth factors and steroids. Several reports

have shown that c-Src kinase activity is required for E2-induced stimulation of MAPK and PI3K activity in breast cancer and bone cells (46, 50, 317).

Microinjection of kinase-dead c-Src into breast cancer cells (MCF-7 and T47D) prevented stimulation of cell proliferation by either E2 or progestins (48). In addition, the essential role for c-Src in E2-dependent activation of MAPK pathway was supported in studies using embryonic fibroblasts derived from Src^{-/-} mice, which failed to support rapid activation of the MAPK pathway in response to E2, whereas wild-type c-Src^{+/+} cells did (172). ER α has been shown to interact with the SH2-domain of c-Src (21, 36, 218), and the phosphorylated tyrosine at position 537 in the LBD of ER α is required for this interaction (25). It has also been reported that moderator of nongenomic activity of ER (MNAR) which is identified from a breast cancer cell library (367) mediated or stabilized the interactions between ER and c-Src. MNAR associates with ligand-bound ER α and ER β through LXXLL motifs, and binds to the c-Src SH3 domain via a proline-rich region (25). It is suggested that MNAR brings the ER into proximity with c-Src and helps enhance activation of c-Src by providing more effective interaction of ER and c-Src relieving c-Src inhibition through binding to the SH3 domain.

In endothelial cells, as in other cell types, ER has been found in caveolae where they activate endothelial nitric oxide synthase (eNOS) through protein kinase-mediated phosphorylation (56, 57). Caveolae are specialized membrane invaginations enriched in the scaffold protein caveolin-1. Caveolae facilitate

signal transduction by providing a subcellular location for various signaling molecules (302). It has been reported that Serine 522 of ER α is necessary for the physical interactions with N-terminal scaffolding domain of caveolin-1 protein (270). Marino and colleagues (6, 7) have shown that cysteine 447 a residue which is crucial to steroid-independent palmitoylation of the receptor is also important for association with the caveolin-1. Mutation of this single amino acid or inhibition of palmitoylation with 2-bromo-palmitate results in a significantly decreased expression of membrane receptors, compared to wild-type ER expression. Furthermore, cysteine 447-mutated ER α does not support E2-induced proliferative signaling through ERK and PI3K (6).

Some motifs in the E domain of ER α are also critical for membrane localization and function and this includes residues necessary for dimerization of the endogenous membrane ER α and ER β (55, 275). Mutation of these motifs prevents both receptor dimerization and E2-dependent signaling through ERK, PI3K, and cAMP in breast cancer cells (275). In contrast, eNOS activation in COS cells transfected with ER may not require membrane ER dimerization (55). Very recently, it was reported that elements within the nuclear localization sequence (NLS) of ER α (D domain) were required for E2-induced activation of ERK and PI3K and nitric oxide production through eNOS activation in transfected COS cells (385).

1.4.4 Genomic ER Activity

1.4.4.1 Classical Genomic ER Activity

The classical mechanism of ER action involves estrogen binding to receptors in the nucleus, after which the receptors dimerize and bind to specific response elements known as estrogen response elements (EREs; GGTCANNNTGACC) located in the promoters of target genes as shown in Figure 1-11 (229). This consensus sequence was first identified in the vitellogenin genes from xenopus and chicken (166). Hormone binding also induces a conformational change within the ligand binding domain of ER, and this conformational change allows coactivator proteins to be recruited (280). This leads to alteration of chromatin, histone unwinding, interactions with components of the basal transcription machinery complex, and subsequent mRNA expression.

1.4.4.2 Non-Classical Genomic ER Activity

A number of studies have shown that ER can regulate transcription without binding directly to DNA. The receptors in such cases are tethered through protein-protein interactions to a transcription factor complex that contacts the DNA as shown in Figure 1-11. Through this mechanism, ER regulates expression of a large number of estrogen-responsive genes that do not contain EREs. This mechanism is also used by other members of the nuclear receptor

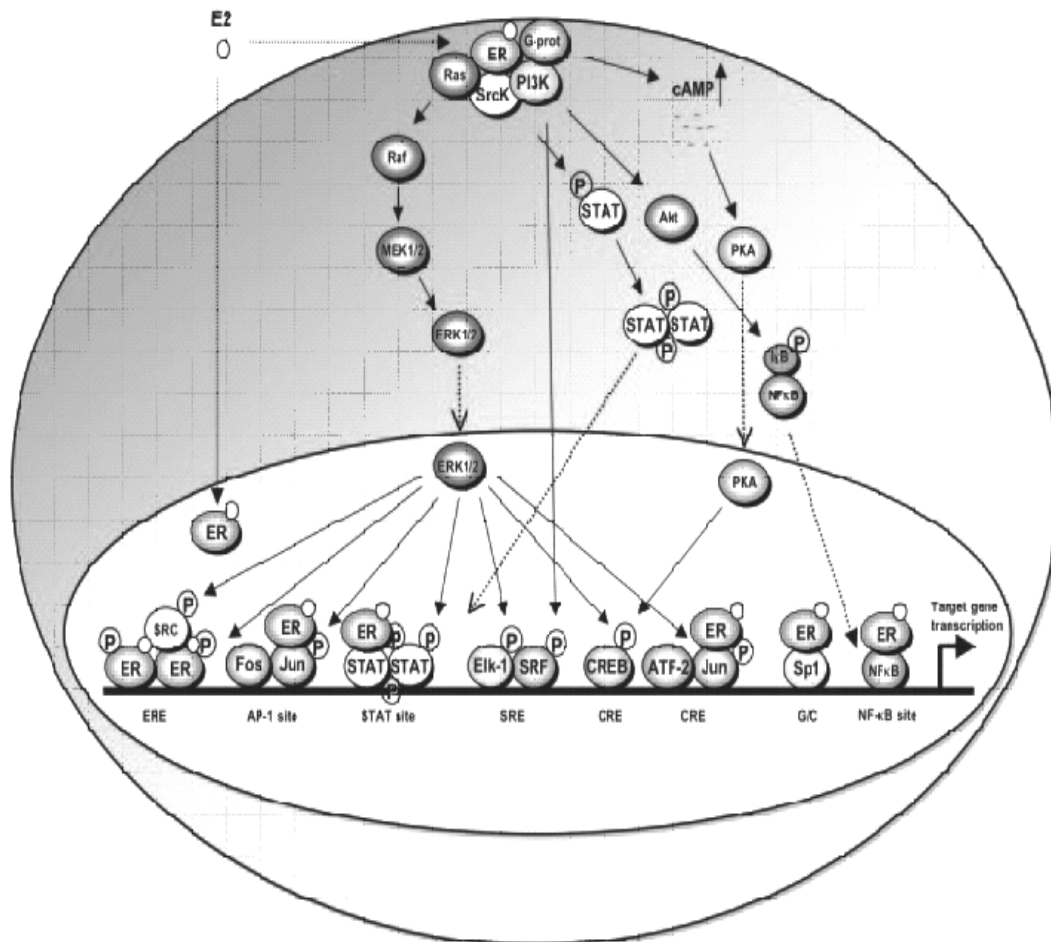


Figure 1-11 Genomic and nongenomic actions of ER on a target gene promoter (30).

superfamily and is often referred to as transcriptional cross talk (113). ER/AP-1 and ER/Sp1 are two major pathways of ER nonclassical genomic actions.

ER/AP-1 Pathway

An E2-responsive AP-1 element was initially identified in the proximal promoter of the ovalbumin gene (336) and other E2-responsive AP-1 elements have been identified in the collagenase, insulin-like growth factor 1, quinone reductase, and cyclin D1 gene promoters (111, 345, 346). Fos and Jun family proteins bind AP-1 elements as homo- or heterodimers. These proteins contain leucine zipper domain that mediates DNA binding and are typically associated with genes that rapidly responded to various extracellular stimuli (303).

Mechanistic studies of ER/AP-1 actions have shown that the requirement for ER structural domains is dependent on the receptor subtype and on ligand structure. For example, E2-dependent activation of ER α /AP-1 complexes requires the AF-2 domain of the receptor, which binds p160 coactivators and stabilizes formation of a multiprotein complex containing c-Jun, ER α , and transcriptional coactivators at the promoter. However, the ER DBD is required for tamoxifen-activated ER α /AP-1 dependent activity (361). Furthermore, ICI, 182,780, an inhibitor of ER dimerization and ERE binding, activates an AP-1 reporter construct (254). Interestingly, full length ER α containing mutations in AF1 also compromised E2-mediated AP-1 activity, indicating that ER α /AP-1 action requires both AF1 and AF2 (363).

ER β also activates transcription from an AP-1 element. However, the effects of estrogen and antiestrogen on ER β /AP-1 contrasts to those observed for ER α /AP-1. E2, ICI, 162,576, tamoxifen, and raloxifene all activate an AP-1 reporter construct in cells cotransfected with ER α whereas, in the presence of ER β , E2 not only acts as antagonist but also inhibits the activity of tamoxifen and raloxifene dependent induction of ER β /AP-1. However, either tamoxifen or raloxifene alone behave as full agonists (240).

ER/Sp1 Pathway

E2-responsive GC-rich elements were initially identified in the c-myc gene promoter (91). This site contains a nonconsensus ERE-half site (ERE $\frac{1}{2}$) and a Sp1 binding site that was required for estrogen-mediated induction. Similar ERE $\frac{1}{2}$ /Sp1 elements have been subsequently characterized in the cathepsin D (177), heat shock protein 27 (Hsp27) (262), TGF α (353), prothymosin α (208), and human PR A (253), gene promoters. However, mutation of the ERE $\frac{1}{2}$ in the Hsp27 promoter did not result in the loss of E2 responsiveness, and the E2-dependent ER α /Sp1 action is still observed in cells transfected with a DBD deletion ER α mutant. The data suggested that GC-rich site alone was sufficient for E2-responsiveness and ER α binding to DNA was not required. The ERE-independent ER α /Sp1 action has also been observed for several genes including retinoic acid receptor α (327), c-Fos (87), insulin-like growth

factor-binding protein-4 (266), bcl-2 (85), adenosine deaminase (371), thymidylate synthase (370), cyclin D1 (51), cad (161), E2F-1 (228).

Although both ER α and ER β form complexes with Sp1 protein, only ER α induces consensus Sp1 element-linked reporter gene activity whereas ER β , exhibits minimal or decreased the basal reporter gene activity and these responses are ligand- and cell type-specific. Interestingly, it was recently reported that both ER α and ER β regulate EGF receptor gene expression through GC-rich elements and, depending on ligand, ER β exerts full agonist activity on this promoter, indicating that promoter context is also an important factor in ER β /Sp1 action (292).

ER α /Sp1 protein-protein interactions were investigated in vitro using GST pull-down assays, which showed interaction between the C-terminal end of Sp1 and multiple regions of ER α (261). Additionally, it has been shown using a series of ER α deletion mutants and ER α /ER β chimeric mutants that the AF1 domain of ER α is critical for ER α /Sp1-mediated transactivation. Recent studies indicated that E2-dependent activation of ER α /Sp1 also required the C-terminal F domain of ER α , which was not required for antiestrogen activation of ER α /Sp1 and overexpression of a C-terminal F domain peptide (aa 575-595) specifically blocked E2-mediated ER α /Sp1 transactivation, suggesting that other nuclear cofactors interacting with the F domain may be important for ER α /Sp1 action (163).

ER α not only interacts with Sp1 but also with Sp3 protein, another member of Sp protein family that can also act as transcriptional repressor. It was reported that vascular endothelial growth factor (VEGF) gene expression is regulated by ER α /Sp1 or ER α /Sp3 either positively or negatively and Sp1/Sp3 ratios maybe critical for VEGF gene regulation. By using Sp protein deficient SL2 cells, upregulation of the VEGF promoter activity with E2 treatment was observed in cells cotransfected with ER α and Sp1 expression plasmid whereas downregulation of the same promoter activity was observed when cells were cotransfected with ER α and Sp3 expression plasmid (320, 321).

1.4.5 Nongenomic ER Activity

Evidence is accumulating that estrogens exert nongenomic actions that are too rapid to be accounted for by the activation of RNA and protein synthesis. Nongenomic actions are a common property of steroid hormones and are frequently associated with the activation of various protein-kinase cascades (198).

The nongenomic mechanism of estrogen action can be grouped into two types as shown in Figure 1-9 and 1-11. First, some models propose that the rapid membrane events are mediated by the classical ER α , which initiates signaling cascades by associating with membrane structures including G proteins, caveolins, and receptor tyrosine kinases. In the second type of model, the ER α is

not involved and another membrane-associated estrogen-binding protein is believed to mediate the response to estrogen.

1.4.5.1 ER-mediated Nongenomic Action

Mitogen Activated Protein Kinase (MAPK)

Mitogen activated protein kinases (MAPKs) are important enzymes in signal transduction and are highly conserved among eukaryotes. In mammalian cells, MAPKs include the extracellular signal-regulated kinase (ERK) 1/2, the p38 kinase and the stress-activated protein kinase (SAPK) or c-Jun NH₂-terminal kinase (JNK) cascades (58, 245). ERK primarily responds to mitogenic signals, while JNK and p38 are predominantly activated by stress signals.

Although MAPKs are a diverse group of kinases, they share an evolutionarily conserved model of activation, which consists of the sequential phosphorylation of three kinases. The MAPK kinase kinase (MAPKKK/MEKK) phosphorylates the serine/threonine residues on MAPK kinase (MAPKK/MEK). The phosphorylated MAPKK in turn activates MAPK through phosphorylation (58). A hallmark of MAPK is a dual-phosphorylation Thr-X-Tyr motif in the activation loop and both threonine and tyrosine phosphorylation are required for the full activity of MAPK (181). MAP kinase phosphatase (MKP) can dephosphorylate the threonine/tyrosine and thereby attenuate MAPK-dependent responses (106).

It has been reported that exposure to estrogens leads to rapid activation of the ERK 1/2 module in various cell types. In nerve cells, membrane-impermeable E2 rapidly induces ERK 1/2 activation, and results in c-Fos-dependent activation of immediate early genes (359). It has also been found that in MCF-7 breast cancer cells, E2 activates ERK1/2 in an ER-dependent manner (88); however, the upstream component involved in E2-dependent activation of this pathway is still unclear. Recently a study showed that in MCF-7 breast cancer cells, E2-induced ERK activation is mediated by a heregulin/human epidermal growth factor receptor-2 (ErbB2)/PKC δ /Ras pathway. In this model, HRG (Heregulin) is synthesized and secreted into extracellular environment upon E2 stimulation. HRG binds to ErbB2 resulting in activation of PKC δ , which in turn activates Ras and initiates downstream MAPK signaling (157). As mentioned in section 1.4.3, interactions between ER α , shc, and IGF-IR are also involved in E2-dependent activation of MAPK in MCF-7 cells.

The p38 kinase is activated by estrogen in endothelial cells (273). In this cell type, E2 rapidly activates p38, leading to MAPKAP-2 kinase activation and phosphorylation of Hsp27. Through this pathway, E2 preserves stress fiber formation, and actin and membrane integrity. Moreover, E2-induced p38 activation prevents hypoxia-induced apoptosis, and induces the migration of endothelial cells and the formation of primitive capillary tubes (273).

In contrast to the other MAPK modules, E2 inhibits the JNK activity in breast cancer cells. In MCF-7 and ZR-75 cells, paclitaxel (taxol) or UV irradiation

induce apoptosis through activation of JNK. However, E2 inhibits taxol- or UV-stimulated JNK activity, therefore abrogating Bcl-2 and Bcl-xl phosphorylation and caspase activation (274). These molecular events may play a role in E2-dependent prevention of chemotherapy or radiation induced apoptosis in breast cancer cells.

Phosphatidylinositol 3-Kinase (PI3K)

ER α physically and functionally interacts with the regulatory subunit (p85 α) of the lipid kinase PI3K (see section 1.4.3), and triggers activation of the catalytic subunit and increased intracellular production of phosphoinositides (311). PI3K phosphorylates the D-3 position of the phosphatidylinositol ring, catalyzing the synthesis of lipid mediators that act as second messengers transferring the signaling cascade to intracellular protein kinases. One of the principal targets of this cascade is the serine-threonine protein kinase Akt/protein kinase B. Activation of Akt mediates many of the downstream cellular effects of PI3K triggered by E2. For example, in vascular endothelial cells, E2 induces eNOS in an ER α -dependent manner via the AKT pathway (311). Activation of PI3K by estrogens is also important in breast cancer cells, where E2 rapidly triggers association of ER α with Src and p85 (50). This ternary complex probably favors hormone activation of Src- and PI3K-dependent pathways, which converge on cell cycle progression (50). There is evidence that PI3K activation by estrogen can also occur in the absence of ER. For example, estrogen activates PI3K in ER-negative MDA-MB-435 and MDA-MB-231 breast cancer cell lines and this

activation can be inhibited by Src kinase inhibitor PP2 but not by antiestrogen ICI 182,780, suggesting that ER-independent pathway exists for PI3K activation (341).

Other Signaling Pathways

Protein kinase A (PKA) plays a regulatory role in mammary tumorigenesis. Cholera toxin (CT), a PKA activator, induces breast cancer cell growth in vitro and in vivo (304) and constitutive activation of PKA is associated with increased tamoxifen resistance in breast cancer cells (217). PKA can prevent ubiquitin-proteasome-dependent ER α degradation induced by the ligand-binding (342).

Estrogen rapidly induces cAMP levels and subsequently activates PKA in breast cancer and uterine cells through activation of adenylyl cyclase (18); however, the mechanism of estrogen-induced adenylyl cyclase is still unknown. In rat pulmonary vascular smooth muscle cells, calcium removal blocked induction of cAMP by E2, suggesting that the intracellular calcium may have an important role.

Although protein kinase C (PKC) has been identified as a target of nongenomic actions of E2, little is known about the mechanism (156, 330). Both G-protein inhibitor GDP β S and phospholipase C inhibitor U73122 block E2-induced PKC activity, suggesting that this process is dependent on G proteins as well as phospholipase C (330). Estrogen causes a rapid increase of

intracellular calcium levels (221), and presumably this process leads to the activation of calcium calmodulin-dependent kinases (CaMKs). Qin and coworkers reported that in MCF-7 breast cancer cells, E2 stimulates CaMKIV activity but not CaMKII (265), suggesting a certain specificity in E2-dependent activation of calcium signaling.

1.4.5.2 ER-independent Activation of Non-genomic Pathway by Estrogens

Several reports suggest that of E2 activation of G proteins by E2 is mediated directly through an orphan G protein-coupled receptor, GPR30. A recent report showed that E2 bound with high affinity to membranes of SKBR3 breast cancer cells, which lack ER α and ER β but express GPR30, and this resulted in activation of cAMP-dependent responses (334). Membranes from human embryonic kidney cells (HEK293 cells), which lack ER α , ER β and GPR30, regain E2-binding activity when cells stably transfected with GPR30. GPR30 binding is selective for E2 and the ER antagonists tamoxifen and ICI 182,780 (105). However, it was not determined whether E2-GPR30 interactions contribute to the overall E2-dependent signaling or the associated downstream responses in breast cancer cells.

In contrast, Revankar et al. identified GPR30 in the endoplasmic reticulum of COS7 monkey kidney cells expressing GPR30 linked to a fluorescent marker, as well as expression of endogenous GPR30 in several other cell lines (277). However, the process of how newly synthesized GPCRs exit the endoplasmic

reticulum and move to the plasma membrane was not fully characterized. GPCR processing in the endoplasmic reticulum is considered critical for eventual signaling activity and E2-initiated G protein signaling in the endoplasmic reticulum would be a unique mechanism to explain some of the physiological effects associated with E2 (199).

1.5 Research Objectives

1.5.1 Objective 1

Treatment of mammalian cells with mitogens, cytokines and differentiation inducing agents is accompanied by alterations in expression of multiple genes that play integral roles in mediating cell-specific responses (325, 326). For example, immediate-early genes such as *c-fos* are rapidly induced (0.3 - 2 hr) in several mammalian cell lines after treatment with mitogens. *c-fos* protein is a nuclear transcription factor that forms part of the activating protein-1 (AP-1)

complex and regulates AP-1-dependent gene expression. The early growth response-1 (Egr-1) gene is also a member of the immediate-early gene group of transcription factors and at least 4 Egr genes have been identified. Egr-1 protein contains a highly conserved DNA-binding domain composed of three zinc fingers, and binds GC-rich promoter DNA sequences (66, 355). Egr-1 modulates transcription of multiple genes and the overall genomic and cellular responses to Egr-1 are complex and dependent on both promoter- and cell-context.

In prostate cancer, Egr-1 plays a role in cancer progression. Expression levels of Egr-1 mRNA and protein are much higher in prostate adenocarcinoma compared to levels in normal prostate tissue (332). Moreover, levels of Egr-1 protein expression correlate with Gleason scores and inversely correlate with the degree of differentiation of carcinoma cells (95, 332). Abdulkadir and co-workers used the transgenic mouse model to study the function of Egr-1 over-expression in prostate tumors *in vivo*. Their data showed that tumor formation was significantly delayed in Egr-1 deficient mice, but tumor initiation and tumor growth rates were not affected by loss of Egr-1(5). Baron and co-workers using Egr-1 antisense oligonucleotides and successfully inhibited transformation of prostate cancer cells (26). These results indicated a unique role for Egr-1 in regulating the transition from localized carcinoma *in situ* to invasive carcinoma. Egr-1 is induced in some but not all cancer cell lines after treatment with serum, ultraviolet light or phorbol esters, and there were differences in Egr-1 inducibility even among ER-positive MCF-7 (inducible), ZR-75 and T47D breast cancer cell lines (137).

Egr-1, like several other immediate-early genes is induced by E2 in the rodent uterus and in MCF-7 breast cancer cells (67, 329). Pratt and coworkers reported that E2 activates Egr-1 expression in MCF-7 cells (263), and this is accompanied by rapid autophosphorylation of raf-1 suggesting that hormonal regulation of Egr-1 may involve rapid non-genomic pathways of estrogen action which have been extensively described in multiple cell types (189, 358). The -600 to +12 region of the Egr-1 promoter contains several potential E2-responsive motifs including a distal GC-rich motif that could be activated by nuclear ER α /Sp1, and multiple SREs (SRE1-4) and cAMP response element (CRE) that can be hormonally activated through non-genomic pathways. The first objective of this study was to investigate the molecular mechanism of E2-dependent activation of Egr-1 in MCF-7 breast cancer cells and identify which *cis*-response elements and *trans*-acting factors in the Egr-1 promoter are required for E2 induced transactivation.

1.5.2 Objective 2

Chicken ovalbumin upstream promoter transcription factors (COUP-TFs) are orphan receptors and members of the nuclear receptor superfamily. Two genes called COUP-TFI (also termed EAR3) and COUP-TFII (also termed ARP-1) have been identified in mammals. These receptors are closely related transcription factors that are widely expressed and are involved in the regulation of several important biological processes, such as neurogenesis, organogenesis,

cell fate determination, and metabolic homeostasis (242). Both genes show an exceptional homology and overlapping expression patterns, suggesting that they may serve redundant functions. However, each factor possesses its own distinct expression profile during development (197). A null mutation of COUP-TFI resulted in defects in neurogenesis, axon guidance, and arborization (267), whereas deletion of COUP-TFII resulted in striking defects in angiogenesis, vascular remodeling, and fetal heart development (248).

Like most nuclear receptors, as transcription factors, COUP-TFI regulates transcription by binding to a variety of response elements, such as DR1, DR3, DR4 DR5 and DR7 containing the AGGTCA motif in target gene promoters. COUP-TFI was originally identified as an activator of the chicken ovalbumin gene. In the arrestin gene promoter, a DR-7 element mediated the positive transcriptional effect of COUP-TF (200) and recently COUP-TFI was shown to activate aldosterone synthase (*CYP11B2*) expression by binding to the -129/-124 element of human *CYP11B2* promoter (306). However, in the other genes COUP-TFI activated transcription through protein-protein interactions with other transcription factors. For example in the HNF-1 α gene COUP-TFI interacted with HNF-4 in the promoter (213) and in the Egr-1 gene COUP-TF enhances transcription by recruiting coactivator SRC-1 through its interaction with Sp-1 (260). It has also been reported that COUP-TFI can function as a transcriptional repressor of many target genes and several mechanisms have been proposed to explain the COUP-TF-mediated repression. COUP-TFs inhibit the transcription of

other nuclear receptor such as retinoic acid receptor and thyroid hormone receptor by competing for binding to the response elements of these receptors, thus acting as passive repressors of their transcriptional activation (343). Another mechanism of passive repression by COUP-TFs involves their ability to heterodimerize with the 9-*cis* retinoic acid receptor, reducing its availability for other nuclear receptors that use it as a partner (384). Furthermore, COUP-TFI represses basal transcriptional activity by interacting with transcriptional corepressors, such as N-CoR and SMRT (308).

It has also been reported that COUP-TFI enhanced human ER activity as a transcription coactivator. The formation of a tight ER α -COUP-TFI intermediate complex resulted in an increased recruitment of ERK2/p42 MAPK, phosphorylation of ER α on Ser 118 and enhanced transcriptional activity (215). However, the functional domains of COUP-TFI required for enhancement of ER α activity, and interactions with ER α have not been determined. The second objective of this study was to investigate COUP-TFI coactivation activity on ER and ER/Sp1 genomic transactivation pathway and also identify the functional domains of COUP-TFI required for this enhanced activity, and for interactions with ER α and Sp1.

CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals, Cells, and Antibodies

MCF-7, ZR-75, and MDA-MB-231 breast cancer cells, HeLa, and COS-7 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). MCF-7 and MDA-MB-231 cells were routinely maintained in DME/F12 medium with phenol red and supplemented with 5% fetal bovine serum (FBS) plus antibiotic antimycotic solution (Sigma, St. Louis, MO). COS-7 and HeLa cells were maintained in Dulbecco's Modified Eagle Media (DMEM) (Gibco Invitrogen Corporation, Carlsbad, CA) medium with phenol red and supplemented with 5% FBS plus antibiotic antimycotic solution. ZR-75 cells were maintained in RPMI 1640 media (Sigma) supplemented with 2.2 g/L sodium bicarbonate, 2.38 g/L HEPES, 0.11 g/L sodium pyruvate, 4.5 g/L glucose, and 7.5 % FBS plus antibiotic antimycotic solution. Cells were cultured and grown in an air-carbon dioxide (95:5) atmosphere at 37°C. For transient transfection studies, cells were grown for 1 day in DME/F12 medium without phenol red and 2.5% FBS stripped with dextran-coated charcoal. ICI 182780 was kindly provided by Dr. Alan Wakeling (AstraZenaca Pharmaceuticals, Macclesfield, UK). The kinase inhibitors PD98059, LY294002, SB202190 and SP600125 were purchased from Cal-Biochem (La Jolla, CA). ER α , Sp1, Elk-1, actin, phospho-Elk-1, and SRF antibodies were obtained from Santa Cruz

Biotechnology, Inc. (Santa Cruz, CA). Myc-tag and Egr-1 antibodies were obtained from Cell Signaling Technology (Beverly, MA). His-tag antibody was obtained from Invitrogen (Carlsbad, CA). All other chemicals and biochemicals were the highest quality available from commercial sources.

2.2 Cloning and Plasmids

2.2.1 Egr-1 Experiment

Wild-type human ER α (ER α) expression plasmid was provided by Dr. Ming-Jer Tsai (Baylor College of Medicine, Houston, TX). The SRF-luc construct contains five tandems SRF elements linked to bacterial luciferase and was purchased from Stratagene (La Jolla, CA). Dominant negative (dn) Elk-1 was provided by Dr. Roger Davis (University of Massachusetts, Worcester, MA). This construct encodes amino acid residues 1 - 168 of Elk-1 and lacks the activation domain. The plasmid Gal-Elk-1C was obtained from Dr. Roger Treisman (Imperial Cancer Research Center, London, UK). pEgr1-CAT plasmid, which contains the -600 to +12 5' flanking sequence from the human Egr-1 gene was kindly provided by Dr. Kathy Sakamoto (UCLA School of Medicine, Los Angeles, CA). pEgr-1A (-600/+12), pEgr-1B (-460/+12), pEgr-1C (-164/+12), pEgr-1D (-480/-285), pEgr-1E (-480/-324), and pEgr-1F (-480/-348) were made by PCR amplification using pEgr1-CAT as template. The PCR products were purified and ligated into pGL2 basic vector (Promega Corp., Madison, WI). Site-directed

mutagenesis was performed using the two-step overlap extension PCR method. Oligonucleotides used for site-directed mutagenesis in this study are listed as Table 2-1.

Table 2-1 Oligonucleotides used for site-directed mutagenesis.

pEgr-1Em1	5' GCA GCA CCT <u>TCC</u> TTG GAG TGG C 3'
pEgr-1Em2	5' GAA CAA CCC TTG CTT <u>GGG C</u> AG CAC 3'
pEgr-1Em3	5' GAT CCC CCG CCT <u>AGC T</u> AA CCC TTA TTT GG 3'
Elk-1c (S383A)	5' GAG CAC CCT <u>GGC</u> TCC CAAT TGC GC 3'
Elk-1C (S389A)	5' TGC GCC CCG <u>TGC</u> CCC GGC CAA GC 3'

Note. Mutations are underlined and substituted bases are indicated in bold.

2.2.2 COUP-TFI Experiment

Taf1-ER α and Null-ER α expression plasmids were provided by Dr. D. McDonnell (Duke University, Durham, NC). The human ER deletion construct 19c-ER α was provided by Dr. Pierre Chambon (Institut de Genetique et de Biologie Moleculaire et Cellulaire, Illkirch, France). The SRC-1 expression plasmid was graciously provided by Dr. B. O'Malley (Baylor College of Medicine, Houston, TX). The pERE₃ reporter containing three consensus ERE sites linked to a luciferase gene was created by cloning an oligonucleotide with three ERE elements into

BamHI-HindIII cut pXP-2 plasmid (298). The wild-type mouse COUP-TFI expression vector (pCR3.1-COUP-TFI) was originally provided by Dr. Ming-Jer Tsai (Baylor College of Medicine) and used as the PCR template for further cloning. The COUP-TFI expression plasmids used in the transfection assay including WT, N-terminal deletion mutants (dN1, and dN2), and C-terminal deletion mutants (dC1 and dC2) were generated by PCR amplification and ligated into pCDNA3.1/His or pCDNA3.1/HIS/-Myc vectors (Invitrogen). The COUP-TFI dN3 mutant were generated by PCR amplification and cloned into pM vector (BD Biosciences Clontech, Palo Alto, CA). For mammalian two-hybrid assay, the expression plasmids of GAL4-DBD-ER α chimeras including pM-ER, pM-ER (A/B) and pM-ER (C/F) were made by Dr. B. Saville in this lab as previously described (298). The VP-16-COUP-TFI chimera expression plasmid was generated by PCR amplification and ligated into pACT vector (Promega). The expression plasmids of point mutation of COUP-TFI (m83, m103 and m138) were generated by site-directed mutagenesis using the two-step overlap extension PCR method and ligated into pCDNA3.1/His vector. Oligonucleotide primers used for cloning or site-directed mutagenesis in this study are listed in Table 2-2.

Table 2-2 Summary of primers used for cloning the COUP-TFI constructs.

Clones	Primers
WT	F, 5' GCGGCCGCATGGCAATGGTAGTTAG 3' R, 5' GCTGCTCGAGGGGAACACTGGATGGACATGT 3'
dN1	F, 5' TATAGGTACCTGGTTCAGGCCAGAGCCAGCAGCA 3' R, 5' GCTGCTCGAGGGGAACACTGGATGGACATGT 3'
dN2	F, 5' TAGCGGTACCTAGGAGCGTCCGCAGGAACTTAAC 3' R, 5' GCTGCTCGAGGGGAACACTGGATGGACATGT 3'
dN3	F, 5' TCTAGAATGGAAGCGTTTCAGCGAGGAA 3' R, 5' GCGGCCGCCTAGGAACACTGGATGG 3'
dC1	F, 5' AGCTAAGCTTATGGCAATGGTAGTTAGCAG 3' R, 5' AGCTCTCGAGCAGCAGTTTGCCAAAGCGGC 3'
dC2	F' 5' AGCTAAGCTTATGGCAATGGTAGTTAGCAG 3' R' 5' AGCTCTCGAGCAGCGGCATGGAGCAC 3'
VP16-COUP	F, 5' ACGCGTCAATGGCAATGGTAGTTAG R, 5' TCTAGACTAGGAACACTGGATGG
m83 COUP-TFI	F, 5' GCACATCGAG GC CGTGGTGTGCG 3' R, 5' CGCACACCAC GC CTCGATGTGC 3'
m103 COUP-TFI	F, 5' CTGCGAGGG GC CAAAAGTTTCT 3' R, 5' AGAACTTTT GC GCCCTCGCAG 3'
m138 COUP-TFI	F, 5' GTGCCAATAC GC CCGCCTCAAGAAG 3' R, 5' CTTCTTGAGGCG GC GTATTGGCAC 3'

Mutations are underlined and substituted bases are indicated in bold.

2.3 Transient Transfection and Luciferase Assay

2.3.1 Egr-1 Experiment

For transfection experiments, 175,000 MCF-7 cells were initially seeded in 12-well plates. Twenty-four h after seeding, MCF-7 cells were transfected by the calcium phosphate method with Egr-1 promoter-luciferase reporter constructs, ER α expression vector and pCDNA3/*His/lacZ* (Invitrogen) that was used as a standard reference control plasmid for determining transfection efficiencies. After 5 h, cells were shocked with 25% glycerol and washed with PBS. Fresh DME/F12 without phenol red and charcoal-stripped FBS containing DMSO or 1 nM E2 in DMSO were added to the cells and incubated for 24 h.

2.3.2 COUP-TFI Experiment

MCF-7, MDA-MB231, HeLa, COS-7 and ZR-75 cells were seeded in DME/F-12 medium without phenol red containing 2.5 % dextran/charcoal-stripped FBS. After 24 h cells were transfected with GeneJuice transfection reagent (Novagen, Madison, WI) according to manufacture's recommendation. Five hours after transfection, cells were replaced with fresh DME/F12 without phenol red and treated with DMSO or 10 nM E2 for 36 h.

Cells from each experiment were then harvested in 100 μ l of 1X Reporter lysis buffer (Promega). Luciferase assays were performed on 30 μ l of the cell extract using the Luciferase assay system (Promega). Light emission was

detected on a Lumicount luminometer (Packard, Meriden, CT). β -Galactosidase assays were performed on 20 μ l of cell extract using the luminescent Galacton-Plus assay kit (Tropix, Bedford, MA). The luciferase activity observed in each treatment group was normalized to β -gal activity obtained from the same sample to correct for transfection efficiencies. Data are expressed as fold induction (by E2 or other chemicals) compared to the solvent (DMSO) control.

2.4 Western Blot Assay

Cells were seeded into 60 mm tissue culture plates in DME/F-12 medium without phenol red containing 2.5% charcoal-stripped FBS. After 24 h, cells were treated with 10 nM E2 and harvested at designated time points and lysed in ice-cold lysis buffer (50 mM HEPES [pH 7.5], 500 mM NaCl, 10% [vol/vol] glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA) supplemented with protease inhibitor cocktail (Sigma). Equal amounts of protein from each treatment group were boiled in 1x Laemmli buffer (50 mM Tris-HCl, 2% sodium dodecyl sulfate [SDS], 0.1% bromphenol blue, 175 mM β -mercaptoethanol), separated by SDS-10% polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with Blotto (5% milk, Tris-buffered saline [10 mM Tris-HCl, pH 8.0, 150 mM NaCl], and 0.05% Tween 20) and probed with primary antibodies. Following incubation with

peroxidase-conjugated secondary antibody, immunoglobulins were visualized using the ECL detection system (Perkin Elmer Foster City, CA).

2.5 Nuclear Extract Preparation and EMSA

Cells were seeded in 100 mm tissue culture plates using DME/F12 without phenol red, supplemented with 2.5% charcoal-stripped FBS. After 24 h, cells were treated for 1 h with DMSO or 10 nM E2. Nuclear extracts were obtained using the NE-PER nuclear and cytoplasmic extraction kit (Pierce) according to the manufacturer's instructions. Nuclear extracts obtained from different treatment groups were incubated for 20 min in HEGD buffer with poly-(dI-dC), unlabeled oligonucleotides or antibodies for supershift assays. The mixture was then incubated for additional 20 min after addition of ³²P-labeled oligonucleotide. Reaction mixtures were separated on 5% polyacrylamide gels (acrylamide:bis-acrylamide 30:0.8) at 140 V in 1X TBE (0.09 M Tris-HCl, 0.09 M boric acid and 2 mM EDTA, pH 8.3). Gels were dried and protein-DNA complexes were visualized using a Storm 860 instrument (Amersham Biosciences, Piscataway, NJ). Oligonucleotides used for EMSA in this study are summarized as follows (mutations are underlined and substituted bases are indicated in bold).

SRE3	5' AGG ATC CCC CGC CGG AAC AAC CCT TAT TTG GGC AG 3'
mTCF	5' AGG ATC CCC CGC <u>CTA GCT</u> AAC CCT TAT TTG GGC AG 3'
mSRF	5' AGG ATC CCC CGC CGG AAC AAC CCT <u>TGC</u> TTG GGC AG 3'

2.6 RT-PCR Assay

Total RNA was extracted using Nucleospin RNA purification kit (BD Biosciences Clontech), following the manufacturer's instructions. An aliquot of 750 ng RNA was used as the template for cDNA synthesis by incubating with oligo-d(T) primer and multiscribe reverse transcriptase (Perkin Elmer) at 48°C for 40 min. PCR amplification was performed with Taq PCR Master Mix (Promega, Madison, WI). The following conditions were used for the PCR assays: one cycle of 2 min at 95°C; 34 cycles of 30 sec at 95°C; 30 sec at 57.5°C; 1 min at 72°C; one cycle of 5 min at 72°C. PCR products were analyzed by electrophoresis on 1.5% agarose gels containing ethidium bromide. Oligonucleotide primers used for PCR in this study include the following

Egr-1	F' 5' GAG CCG AGC GAA CAA CCC TAC GAG CAC CTG R' 5' GCG CTG AGG ATG AAG AGG TTG GAG GGT TGG
GADPH	F' 5' TGT GTC CGT CGT GGA TCT GA R' 5' CCT GCT TCA CCA CCT TCT TGA
<i>c-fos</i>	F, 5' GCT TCA ACG CAG ACT ACG AG R, 5' TAG AAG GAC CCA GAT AGG TC

2.7 Coimmunoprecipitation Assay

COS-7 cells were seeded into 60 mm tissue culture plates in phenol red-free DME/F-12 medium containing 2.5% charcoal-stripped FBS. After 24 h., transient transfections were performed by using GeneJuice transfection reagent (Novagen) according to the manufacturer's protocol. After 4-6 h., transfected cells

were treated with 10 nM E2 for 24h. Cells were harvested and lysed by using 1 ml of RIPA buffer (1x PBS, 1% Nonidet P-40 or Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml PMSF in isopropanol, aprotinin, 100 mM sodium orthovanadate), and cellular debris was removed by centrifugation at 10,000xg for 10 min at 4°C. The supernatant was transferred to a fresh microcentrifuge tube and precleared by adding 20 µl of protein A-agarose conjugate slurry (Sigma) and incubated at 4°C for 1h. After centrifugation for 1 min, the supernatant was transferred to another new microcentrifuge tube, and 2.5 µg of rabbit polyclonal anti-ER α antibody (Santa Cruz) was added and incubated at 4 °C for 1 hr. After incubation, 20 µl of protein A-agarose conjugate slurry (Sigma) was added and incubated at 4°C for another 1 h. The immunoprecipitate was collected by centrifugation, gently washed with 500 µl RIPA buffer (3X), and resuspended and denatured in 50 µl of 2x Laemmli buffer. The immunoprecipitated sample was analyzed in a western blot assay.

2.8 Statistical Analysis

Statistical significance was determined by ANOVA and Student's t-test, and the levels of probability are noted. The results are expressed as means \pm SD for at least three separate (replicate) experiments for each treatment.

CHAPTER III

RESULTS

3.1 Egr-1 Is Activated by E2 in MCF-7 Cells

3.1.1 Deletion and Mutational Analysis of the Egr-1 Gene Promoter

The results in Figure 3-1 A show that E2 induced Egr-1 protein levels by approximately 8.2-fold, and this complements results of previous studies in MCF-7 cells which show that E2 induces Egr-1 mRNA levels (263). E2 did not induce luciferase activity in MCF-7 cells transfected with pEgr-1A alone; however, in cells cotransfected with ER α expression plasmid (500 ng), E2 induced luciferase activity (> 7-fold), and this response was inhibited by the antiestrogen ICI 182780 (Fig. 3-1 B). Thus, hormone-responsiveness in MCF-7 cells was observed only after cotransfection with ER α . Similar results have previously been reported for multiple E2-responsive plasmids activated through nuclear or extranuclear pathways of estrogen action, and this is related to limiting levels of ER α in transfected cells that overexpress the plasmids (88-90, 297, 331). However, it was also observed that higher concentrations of E2 also significantly induced luciferase activity in MCF-7 cells transfected only with pEgr-1A (no hER α cotransfection) (Fig. 3-1 C). This has also been reported for hormone-dependent activation of constructs containing c-fos promoter inserts which are activated through kinase-dependent pathways in MCF-7 cells (88, 89).

The -600 to +12 region of the *Egr-1* promoter contains several potential E2-responsive motifs including a distal GC-rich motif that could be activated by ER α /Sp1 (nuclear), multiple SREs (SRE1-4), and cAMP response element (CRE) that can be hormonally activated through non-genomic (extranuclear) pathways (51, 85, 88, 189, 294). Transfection studies in MCF-7 cells with pEgr-1A (-600 to +12), pEgr-1B (-460 to +12), and pEgr-1C (-164 to +12) (Fig. 3-2 A) show that the upstream GC-rich and downstream CRE and SRE1 motifs are not necessary for hormone-inducibility suggesting the SREs 2 – 4 are necessary for this response. The E2-responsiveness of several 3'-deletion constructs containing SREs 2 – 4 (pEgr-1D, -480 to -285), SRE4 and 3 (pEgr-1E, -480 to -324), and SRE4 (pEgr-1F, -480 to -376) were also investigated in MCF-7 cells (Fig. 3-2 B) and induction by E2 was observed only for the former two constructs. These results suggest that SRE3 was required for E2-induced transactivation. Mutation analysis of SRE3 was determined using constructs containing selective mutations in the SRF (SRE2 and SRE3) and TCF (SRE3) motifs. E2-induced transactivation was observed in cells transfected with pEgr-1Em1 which contained a mutation in an adjacent SRF binding site. However, induction was not observed in cells transfected with constructs containing SRE3 mutations in the SRF or TCF sites (pEgr-1Em2 and pEgr-1Em3) (Fig. 3-2 C). These results indicate that SRE3 is the major E2-responsive motif in the *Egr-1* gene promoter and that TCF and SRF motifs are required.

A.

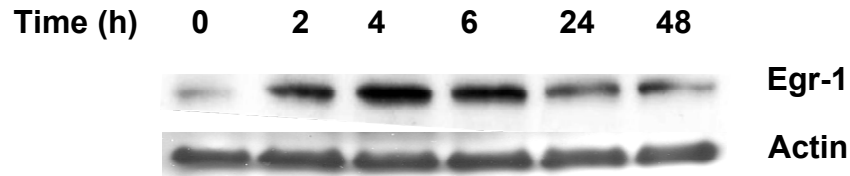
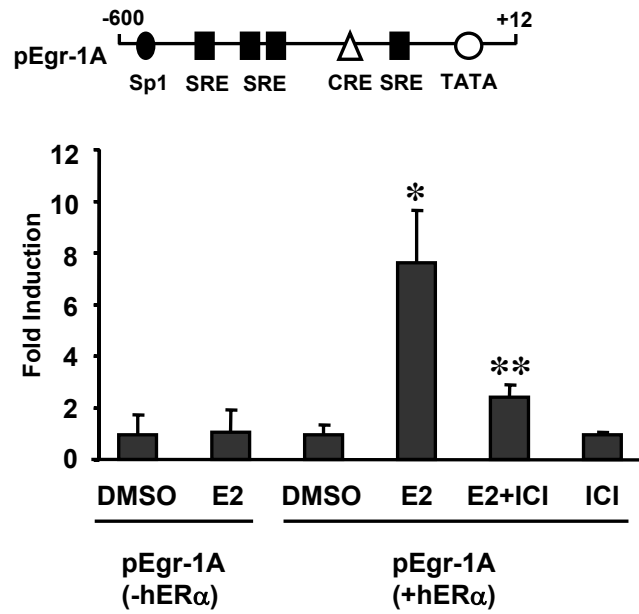


Fig. 3-1. Hormone-responsiveness of Egr-1 in MCF-7 cells. (A) Induction of Egr-1 protein by E2. MCF-7 cells were treated with 10 nM E2 for different times and levels of Egr-1 protein were determined by Western blot analysis as described in the Materials and Methods. Actin protein was used as a loading control, and these cells were not cotransfected with ER α . (B) Hormone activation of pEgr-1A. MCF-7 cells were transfected with pEgr-1A with or without ER α expression plasmid, treated with DMSO, E2, ICI 182780, or their combination, and luciferase activity was determined as described in the Materials and Methods. Results are expressed as means \pm SD for three replicate determinations for each treatment group, and significant ($p < 0.05$) induction by E2 (*) or inhibition by ICI 182780 (**) is indicated. (C) Activation of pEgr-1A in the absence of cotransfected ER α . Cells were transfected with pEgr-1A, treated with 1 – 1000 nM E2, and luciferase activity was determined as described in the Materials and Methods. Results are expressed as means \pm SD for three replicate determinations for each treatment group, and significant ($p < 0.05$) induction by E2 (*) is indicated..

B.



C.

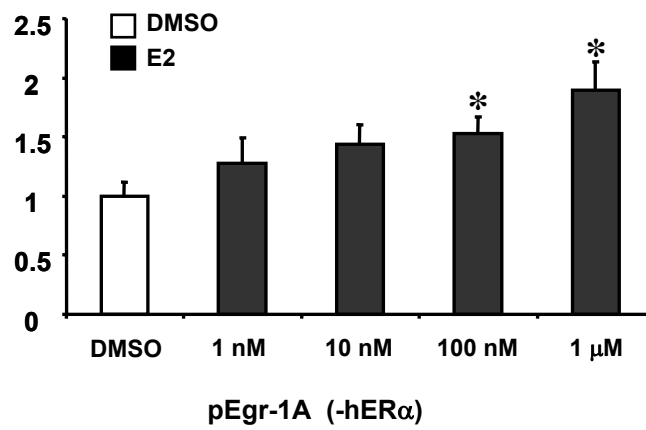


Fig. 3-1. continued.

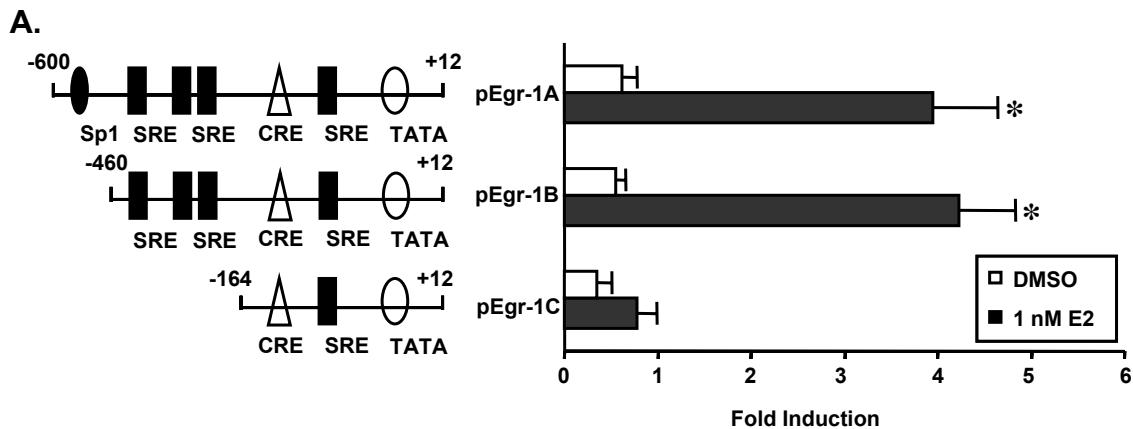
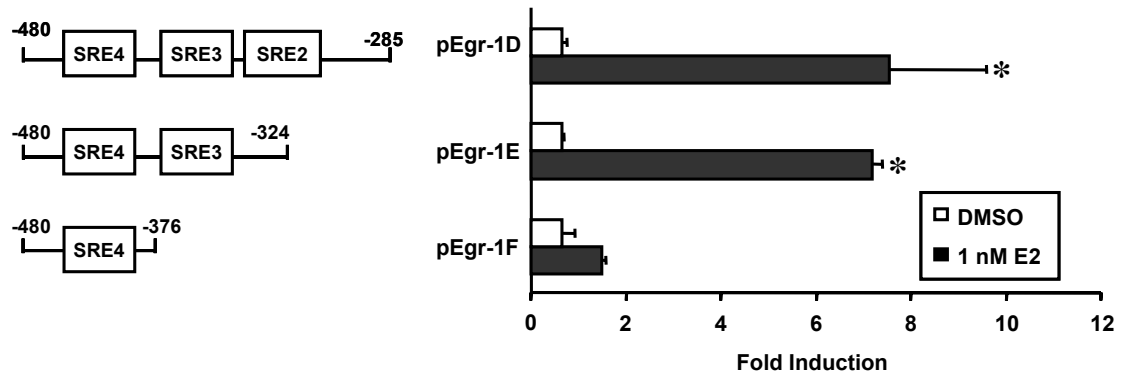


Fig. 3-2. Deletion and mutation analysis of the Egr-1 gene promoter. Deletion (A, B) and mutation (C) analysis of pEgr-1A. MCF-7 cells were transfected with pEgr-1 constructs and ER α expression plasmid, treated with DMSO or 1 nM E2, and luciferase activity was determined as described in the Materials and Methods. Results are expressed as means \pm SD for three replicate determinations for each treatment group, and significant ($p < 0.05$) induction by E2 is indicated by an asterisk.

B.



C.

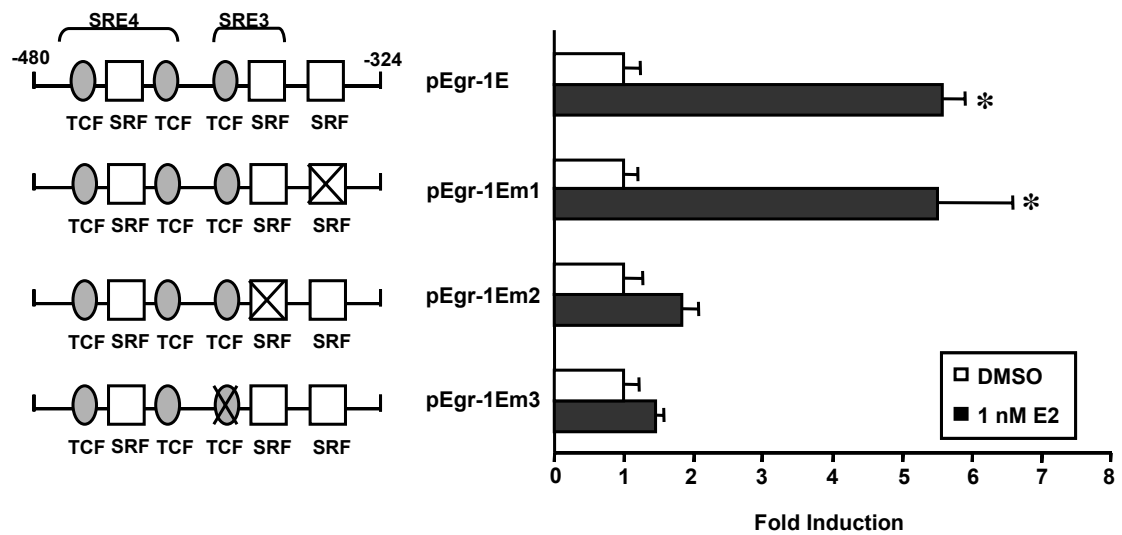


Fig. 3-2. continued.

3.1.2 Protein Interactions with SRE3

Nuclear extracts from DMSO and E2-treated MCF-7 cells were incubated with [³²P]SRE3 and analyzed in a gel mobility shift assay (Fig. 3-3). In this gel, two major specifically-bound retarded bands were formed (see arrow) using DMSO and E2-treated extracts (lanes 2 and 6). Coincubation of both extracts with [³²P]SRE3 and Elk-1 (lanes 3 and 7) or phospho-Elk-1 (lanes 4 and 8) antibodies gave supershifted bands (SS →) indicating that both forms of Elk-1 were associated with the SRE oligonucleotide. Although the overall intensities of the retarded bands were comparable using both E2- and solvent (DMSO)-treated nuclear extracts, the supershifted phospho-Elk-1 complex was more intense using the hormone-treated extracts (lane 4 vs. lane 8). In competition experiments with unlabeled oligonucleotides (lanes 9 - 11), mutant oligonucleotides in the TCF (m1-SRE3) and SRF (m2-SRE3) sites only slightly decreased the more and less mobile retarded bands, respectively. In contrast, competition with the wild-type SRE3 oligonucleotide resulted in complete loss of both retarded bands. Antibody supershift experiments were also carried out using [³²P]SRE3 and nuclear extracts from DMSO- and E2-treated cells with SRF antibody. SRF antibody can also induce formation of a supershifted complex in both treatment groups (data not shown). These data are consistent with E2-induced phosphorylation of Elk-1 and interaction of SRF and phospho-Elk1

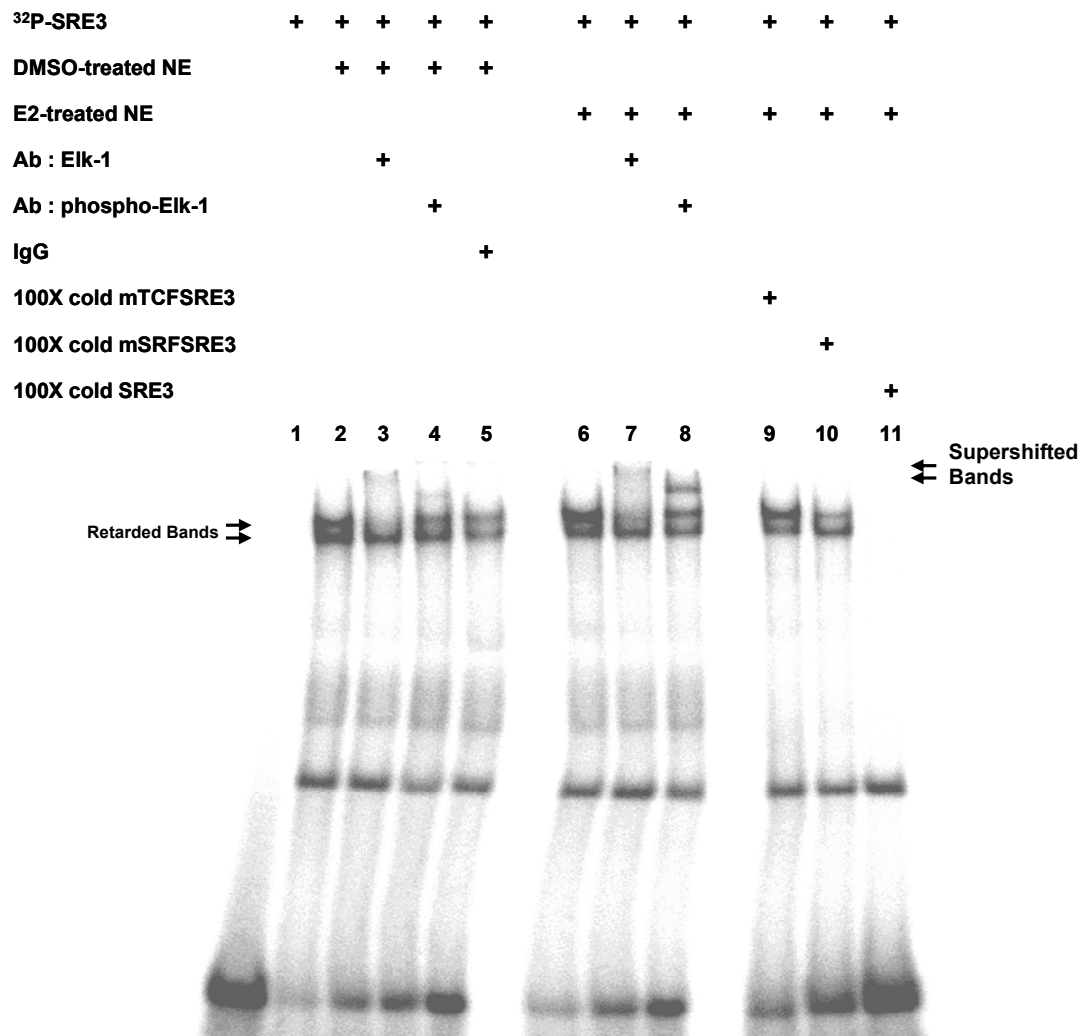


Fig. 3-3. Gel mobility shift assay of SRE3-protein interactions. Interactions of nuclear extracts with [³²P]SRE3. [³²P]SRE3 was incubated with nuclear extracts from MCF-7 cells treated with DMSO or E2 and coincubated with Elk-1, phospho-Elk-I antibodies, or non-specific IgG or unlabeled oligonucleotides (100-fold excess), and analyzed by gel mobility shift assay as described in the Materials and Methods. Retarded and supershifted bands are indicated with arrows.

on SRE3 in the *Egr-1* gene promoter, and this was comparable with SRF/Elk-1 interactions with the SRE in the *c-fos* gene promoter (88).

3.1.3 Role of Elk-1 in Activation of *Egr-1* Gene Expression

The role of Elk-1 in activation of *Egr-1* was further investigated in the MCF-7 cells transfected with pEfg-1D and increasing amounts of dominant negative (dn) expression plasmid for Elk-1 (50 - 500 ng) (Fig. 3-4 A). dn-Elk-1 inhibits E2-induced activation of pEgr-1D and confirms the role of Elk-1 in activation of SRE3. GAL4-Elk-C contains the C-terminal region of Elk-1 (amino acids 307 - 428) fused to the DNA binding domain of the yeast GAL4 proteins. The Elk-C region can serve as a transactivation domain (206, 338), and in the presence of cotransfected ER α , E2 induces reporter gene activity in MCF-7 cells transfected with GAL4-Elk-C and a construct containing 5 copies of the GAL4 response element linked to a bacterial luciferase reporter gene (pGAL4₅) (Fig. 3-4 B). This construct is also induced by E2 alone (ca. 2-fold), but is enhanced by cotransfection with ER α due to overexpression of the reporter construct and limiting levels of endogenous ER α (88). This hormone-induced response was inhibited by the MAPK kinase inhibitor PD98059 but not by 20 μ M SB202190 or 25 μ M SP600125 which inhibit p38 and jun N-terminal kinase, respectively. These results are consistent with hormonal activation of the ras-MAPK pathway in breast cancer cells (219, 220). The results illustrated in Figure 3-4 C compared the differences in hormone-induced activation of wild-type Elk-C and constructs

containing S383A and S389A mutations. E2 activates wild-type GAL4-ElkC and partially activates the S389A mutant but not the S383A mutant, and this pattern of activation was similar to that observed for serum activation of Elk-1 in NIH 3T3 cells (206). The results in Figure 3-4 D demonstrate that E2 induces phosphorylation of Elk-1, and this is inhibited by PD98059 but not SB02190, confirming the role of MAPK in this response.

However, previous studies indicate that the SRE in the *c-fos* gene promoter is also activated through phosphatidylinositol-3-kinase (PI3-K) which is upregulated by E2 in MCF-7 cells (88). Results in Figure 3-5 A show that the MAPK inhibitor PD98059 inhibits E2-induced transactivation in MCF-7 cells transfected with pEgr-1E, whereas this response is not blocked by LY294002, an inhibitor of PI3-K. As a positive control, LY294002 but not PD98059 inhibited E2-dependent activation of a construct containing 5 SRF elements (SRF-luc) in MCF-7 cells (Fig. 3-5 B) as previously reported (89). Induction of Egr-1 mRNA levels by E2 (Fig. 3-5 C) were also inhibited by PD98059 and not LY294002 confirming that hormonal activation of Egr-1 is dependent on ER α and kinase-dependent activation of MAPK. In contrast, hormone-dependent activation of *c-fos* is due to activation of both MAPK and PI3-K pathways (88, 89), and inhibitors of these pathways decrease induction of *c-fos* mRNA in MCF-7 cells (Fig. 3-5 C).

A.

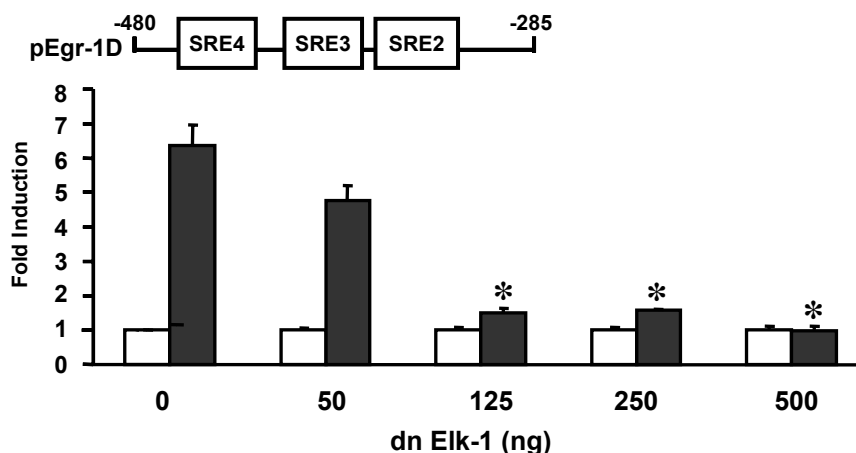
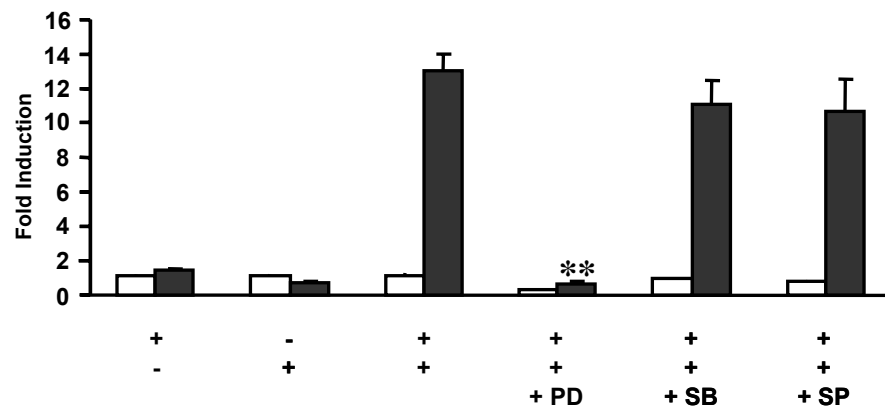
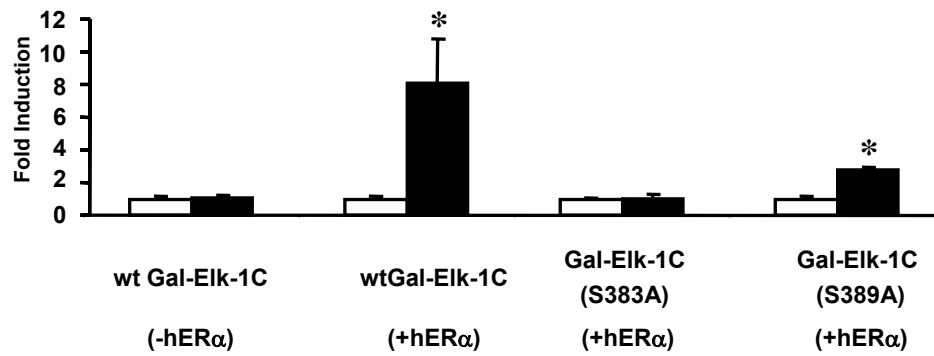


Fig. 3-4. Role of Elk-1 phosphorylation in activation of Egr-1. (A) Effects of dnElk-1. MCF-7 cells were transfected with pEgr-1D, treated with DMSO or 1 nM E2, cotransfected with different amounts of dnElk-1 expression plasmid, and luciferase activity was determined as described in the Materials and Methods. Significant ($p < 0.05$) inhibition of E2-induced luciferase activity by dnElk-1 is indicated by an asterisk. (B) Hormone activation of GAL4-Elk-1C/pGAL4₅. MCF-7 cells were transfected with GAL4-Elk-1C/pGAL4₅, ER α expression plasmid, DMSO, 10 nM E2 and kinase inhibitors (50 μ M PD98059, 20 μ M SB202190, and 25 μ M SP600125), and luciferase activities were determined as described in the Materials and Methods. Significant ($p < 0.05$) inhibition of E2-induced activity is indicated (**). (C) Activation of GAL4-Elk-1C. MCF-7 cells were transfected with pGAL4₅, wild-type and mutant GAL4-Elk-1C, (\pm) ER α expression plasmid, treated with DMSO or 10 nM E2, and luciferase activity was determined as described in the Materials and Methods. Significant ($p < 0.05$) induction by E2 is indicated by an asterisk. (D) Phosphorylation of Elk-1. MCF-7 cells were treated with DMSO, 10 nM E2 alone, or in combination with 50 μ M PD98059 or 20 μ M SB202190, and whole cell lysates were examined by Western blot analysis for Elk-1 and phospho-Elk-1 proteins. Similar results were observed in duplicate experiments. Results in (A), (B) and (C) are means \pm SD for three replicate experiments for each treatment group.

B.



C.



D.

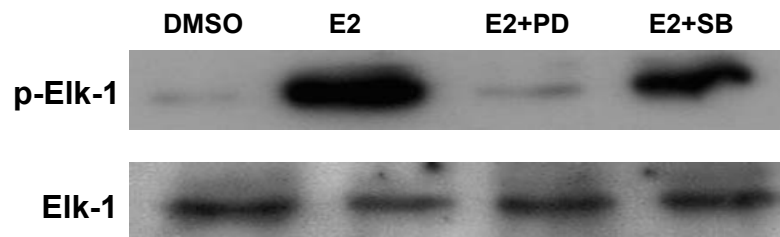


Fig. 3-4. continued.

A.

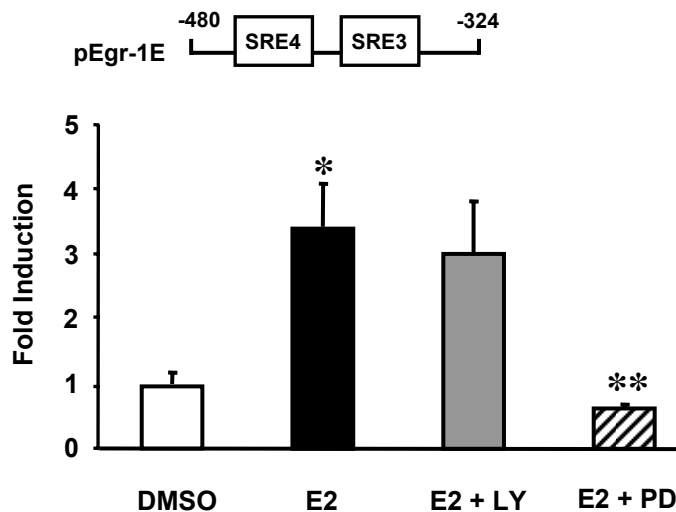
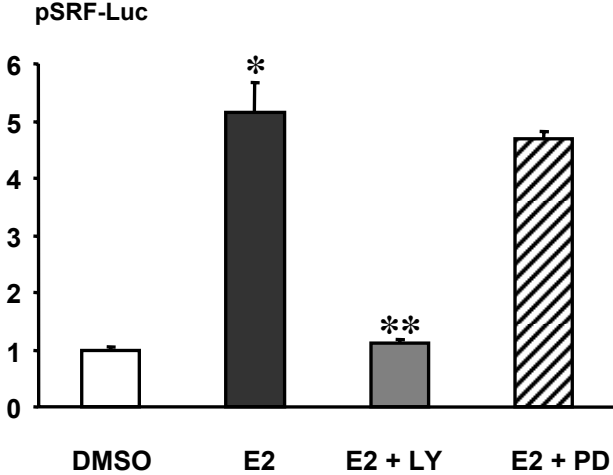


Fig. 3-5. Effects of PD98059 and LY294002 on hormone-induced activation of Egr-1 and comparative SRE sequences. (A) Activation of pEgr-1E. MCF-7 cells were transfected with pEgr-1E, treated with DMSO, 10 nM E2 alone or in combination with 25 μ M PD98059 or 50 μ M LY294002, and luciferase activity was determined as described in the Materials and Methods. Results are means \pm SD for three replicate experiments for each treatment group, and significant ($p < 0.05$) inhibition in cotreatment groups is indicated (*). (B) Induction of SRF-luc by E2. MCF-7 cells were transfected with SRF-luc, treated with E2, DMSO or kinase inhibitor as indicated in Fig. 3-5 A (above), and luciferase activity determined as described in the Materials and Methods. Results are means \pm SD for three replicate determinations for each treatment group, and significant ($p < 0.05$) induction (*) or inhibition (**) is indicated. (C) Induction of pEgr-1 and c-fos mRNA. MCF-7 cells were treated with DMSO, 10 nM E2 alone or in combination with 25 - 50 μ M PD98059 or 25 - 50 μ M LY294002, and induction responses were determined by RT-PCR as described in the Materials and Methods. Similar results were observed in duplicate experiments.

B.



C.

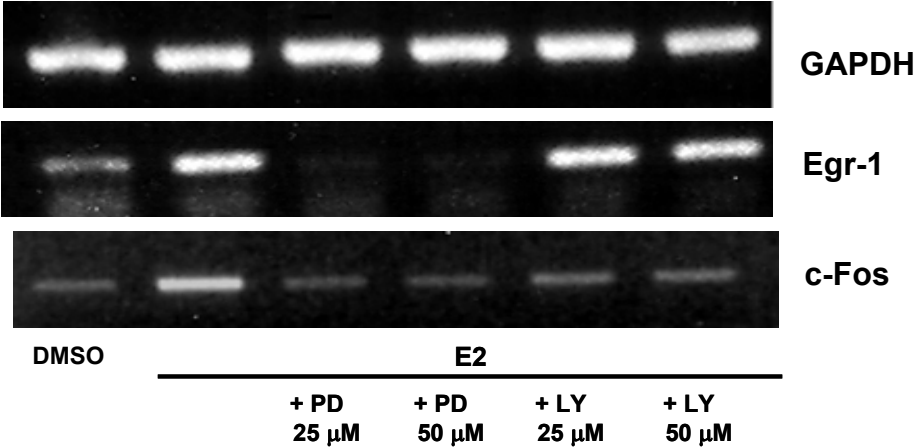


Fig. 3-5. continued.

3.2 COUP-TFI Coactivates ER α -Mediated Transactivation

3.2.1 Coactivation of ER α by COUP-TFI

Coactivation of ER α -dependent transactivation by COUP-TFI was initially examined in ER α -positive MCF-7 breast cancer cells. MCF-7 cells were transfected with pERE₃, which contains three tandem EREs in a minimal TATA-luciferase construct, and ER α expression plasmid. The transfected pERE₃ construct is overexpressed in the transfected cells and minimal E2-inducibility is observed in MCF-7 cells in the absence of co-transfected ER α . This system is ideal for investigating coactivation of ER α and determining domains of ER α and coactivators required for E2-dependent transactivation in a breast cancer cell context. The results in Figures 3-6 A show that E2 causes a 1.6-fold increase in reporter gene activity in MCF-7 cells transfected with 2.5 ng ER α expression plasmid, and cotransfection with 50, 100 and 200 ng COUP-TFI expression plasmid resulted in a 7.9-, 5.5- and 9-fold enhancement of E2-induced luciferase activity. COUP-TFI alone also increased basal activity and this modified the overall enhanced activity by COUP-TFI. To determine the cell specificity of coactivation by COUP-TFI, we also tested ER α -negative breast (MDA-MB 231) and non-breast cancer (COS-7 and HeLa) cells. In MDA-MB 231, COS-7 and HeLa cells, E2-induced luciferase activity was observed only after cells transfected with ER α , and COUP-TFI expression significantly enhanced E2-dependent activity up to 30.7-, 10.3- and 3.7-fold respectively (Figs. 3-6 B, C

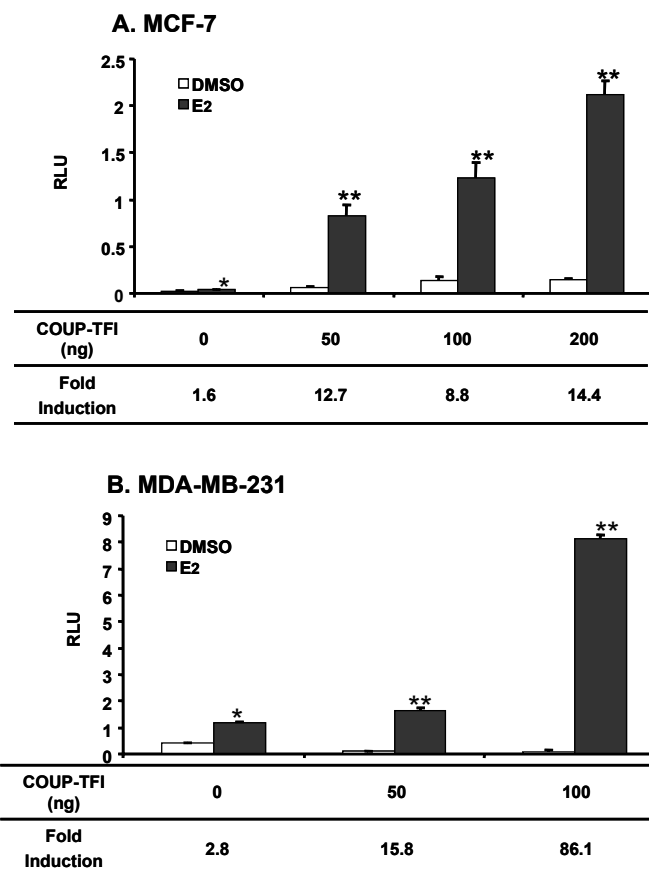


Fig. 3-6 Coactivation of ER α by COUP-TFI. (A) MCF-7, (B) MDA-MB-231, (C) COS-7 and (D) HeLa cells were transfected with pERE3, ER α , β -galactosidase and increasing amounts of pCDNA3-COUP-TFI (0, 50, 100 and 200 ng) expression plasmid. After transfection, cells were treated with DMSO or 10 nM E2 for 36 h., and luciferase activity normalized to β -galactosidase activity was determined as described in the Materials and Methods and presented as relative luciferase units (RLU). Significant ($p < 0.05$) induction by E2 (*) or coactivation of E2-induced activity by COUP-TFI (**) is indicated.

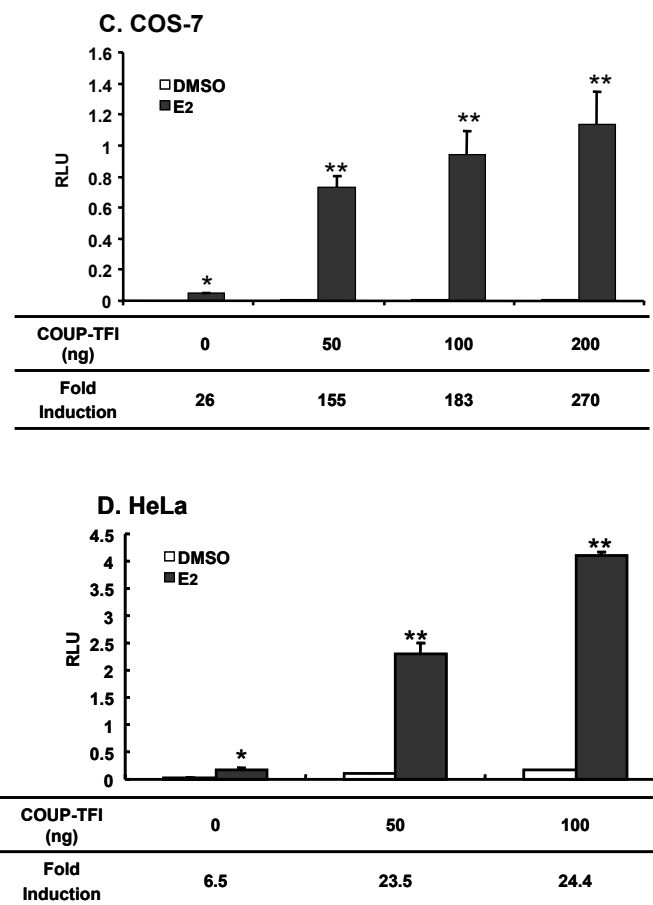


Fig. 3-6. continued.

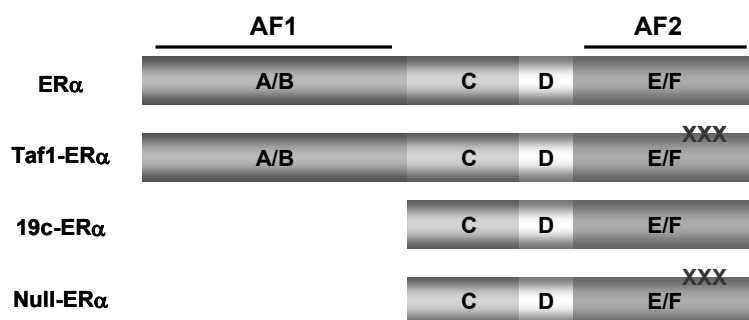
and D). These results show that COUP-TFI significantly coactivated ER α -mediated transactivation in ER α -positive and negative breast cancer cells and in ER α -negative COS-7 and HeLa cells and suggest that this coactivation response is primarily due to COUP-TFI and commonly expressed cofactors.

3.2.2 Coactivation of Variant ER α by COUP-TFI

ER α contains two major activation domains and we therefore investigated the coactivation activity of COUP-TFI with three ER α variants as shown in Figure 3-7 A. The Taf1-ER α contains three mutations in helix 12 (D538N, E542Q, and D545N) that block AF2-dependent interaction with coactivators and inactivates AF-2-dependent transcriptional activation. The 19c-ER α is an A/B domain deletion mutant which lacks AF1. The Null-ER α which contains mutations on AF2 and deletion of AF1 has the minimal hormone response (74).

When COS-7 cells transfected with pERE₃ and COUP-TFI plasmid alone, COUP-TFI did not affect basal luciferase activity after treatment with E2 (Fig. 3-7 B), suggesting that enhancement of E2-induced luciferase activity by COUP-TFI is ER α dependent. In cells transfected with Taf1-ER α and pERE₃, E2 induced a 3.8-fold increase in reporter gene activity and cotransfection with 50 and 100 ng COUP-TFI expression plasmid significantly enhanced E2-induced luciferase activity. COUP-TFI coactivation of Taf1-ER α was comparable to that observed in cells transfected with wild-type ER α and COUP-TFI (Fig. 3-7 C). E2 also induced luciferase activity in COS-7 cells transfected with pERE₃ and 19c-ER α ;

A



B.

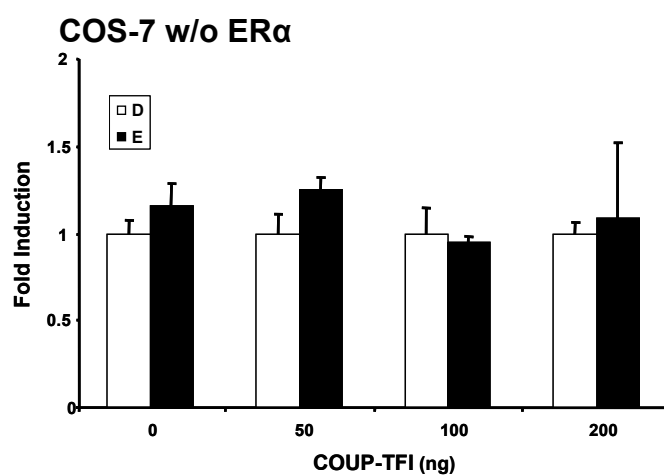


Fig. 3-7 Coactivation of mutant ER α by COUP-TFI. (A) ER α variants. COS-7 cells transfected without ER α (B) or with Taf1-ER α (C), 19c-ER α (D), Null-hER α variant (E). After transfection cells were treated with DMSO or 10 nM E2 for 36 h., luciferase activity normalized to β -galactosidase activity was determined as described in the Materials and Methods. Fold induction was calculated relative to activity observed in cells treated with DMSO. Significant ($p < 0.05$) induction by E2 (*) or coactivation of E2-induced activity by COUP-TFI (**) is indicated.

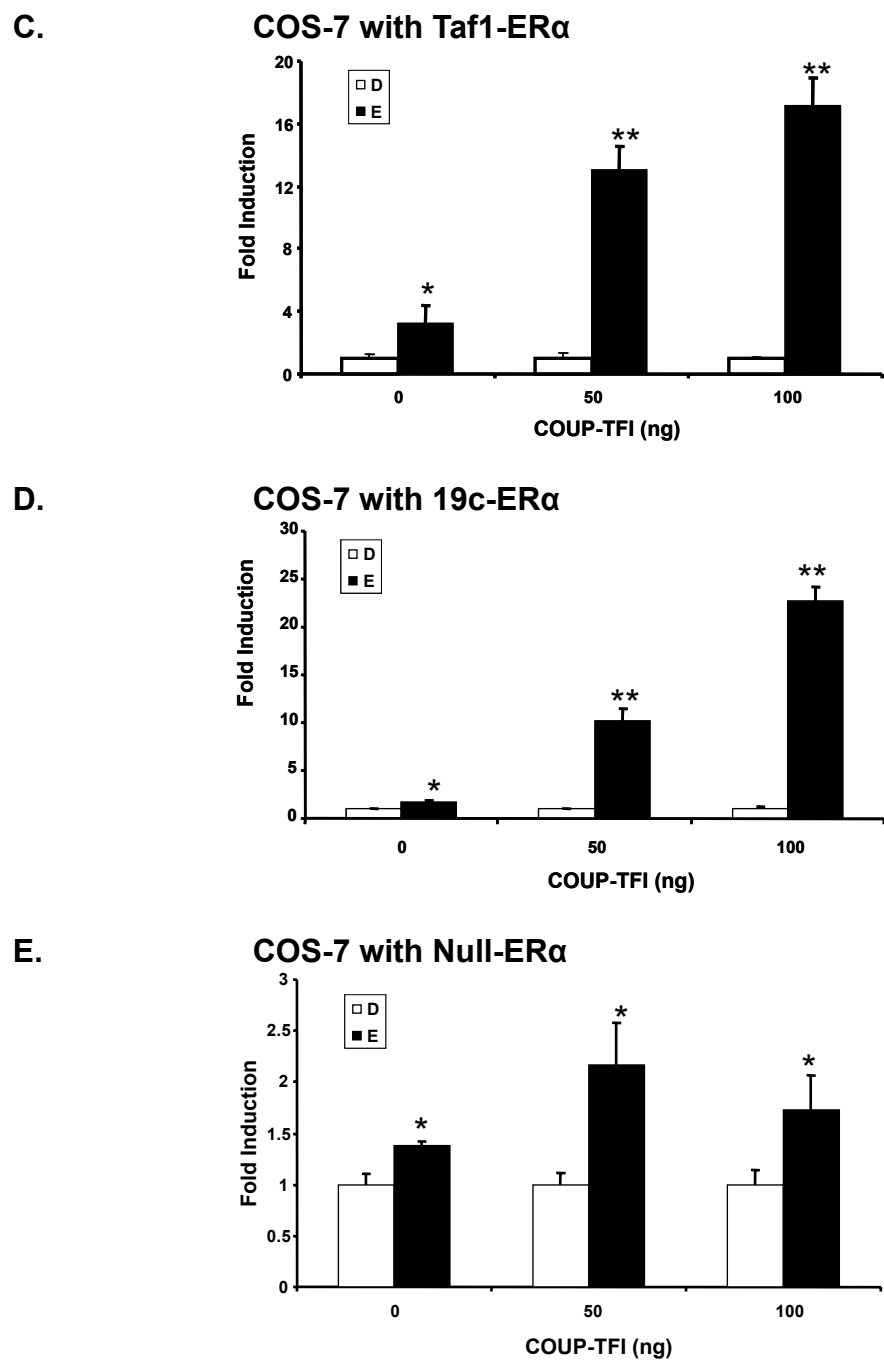


Fig. 3-7. continued.

cotransfection with COUP-TFI also significantly enhanced E2-induced luciferase activity as shown in Figure 3-7 D. However, cells cotransfected pERE₃, COUP-TFI and null-ER α , only minimal coactivation activity of COUP-TFI was observed (Fig.3-7 E). These data suggest that the functional helix 12 on AF-2 is not required for coactivation by COUP-TFI when the functional AF-1 of ER α is intact. However, when the AF-1 domain of ER α is deleted (i.e. 19c-ER α), the intact AF2 function is required for coactivation by COUP-TFI, and mutation of helix 12 amino acids (i.e. null-ER α) resulted in loss of coactivation activity.

Metivier and coworkers previously reported that interactions between COUP-TFI and ER α enhanced the phosphorylation of ER α at Ser118 by increasing the affinity of ER α for interactions with ERK2, resulting in enhanced ER α transcriptional activity by COUP-TFI (215). However when COS-7 cells were transfected with pERE₃ and an m118-ER α mutant which contains a Ser/Ala point mutation on Ser118 of ER α , E2 induced a 3.8-fold increase in reporter gene activity and cotransfection with 100 ng COUP-TFI expression plasmid significantly enhances E2-induced luciferase activity by 25-fold (Fig. 3-8 A). Furthermore, in COS-7 cells transfected with ER α , COUP-TFI and pERE₃, treatment with the MAPK inhibitor PD98059, did not affect the enhancement of ER α -mediated transactivation by COUP-TFI (Fig. 3-8 B). These results show that mutation of the critical MAPK-dependent phosphorylation site (S118) in ER α did not result in loss of COUP-TFI coactivation activity, suggesting that there must be

another mechanism of ER α coactivation by COUP-TFI other than recruitment of ERK2/p42 MAPK and phosphorylation of the Ser 118 as previously reported in CHO-K1 cells (215).

3.2.3 Interactions of ER α with COUP-TFI

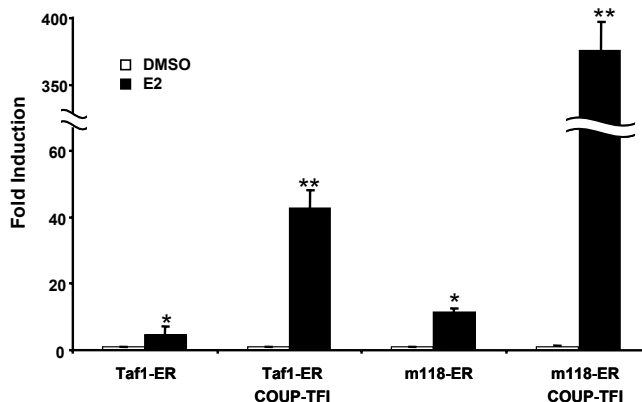
Interactions between COUP-TFI and ER α were investigated in a mammalian two-hybrid assay. HeLa cells were transfected with expression vectors for the GAL4 DBD (pM) or the chimeras of DBD fused to ER α (pM-ER) in the presence of the VP16 activation domain alone (VP16) or VP16 fused to the COUP-TFI (VP16-COUP), and pGAL4₅ (five tandem GAL4 response elements linked to a luciferase reporter gene). The results (Fig. 3-9 A) show that in the absence or present of E2 stimulation when cells transfected with pM-ER and VP16-COUP, the luciferase activity was significantly increased compared to the control luciferase values obtained with cells transfected with pM in the presence of VP16 or VP16-COUP, or pM-ER with VP16. The results show that in the absence of ligand VP16-COUP interacted with pM-ER and after addition of E2 this interaction was increased. Thus results of the mammalian two-hybrid suggest that the interactions of COUP-TFI and ER α are ligand-independent but are also enhanced by E2.

In this study we have shown that coactivation of ER α by COUP-TFI requires either AF-1 or AF-2 of ER α (Figs. 3-7 C-E). Therefore we used the mammalian two-hybrid assay to investigate interactions of COUP-TFI with

different domains of ER α in HeLa cells using VP-COUP and GAL4 DBD fusion proteins with the N-terminal A/B domains of ER α (pM-ER A/B) or with the C to F domains of ER α (pM-ER C/F). When cells were transfected with VP-COUP and pM-ER A/B, the luciferase activity was significantly increased compared to the control luciferase value in cells treated with DMSO or E2, suggesting that COUP-TFI interacts with A/B domain of ER α and the interactions are ligand-independent (Fig. 3-9 B). Furthermore, when VP-COUP was cotransfected with pM-ER E/F, the luciferase activity was only significant increased when cells were treated with E2, suggesting that COUP-TFI interacts with E/F domains of ER α and the interactions are ligand-dependent (Fig. 3-9 C).

Interactions between ER α and COUP-TFI were also investigated in coimmunoprecipitation studies. His-tagged full-length COUP-TFI and ER α were cotransfected in COS-7 cells and treated with 10 nM E2. After 24-h, the cells were lysed and immunoprecipitated with an ER α antibody, and the presence of COUP-TFI in the immunoprecipitate was determined by Western blotting with a monoclonal antibody against His-tag. The results (Fig 3-9 D) show that COUP-TFI is coimmunoprecipitated by ER α antibodies; however COUP-TFI was not immunoprecipitated by ER α antibodies in cells transfected with either ER α or COUP-TFI alone or using control rabbit IgG Ab for immunoprecipitation (data not shown). These data further confirmed that COUP-TFI interacts with ER α in mammalian cells.

A.



B.

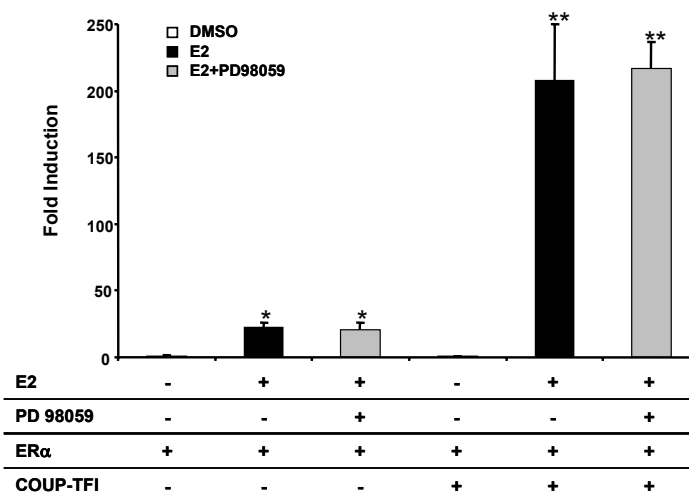


Fig. 3-8 Coactivation of ER α by COUP-TFI is not dependent on the ERK1/2 pathway. (A) Coactivation of Taf1-ER α by COUP-TFI. COS-7 cells were transfected with pERE3, COUP-TFI, β -galactosidase and Taf1-ER α or serine 118 ER α point mutant (m118-ER) expression plasmid. After transfection, cells were treated with DMSO or 10 nM E2 for 36 h., and luciferase activity normalized to β -galactosidase activity was determined as described in the Materials and Methods. (B) Effect of MAPK inhibitor PD 98059. COS-7 cells were transfected with pERE3, COUP-TFI, β -galactosidase and ER α expression plasmid as indicated. After transfection cells were treated with DMSO, 10 nM E2 or cotreated 10 nM E2 with 20 μ M PD 98059 for 36 h., and luciferase activity normalized to β -galactosidase activity was determined as described in the Materials and Methods. Fold induction was calculated relative to activity observed in cells treated with DMSO. Significant ($p < 0.05$) induction by E2 (*) or coactivation of E2-induced activity by COUP-TFI (**) are indicated.

A.

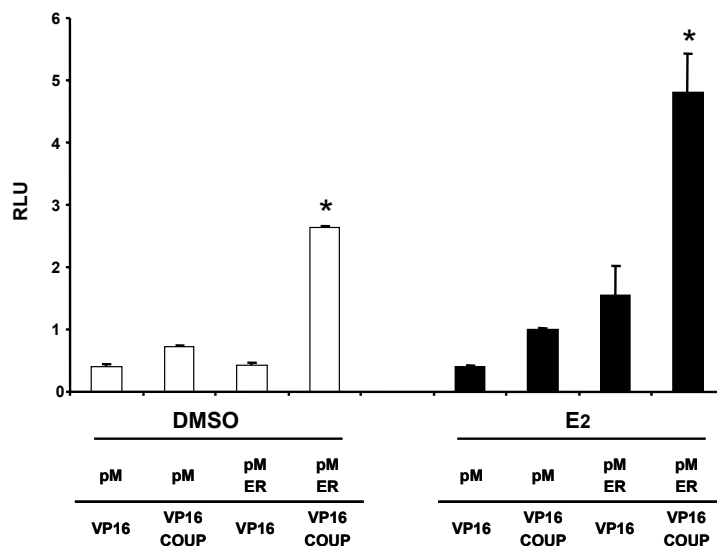
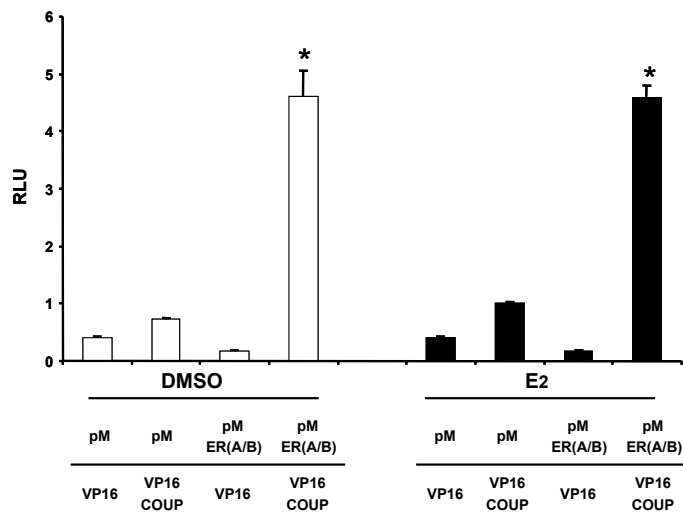
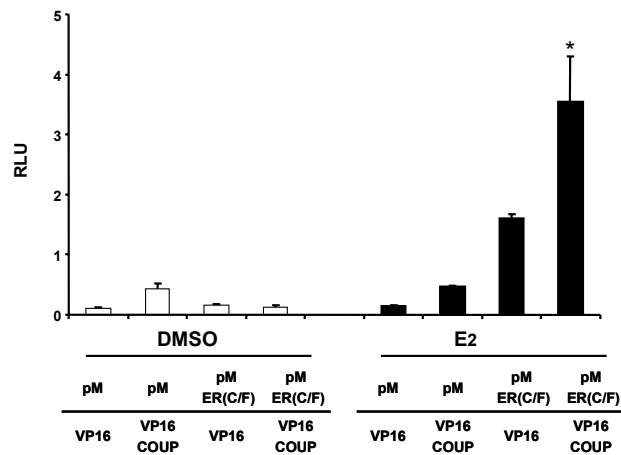


Fig. 3-9 Interactions of COUP-TFI and multiple regions of ER α . Interactions of COUP-TFI with wild-type ER α (A), ER α A/B domains variant (B) or ER α C/F domains variant (C) in mammalian two-hybrid assay. HeLa cells were cotransfected with 5XGAL reporter plasmid, β -galactosidase, either pVP16 (Vp16) or pVp16-COUP-TFI (VP16-COUP) and pM, pM-ER, pM-ER A/B or pM-ER C/F as indicated. After transfection cells were treated with DMSO or 10 nM E2 for 36 h., and luciferase activity normalized to β -galactosidase activity was determined as described in the Materials and Methods and presented as relative luciferase units (RLU). Significant ($p < 0.05$) interactions (*) are indicated. (D) Interactions of COUP-TFI and ER α in a co-immunoprecipitation assay. His-tagged COUP-TFI was transfected into COS-7 cells with or without ER α . After transfection, cells were treated with 10 nM E2 for 24 h., and cell extracts were immunoprecipitated (IP) with anti-ER α antibody and precipitates were then analyzed by Western blot (WB) using anti-His tag and anti-ER α antibodies as described in the Materials and Methods.

B.



C.



D.

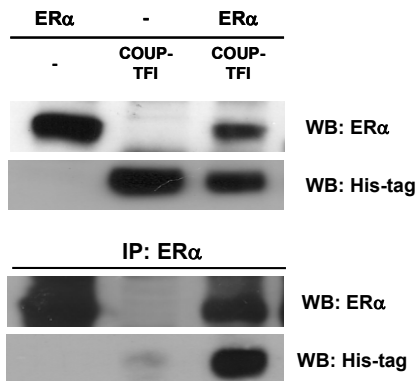


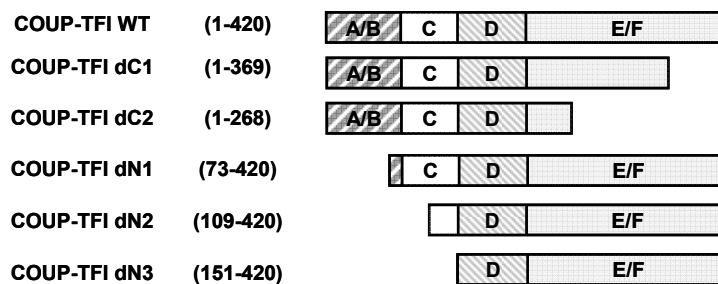
Figure 3-9 continued

3.2.4 Coactivation of ER α by COUP-TFI Deletion Mutants

Previous studies in HeLa and rat urogenital mesenchymal (rUGM) cells showed that the DNA binding domain and the C terminus 35 amino acids of COUP-TFI are important for activation of Egr-1 (260). In contrast, the extreme C terminus region of COUP-TFI can act as a silencing domain and repress transcriptional activity by interactions with the SMRT and N-CoR (308). Domains of COUP-TFI required for coactivation of ER α were determined in MCF-7 and COS-7 cells cotransfected with ER α , pERE₃, and wild-type or deletion mutants of COUP-TFI (Fig. 3-10 A). The mutants include dN1, dN2 and dN3 with N-terminal deletions of amino acids 1-72, 1-108 and 1-150 respectively; the C-terminal mutants dC1 and dC2 containing deletions of amino acids 370-420 and 269-420 respectively. Analysis of the cellular localization of transfected COUP-TFI mutants by western immunoblots indicates that the deletion mutants also accumulate in the nucleus with only minimal levels detected in cytosolic extracts (Fig 3-10 B).

The results in Figures 3-11 A and 3-11 B show that the overall patterns of coactivation of ER α by wild-type and variant COUP-TFI were similar in MCF-7 and COS-7 cells. Decreased coactivation was observed in both cell lines transfected with dC1, dC2 and dN2. However, in MCF-7 cells some coactivation was observed whereas in COS-7 cells these mutations completely abrogated the coactivation response. In contrast dN1 coactivated and dN3 was inactive as a

A.

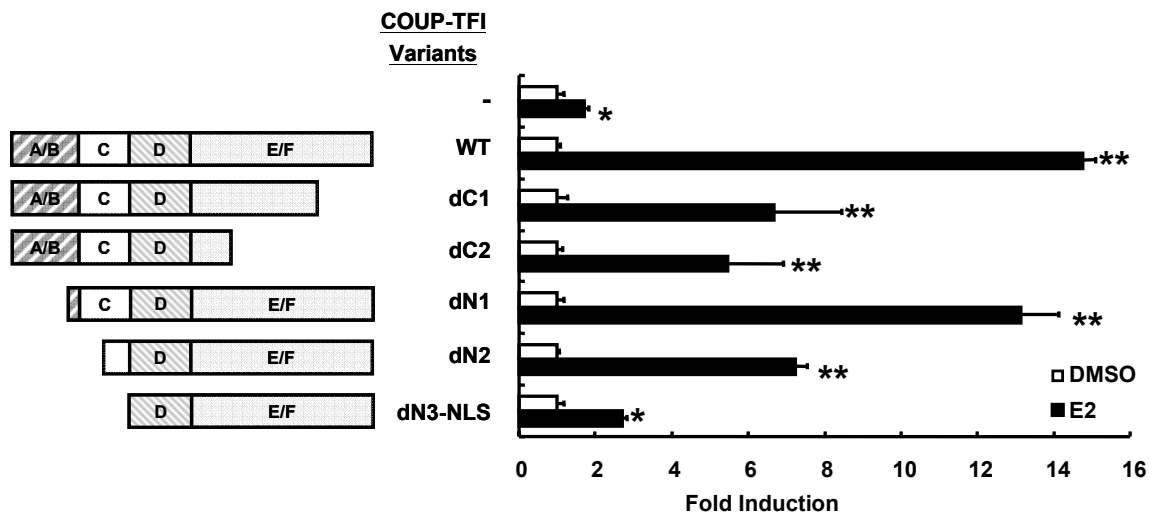


B.



Fig. 3-10 Multiple regions on COUP-TFI are required for coactivation of ER α . (A) Truncation mutants of COUP-TFI. (B) Cytosolic or nuclear localization of COUP-TFI deletion mutants. COS-7 cells were transfected with His-tagged COUP-TFI deletion mutants expressing plasmid, treated with 10 nM E2 for 36 h, harvested and fractionated for cytosolic (C) and nuclear (N) protein as described in Materials and Methods.

A. MCF-7



B. COS-7

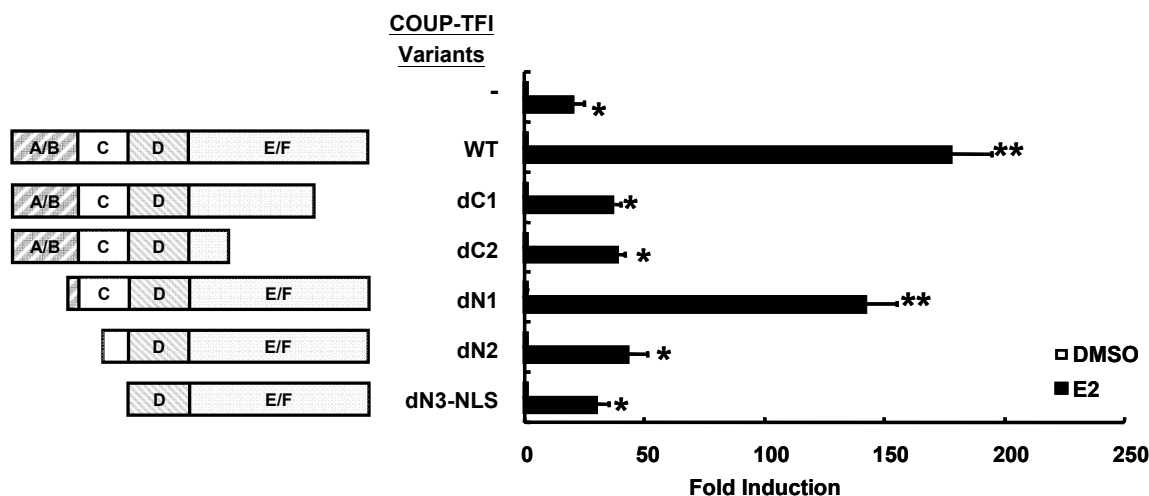


Fig. 3-11 Coactivation of ER α by COUP-TFI deletion mutants. (A) MCF-7 or (B) COS-7 cells were transfected with pERE3, ER α , β -galactosidase and various truncation mutants of COUP-TFI expression plasmid. After transfection cells were treated with DMSO or 10 nM E2 for 36 h, luciferase activity normalized to β -galactosidase activity was determined as described in the Materials and Methods. Fold induction was calculated relative to activity observed in cells treated with DMSO. Significant ($p < 0.05$) induction by E2 (*) or coactivation of E2-induced activity by COUP-TFI (**) is indicated.

coactivator in both cell lines. These data suggest that both the C-terminal aa 370-420 and DNA binding domain of COUP-TFI are important for its activity as a coactivator of ER α .

3.2.5 Coactivation of ER α by COUP-TFI Zinc-Finger Motif Mutants

The role of the DBD of COUP-TFI in its activity as a coactivator of ER α was also investigated. There are two C4-type zinc-finger motifs in the DBD of COUP-TFI and three mutant constructs of COUP-TFI with Cys/Ala point mutations in the zinc finger motifs (m83, m103 and m138) were generated by site-direct mutagenesis (Fig. 3-12 A). The results of coactivation studies with wild-type and point mutant COUP-TFI constructs (Fig. 3-13) show that coactivation was decreased in cells transfected with the mutant constructs, suggesting that both C4-type zinc finger motifs on COUP-TFI are important for coactivation of ER α by COUP-TFI. Analysis of the cellular localization of transfected COUP-TFI mutants by western blots indicates that three point mutants (m83, m103 and m138) accumulated in the nucleus (Fig. 3-12 B). These data suggest that the loss of coactivation activity of COUP-TFI mutants is not caused by their failure to accumulate in the nucleus.

The interactions between ER α and COUP-TFI point mutants were also investigated in coimmunoprecipitation studies. Three His-tagged DBD mutant constructs (m83, m103 and m138) were cotransfected along with ER α into COS-7 cells. Results of co-immunoprecipitation studies (Fig. 3-14) showed that ER α interacted with

lower affinity with the DBD mutants compared to interactions with wild-type COUP-TFI. These data suggest that the zinc finger motifs of COUP-TFI also play an important role in the interaction of this protein with ER α .

3.2.6 Cooperative Coactivation of COUP-TFI with SRC-1

Coactivators are critical nuclear proteins required for the functional activity of NRs, and they serve as bridging molecules between NRs and the basal transcriptional machinery (356). Coactivators may also directly affect chromatin structure or recruit other coactivators which modify chromatin structure and facilitate activation of target genes (20). Previous studies have shown that SRC-1 interacts with COUP-TFI for activation of Egr-1 expression in HeLa cells and SRC-1 also interacts with and coactivates ER α -mediated transactivation, and this response is AF2-dependent (260). Therefore, we investigated the cooperative coactivation of ER α by COUP-TFI and SRC-1 in COS-7 cells transfected with ER α and pERE₃ (Fig. 3-15). The results show that transfection of SRC-1 or COUP-TFI alone coactivated ER α -dependent transactivation by 6.7-fold or 7.9-fold respectively and cotransfection with SRC-1 plus COUP-TFI expression plasmids gave greater than additive response and enhanced transactivation by 18.1-fold indicating that COUP-TFI and SRC-1 cooperatively coactivated ER α . However, cotransfection with SRC-1 and the “inactivated” COUP-TFI deletion mutant dC1 in which aa 370-420 (c-terminal) have been deleted, show that the enhanced coactivation by SRC-1 (8.4-fold) was not significantly higher than observed in cells transfected with SRC-1 alone. These data suggest that the

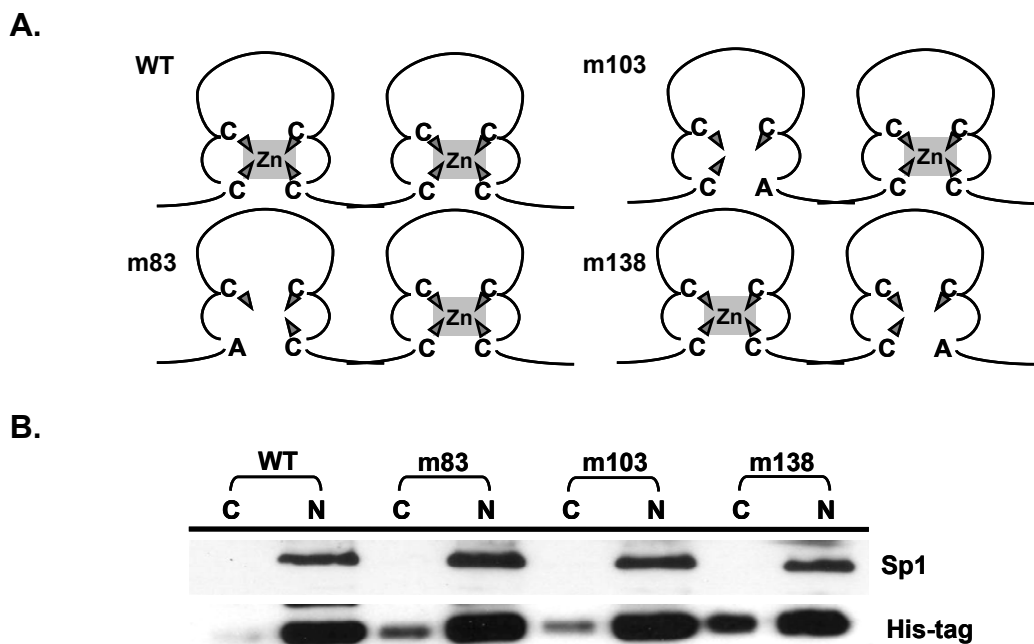


Fig.3-12 Zinc finger motifs in the DNA binding domain of COUP-TFI are important for coactivation of ER α . (A) Three different point mutations were made in the zinc finger motifs of COUP-TFI (C83A, C103A, C138A). (B) Cytosolic and nuclear localization of COUP-TFI variants. COS-7 cells were transfected with wild-type or mutant His-tagged COUP-TFI expressing plasmid, treated with 10 nM E2 for 36 h, harvested and fractionated for cytosolic (C) and nuclear (N) protein as described in Materials and Methods.

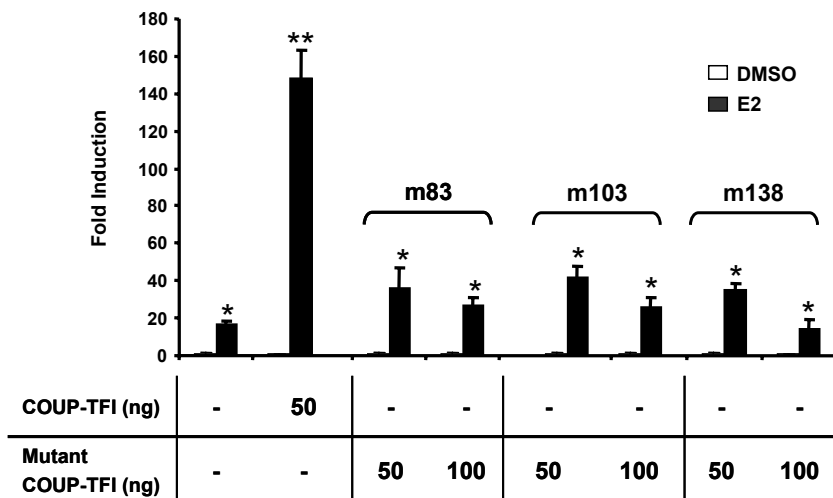


Fig. 3-13 Coactivation of ER α by DBD point mutants of COUP-TFI
 COS-7 cells were transfected with pERE3, ER α , β -galactosidase and various zinc finger mutants of COUP-TFI expression plasmid. After transfection cells were treated with DMSO or 10 nM E2 for 36 h., and luciferase activity normalized to β -galactosidase activity was determined as described in the Materials and Methods. Fold induction was calculated relative to activity observed in cells treated with DMSO. Significant ($p < 0.05$) induction by E2 (*) or coactivation of E2-induced activity by COUP-TFI (**) is indicated.

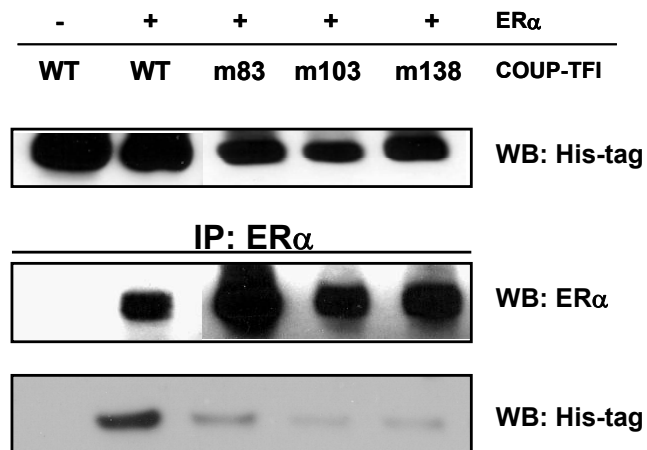


Fig. 3-14 The zinc finger motif of COUP-TFI is critical for protein-protein interactions with ER α . Wild-type or mutants His-tagged COUP-TFI were transfected into COS-7 cells with or without ER α . After transfection, cells were treated with 10 nM E2 for 24h., and cell extracts were immunoprecipitated (IP) with anti-ER α antibody and precipitates were then analyzed by Western blot (WB) using anti-His tag and anti-ER α antibodies as described in the Materials and Methods.

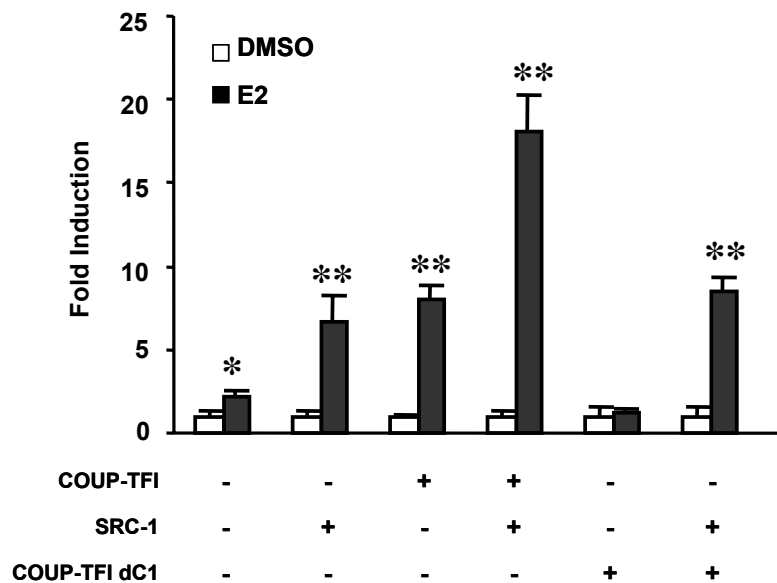


Fig. 3-15 Cooperative coactivation of ER α by COUP-TFI and SRC1. COS-7 cells were cotransfected with pERE₃, ER α , β -galactosidase, COUP-TFI variants and SRC1 as indicated; cells were treated with DMSO or 10 nM E2 for 36 h., Fold induction was calculated relative to activity observed in cells treated with DMSO. Significant ($p < 0.05$) induction by E2 (*) or coactivation of E2-induced activity by COUP-TFI (**) is indicated.

C-terminal region of COUP-TFI is required for cooperative coactivation with SRC-1.

3.2.7 Coactivation of ER α /Sp1 by COUP-TFI

Several hormone-responsive genes in breast cancer cells are regulated through interactions of ER α /Sp1 with GC-rich promoter elements [285-298]. Results in Figure 3-16 show that E2 significantly induced luciferase activity in ZR-75 cells transfected with pSp1₃, a construct containing three consensus GC-rich Sp1 binding sites linked to luciferase. In cells also cotransfected with COUP-TFI expression plasmid (25, 50 or 100 ng) there was a significant increase in basal and E2-induced luciferase activity and there was also a > 3-fold enhanced induction response in cells cotransfected with 50 ng COUP-TFI expression plasmid. This represents one of the first examples of the coactivation of ER α /Sp1 in breast cancer cells.

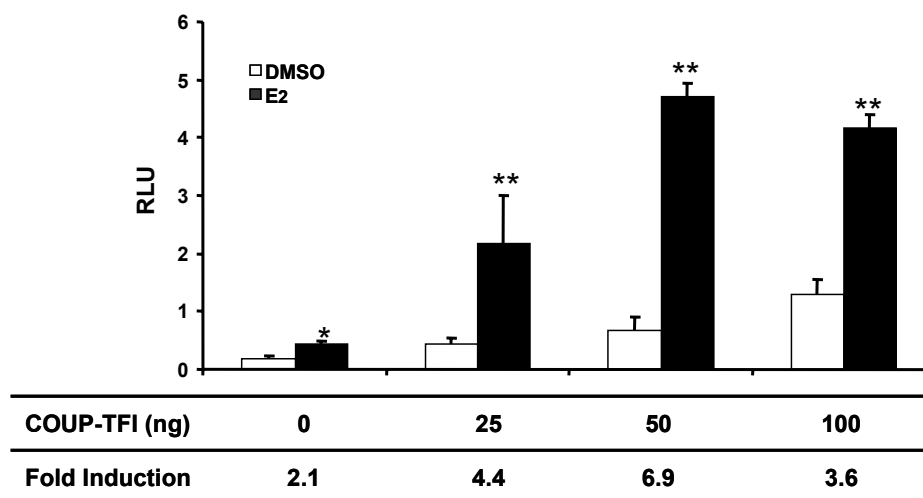


Fig. 3-16 Coactivation of ER α /Sp1 by COUP-TFI. ZR-75 cells transfected with pSp1₃, ER α , β -galactosidase and increasing amounts of pCDNA3-COUP-TFI (0, 25, 50 and 100 ng) expression plasmid. After transfection cells were treated with DMSO or 10 nM E2 for 36 h., luciferase activity normalized to β -galactosidase activity was determined as described in the Materials and Methods and presented as relative luciferase units (RLU). Fold induction was calculated relative to activity observed in cells treated with DMSO. Significant ($p < 0.05$) induction by E2 (*) or coactivation of ER α /Sp1 by COUP-TFI (**) is indicated.

CHAPTER IV

DISCUSSION AND CONCLUSION

4.1 Mechanism of Induction of *Egr-1* by E2 in MCF-7 cells

Egr-1 is an immediate-early gene induced by mitogens in mammalian cells, and *Egr-1* acts as a transcription factor that modulates expression of several genes (69, 72, 325). Several studies also suggest that *Egr-1* can act as a tumor suppressor gene in some cells. For example, in a subclone of human HT1080 fibrosarcoma cells, overexpression of *Egr-1* inhibited transformed growth and [³H]thymidine uptake and suppressed the rate of tumor growth in athymic nude mice bearing HT1080 xenografts (138). Expression of *Egr-1* was also relatively high in non-tumorigenic MCF-10A and 184A1N4 immortalized mammary epithelial cells, but low to non-detectable in ER-negative and ER-positive (ZR-75, T47D and MCF-7) breast cancer cells lines. A similar pattern of *Egr-1* expression was also observed in rat mammary tissue (high) and mammary tumors (low), suggesting that loss of *Egr-1* expression may be required for development of breast cancer.

Pratt and coworkers previously reported that E2 induced *Egr-1* gene expression in MCF-7 cells; this was accompanied by rapid autophosphorylation of raf-1. In this study, E2 also induced *Egr-1* gene expression (mRNA and protein) in MCF-7 cells (Figs. 3-1 A and 3-5 B), and the mechanism of this response was further investigated using a series of constructs containing *Egr-1* promoter inserts.

The -600 to +12 region of the *Egr-1* gene promoter contains a GC-rich site, multiple SREs, and a cAMP response element (CRE). Previous studies indicate that both SRE and CRE motifs are hormone-responsive through ER α -dependent extranuclear induction of the src-ras-MAPK and PKA pathways (51, 85, 88, 219, 220). In contrast, the more distal GC-rich Sp1 binding site could be activated by the non-classical nuclear ER α /Sp1 pathway (261). Deletion analysis (5'- and 3'-) of the *Egr-1* gene promoter (Figs. 3-2 A and 3-2 B) indicates that SRE3 and SRE4 are E2-responsive and further mutation analysis (Fig. 3-2 C) demonstrates that E2-responsiveness is linked to the TCF and SRF motifs within SRE3.

Previous studies in this laboratory showed that E2 also induced *c-fos* gene expression in MCF-7 cells through activation of a proximal SRE through the ras-MAPK pathway (88). Results in Figures 3-3 and 3-4 confirm that hormone-dependent activation of SRE3 in the *Egr-1* promoter is also accompanied by Elk-1 phosphorylation and is inhibited by dominant negative Elk-1 expression. Thus, both immediate-early genes *Egr-1* and *c-fos* are induced by E2 through activation of ras-MAPK by extranuclear pathways in breast cancer cells (88).

Recent studies have shown that *Egr-1* is regulated, in part, through the MAPK pathway in several cell lines (93, 151, 283). For example, in rat granulosa cells, gonadotropin-dependent upregulation of *Egr-1* is dependent on multiple factors including MAPK and protein kinase A-dependent phosphorylation of factors associated with SRE1 and other proximal motifs (283). Activation of the

MAPK pathway is also required for light-induced upregulation of Egr-1 in the suprachiasmatic nucleus of mice (93) and hyperoxia-induced expression of Egr-1 in mouse alveolar carcinoma cells (151). In the latter cell line, the MAPK inhibitor PD98059 blocked hyperoxia-induced expression of Egr-1, whereas PI3-K and p38 MAPK inhibitors had no effect. In the rat anterior pituitary gland and primary neonatal rat cardiomyocytes, hormonal activation of Egr-1 was MAPK-dependent and in cardiomyocytes, SREs were identified as putative hormone-responsive motifs (75, 203). In breast cancer cells, activation of PI3-K by E2 has been identified as an important pathway for proliferation of MCF-7 cells (50, 89, 328). Moreover, E2-mediated induction of c-fos through the SRE involved simultaneous activation of src-MAPK and src-PI3-K pathways where PI3-K activates the serum response factor (50, 88). We therefore investigated the role of PI3-K in the activation of Egr-1 mRNA expression by E2 (Fig. 3-5 C) and luciferase activity in cells transfected with pEgr-1E (Fig. 3-5 A). The results show that for both responses, E2-induced transactivation was inhibited by PD98059 but not by the PI3-K inhibitor LY294002. Since E2 activates both MAPK and PI3-K pathways in breast cancer cells (49, 88, 93, 151, 220), the differential effects of the latter pathway on activation of Egr-1 and fos through their respective SREs may be due, in part, to promoter context.

Ling and coworkers (195) investigated interactions of wild-type and variant Elk-1 and SRF with different SREs to form transcriptional-active ternary complexes. One type of SRE which is characteristic of the motif in the c-fos

promoter contains a "strong" SRF site (CArG) and a weak TCF (ets) site (Fig. 4-1). In this model, SRF binds the SRE and recruits Elk-1, and both MAPK and PI3-K inhibitors block SRE-dependent transactivation. Another type of combined ets and CArG (CECI) motif has a high affinity TCF (or ets) site next to a weak CArG (SRF) site (195) (Fig. 4-1). In this model, Elk-1 binds the SRE and subsequently recruits SRF to form the transcriptional-active ternary complex. The TCF site in the Egr-1 promoter is identical to the corresponding "strong" motif in the CECI promoter. Thus, hormone-induced transactivation of Egr-1 requires MAPK-dependent activation of Elk-1 which interacts with a "strong" TCF site, and subsequent recruitment of SRF is not dependent on activation through the PI3-K pathway. Differential activation of c-fos and Egr-1 is also consistent with the growth-promoting activities of both c-fos and the PI3-K pathway in breast cancer cells (50, 89, 328), whereas Egr-1 is associated with suppression of breast cancer cell growth (138). In contrast, there is evidence that Egr-1 may enhance formation and growth of prostate cancer (26), and current studies are investigating the mechanisms that distinguish between the differential effects of Egr-1 in hormone-dependent breast and prostate cancer.

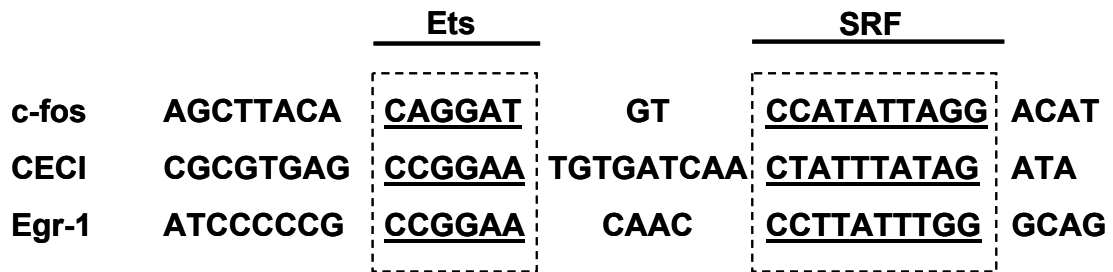


Fig. 4-1 Comparative SREs. The SREs in the c-fos, CECI and Egr-1 gene promoters are given and the Ets (Elk-1 site) and SRF motifs are indicated [367].

4.2 Coactivation of E2-Induced Transactivation by COUP-TFI

The NR superfamily of transcription factors contains ligand-activated and orphan receptors that interact with genomic *cis*-element in target gene promoters to induce or repress gene expression (27, 123, 205, 235). Steroid hormone receptors such as ER α have been extensively used as models for determining the mechanisms of ligand-dependent receptor-mediated transactivation, which requires the assembly and recruitment of a nuclear complex of coactivators/coregulatory proteins (8, 9, 216, 235, 276). The p160/SRC family including SRC1/NCoA1, TIF2/GRIP1 and pCIP/AIB1/RAC3/ACTR/TRAM-1 was first discovered as coactivators of NRs that specifically interact with AF-2 of ligand-bound NRs (211). Sequence analysis of SRC proteins identified a basic helix-loop-helix (bHLH) domain and Per-Arnt-Sim (PAS) domains in the amino-terminal region, a centrally located receptor-interacting domain (RID) and a C-terminal transcriptional activation domain (AD) (Fig. 1-8). The RID which contains three conserved LXXLL motifs (NR box) appear to contribute to the specificity of coactivator-NR interaction. The histone acetyltransferase (HAT) activity was identified in C-terminal region of SRC-1. There are also activation domains that can interact with the CREB-binding protein (CBP) in C-terminal AD of SRC family (211). One mechanism by which the SRC family coactivates NRs resulted from studies on the interactions and recruitment of the CBP/p300 coactivators which have intrinsic HAT activity that mediates the acetylation of nucleosome of histones, a covalent modification generally associated with the

enhancement of transcription [145,146]. Other proteins with the ability to modify histones or remodel chromatin structure include CARM1 (62), an arginine methyltransferase, and ATP-dependent remodeling factors BRG1 and hBrm (84, 143). These proteins have also been identified as coactivators of NRs. The multiprotein Mediator complexes known as the TRAP and DRIP complexes are a class of NR coactivators which also enhance transactivation by interactions with DNA-bound NRs and the RNA pol II transcriptional machinery to stabilize the formation of transcription PICs at the promoter [161]. DRIP205 and DRIP150 have been shown to coactivate ER α -mediated transactivation in ZR-75 breast cancer cells (188, 368). Coactivation of ER α by DRIP205 does not require NR boxes and multiple domains of DRIP205 play a role in coactivation of ER α and in interactions with ER α (188, 368). Coactivation of ER α by DRIP150 also does not require NR boxes and a novel sequence (aa795-804) with putative α -helical structure is required for coactivation of ER α by DRIP150 (188, 368). Some unexpected molecules such as steroid-receptor-RNA activator-1 (SRA1), an RNA transcript, has been reported as a ligand-independent ER α coactivator in COS-1 cells (184). P68 (Ddx5) and p72 (Ddx17) which are RNA helicases have also been reported to act as transcriptional coactivators for ER α through ER α AF-1 by association with SRA and the AF-2 coactivator SRC1/TIF2 in MCF-7 and COS-1 cells (357). Coactivators identified to date are remarkable in both their number and diversity, suggesting that NR coactivation may involve more than one class of coactivators and the potential complexities associated with multiple

pathways are consistent with the subtle ligand-, tissue- and gene-specific action of NRs.

COUP-TFI, one of the most studied of the orphan receptors, is involved in regulation of several important biological processes, such as neurogenesis, organogenesis, cell fate determination, and metabolic homeostasis (204, 205). The target genes for COUP-TF are summarized in Table 4-1. COUP-TFI acts as a transcription factor via COUP-TFI homodimers or as a heterodimer with RXR. In addition COUP-TFI interacts with other NRs such as RAR, TR, VDR,PPAR, and HNF4 and binds to a wide variety of response elements that contain imperfect AGGTCA direct repeats separated by a variable number of nucleotides (70, 343). COUP-TFI was initially identified as an activator of the chicken ovalbumin gene (290). In P19 embryonal carcinoma cells, COUP-TFI up-regulated vitronectin mRNA level and stimulated the vitronectin promoter activity in cells which overexpressed COUP-TFI (10). Recent studies showed that COUP-TFI activated transcription of the human CYP11B2 gene by binding to the -129/-114 promoter region of CYP11B2 in human adrenocortical H295R cells (306). Furthermore, there is increasing evidence showing that COUP-TF activates transcription through protein-protein interactions with DNA-bound transcription factors without a requirement for DNA binding. For example, COUP-TFI enhanced mRNA and protein expression levels of *NGFI-A* gene, also known as Egr-1 or Zif268, in HeLa and rUGM cells by interactions with Sp1. Both the DBD and the C terminus region of COUP-TFI are important for the *NGFI-A*

activation (260). COUP-TFI can also activate transcription as coactivators of other nuclear receptors. For example in HeLa cells, COUP-TFI interacts with another orphan receptor, hepatic nuclear factor 4 (HNF-4), for induction of phosphoenolpyruvate carboxykinase (PEPCK) gene transcription by glucocorticoids and the E/F domains of COUP-TFI are required for coactivation of HNF-4 (324). Recently, studies from different laboratories have shown that there is cross-talk between COUP-TFI and ER α where COUP-TFI modulates ER α -mediated gene expression (167, 169, 215, 252, 309). Melivler and coworkers reported that COUP-TFI formed a tight complex with ER α and enhanced ER α -dependent activity (215). The formation of a tight ER α -COUP-TFI intermediate complex resulted in an increased recruitment of ERK2/p42 MAPK to this complex resulting in phosphorylation of the ER α on Ser 118 in the A/B (N-terminal) region of ER α and this enhanced transcriptional activity of ER α . Coactivation of ER α by COUP-TFI can only take place in AF1 permissive cells such as HepG2, COS-7 and PC3 cells (i.e. cells in which ER α activity is driven mainly by AF1). Mutation of S118 of ER α impaired the effects of COUP-TFI on ER α -dependent activity (215).

Table 4-1 Target genes for COUP-TFs (307).

Gonads	LH receptor, FSH receptor	↓
Adrenal cortex	CYP17 (17 α -hydroxylase/17,20-lyaseP450)	↓
	CYP11B2 (Aldosterone synthase P450)	↑
	CYP19 (Aromatase P450)	↓
	DAX-1	↓
Pituitary gland	Oxytocin	↓
Cerebellum	PCP-2 (Purkinje cell protein-2)	↓
Livers	Angiotensinogen	↓
	HNF-1 (Hepatocyte nuclear factor-1)	↑
Heart	ANF (Atrial natriuretic factor)	↑
	Calreticulin (Ca ²⁺ binding chaperone of the endoplasmic reticulum)	↑
	NHE-1 (Na ⁺ /H ⁺ exchanger-1)	↑
Adipose tissue	PEPCK	↑
	LPL (Lipoprotein lipase)	↑
Prostate	NGFI-A	↑
Others	CaMKIV (Ca ²⁺ / calmodulin-dependent protein kinase IV)	↓
	Vitronectin	↑

The arrows (↑) and (↓) indicate that COUP-TFs upregulate and down-regulate target genes, respectively.

The results of this study show that COUP-TFI coactivated ER α in cells transfected with pERE₃ not only in COS-7 (AF-1 permissive) but also in MCF-7, HeLa and MDA-MB-231 cells (AF-2 permissive). Furthermore, in COS-7 cells cotransfected with pERE₃, COUP-TFI and the S118A mutant of ER α , COUP-TFI enhanced E2-induced luciferase reporter activity (Fig. 3-8 A). Treatment of COS-7 cells with the MAPK inhibitor PD98059 did not affect the coactivation of E2-induced transactivation by COUP-TFI (Fig. 3-8 B). These data suggest that there is another mechanism of coactivation of ER α by COUP-TFI other than recruitment of ERK2/p42 MAPK and phosphorylation of the Ser 118 as previously reported by Melivler and coworkers (215). Results of this study clearly demonstrate that coactivation of ER α by COUP-TFI in both AF-1 and AF-2 permissive cells (Figs.3-6 A-D), and mutation of the critical MAPK-dependent phosphorylation site (S118) in ER α does not result in loss of COUP-TFI coactivation activity.

COUP-TFI, unlike most coactivators which are recruited by AF-2 of ER α , did not require the critical helix 12 region of ER α AF-2 for its coactivation of ER α -mediated transactivation in COS-7 cells since E2-induced activity in cells transfected with Taf-1ER α (Fig 3-7 C). COUP-TFI has minimal effect on coactivation of ER α when cells were transfected with pERE₃ and null-ER α mutant which is an AF-1 deletion mutant also containing three point mutation on helix 12 (Fig. 3-7 E), suggesting that the AF-1 of ER α is involved in coactivation by COUP-TFI. However, when COS-7 cells were transfected with 19c-ER α an AF-1

deletion mutant and pERE₃, interestingly COUP-TFI has the same magnitude of ER α coactivation compared to cells transfected with wild-type ER α (Fig 3-7 D). These results suggest that the AF-2 of ER α is required for coactivation of ER α by COUP-TFI but only when AF-1 of ER α is deleted. The requirements of AF-1 or AF-2 of ER α for activity of COUP-TFI as a coactivator were further investigated for the roles of these regions of ER α for interactions with COUP-TFI. The results from mammalian two-hybrid assays showed that COUP-TFI interacts with the A/B domains of ER α in cells treated with DMSO and E2, suggesting that the interactions of COUP-TFI and A/B domains of ER α are ligand-independent (Fig. 3-9 B). COUP-TFI also interacts with the C/F domains of ER α but only when cells are stimulated with E2 (Fig. 3-9 C), suggesting that the interactions between COUP-TFI and AF-2 ER α are also induced by E2. In summary, both A/B domains which contain AF-1 and C/F domains which contain AF-2 of ER α are involved in coactivation function and physical interactions with COUP-TFI.

We also investigated the functional and physical interactions of several N-terminal and point mutants of COUP-TF1 in transfection and co-immunoprecipitation assays (Figs. 3-11, 3-13 and 3-14). Deletion of amino acid 1-72 (dN1) did not affect the functional activity of COUP-TF1 as a coactivators, however, deletion of amino acids 1-108 resulted in loss of activity (Figs. 3-11 A and B). These results suggest that coactivation of ER α by COUP-TF1 in COS-7 and MCF-7 cells requires an intact DBD of COUP-TFI

and/or a small fragment of the A/B domain. A previous report in CHO cells also showed that the DBD of COUP-TFI was required for coactivation of ER α (215) and these results were consistent with those observed in COS-7 and MCF-7 cells (Fig. 3-11 A and B). The DBD of COUP-TF1 is similar to other nuclear receptors and contains two zinc finger motifs with multiple cysteine residues coordinated by zinc ions (Fig. 3-12 A). The importance of the zinc finger motifs in the activity of COUP-TFI as a coactivator was further investigated using cysteine mutants of COUP-TFI at amino acids 83 and 103 (in zinc finger 1) and 138 (zinc finger 2). In transfection studies, these COUP-TF1 point mutations (C83A, C103A and C138A) resulted in loss of coactivation activity for ER α (Fig. 3-13); moreover, in co-immunoprecipitation assays, these point mutations also reduced the interactions between COUP-TFI and ER α . The results from functional activity and physical interactions assays of COUP-TFI zinc-finger mutants suggest that zinc finger motifs of COUP-TFI are important for coactivation and interactions with ER α .

Cooperative or synergistic coactivator-NR interactions involving two or more coactivators were previously reported (84, 112, 170, 357). CARM1 and PRMT1 enhanced ER α action only in the presence of GRIP1 (60, 170); CBP/p300 further increased coactivation of ER α by GRIP1 in CV-1 cells, and this was dependent on binding of both CBP/p300 and CARM1 to two different domains on GRIP1. Another study showed that ligand-dependent coactivation of ER α by SNURF cooperatively coactivated ER α with TATA-binding protein (TBP)

in ZR-75 breast cancer cells. Loss of cooperatively enhancement of ER α -mediated transactivation by cotransfection of TBP and RING domain mutant of SNURF resulted from interactions of TBP with the C-terminal RING domain of SNURF. Moreover, TBP did not cooperatively coactivate ER α in cells transfected with Δ 1-20 SNURF (interacts with ER α but not DNA) or Δ 31-65 SNURF (interacts with DNA but not ER α), suggesting that cooperative coactivation of ER α by TBP/SNURF is dependent on domains of SNURF that bind TBP, ER α , and DNA (298). Furthermore, coactivation of ER α in MCF-7 cells by SRC-1 and the RNA coactivator SRA are synergistically enhanced by p72, an RNA binding DEAD box protein that interacts with AF1-of ER α (357). COUP-TFI has been reported to cooperate with SRC-1 or p300 to enhance the transactivation of *NGFI-A* in HeLa cells (260). Also COUP-TFI associated with GRIP1 or SRC-1 to coactivate HNF-4-mediated transactivation of PEPCK in HeLa cells and the C-terminal 15 amino acids are required for protein-protein interactions between COUP-TFI and the coactivators identified in a yeast two-hybrid assay (324). The results from this study showed that the deletion of C-terminal amino acids 370-420 (i.e. dC1 COUP-TFI mutant) resulted in loss of coactivation activity (Fig. 3-11), however, this COUP-TFI mutant still interacted with ER α . These results are consistent with previous reports that the C-terminal region of COUP-TFI is important for coactivation with HNF-4 in HeLa cells (324). The function of C-terminal amino acids 370-420 was further investigated in cooperative coactivation with SRC-1 in COS-7 cells transfected with pERE₃. In this study, (Fig. 3-15) it was shown that

SRC-1 and COUP-TFI cooperatively enhanced ER α -dependent transactivation; however, deletion of C-terminal region of COUP-TFI abolishes this cooperative activity, suggesting that aa 370-420 of COUP-TFI which are predicted to form a α -helix structure are important for interactions with SRC-1.

ER α /Sp1-mediated transactivation has been linked to hormone activation of several genes involved in cell cycle progression, DNA synthesis and metabolism of purines and pyrimidines [285-298]. *In vitro* studies show that ER α interacts with both Sp1 and Sp3, and the C-terminal DBD of Sp1 is the major interaction site for ER α (299). Recently Kim et al used the FRET technique to investigate the interactions between ER α and Sp1 in living MCF-7 breast cancer cells. Results from FRET analysis showed that ER α interacts with Sp1 in living breast cancer cells and the interactions are ligand-dependent (162). COUP-TFI has been shown to interact with Sp1 in GST-pull down assay and activate *NGFI-A* expression through Sp1 GC-rich promoter elements in HeLa and rUGM cells (260). Only a few coactivators of ER α such as DRIP205, and DRIP150 have been reported as coactivators for ER α /Sp1 in ZR-75 breast cancer cells and research on identification of ER α /Sp1 coactivators is in progress. The results of transfection assays in ZR-75 cells showed that COUP-TFI increased the basal luciferase activity of pSp1₃ (Fig. 3-16) which is consistent with previous reports showed that COUP-TFI interacted with Sp1 and up-regulated NGFI-A gene expression through interactions with Sp1 in HeLa and rug cells; furthermore COUP-TFI also enhanced E2-induced luciferase activity, suggesting that

coactivation of ER α /Sp1 by COUP-TFI was also observed in ZR-75 cells transfected with pSp1₃ (Fig. 3-16). The molecular mechanisms of this response are currently being investigated.

In conclusion, we have shown here that COUP-TFI interacts with ER α and functions as a coactivator for ER α -mediated transactivation. The DNA binding domain of COUP-TFI is important for interactions with ER α and is also critical for its coactivation activity. COUP-TFI also functions as a coactivator for ER α /Sp1 pathway in ZR-75 breast cancer cells.

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